Protein Detector™ Western Blot Kit

TMB System

For detection of membrane-bound proteins using horseradish peroxidase labeled secondary antibodies and TMB Substrate.

Catalog No.

Catalog No. 54-11-50

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INTRODUCTION

An enzyme immunoassay (EIA) using affinity purified antibodies is a highly specific method for analysis of proteins. Use of an enzyme-linked affinity purified antibody together with a sensitive precipitating substrate provides an excellent method for the characterization of samples bound to membranes through Western or dot blotting. Following attachment of protein to a membrane, a primary antibody is used to selectively bind the protein of interest (1). Alternatively, a known protein is bound to the membrane for screening of specific monoclonal antibodies or serum samples (2). An enzyme-labeled secondary antibody directed against the species of origin of the primary antibody or serum is then applied. This antibody is coupled to horseradish peroxidase (HRP) through a modified periodate method (3). The horseradish peroxidase substrate used is 3,3',5,5'-tetramethylbenzidine (TMB). When HRP reacts with hydrogen peroxide in the TMB substrate, an insoluble blue dye is precipitated onto the site where the enzyme-labeled antibody is bound to the membrane through the antigen-antibody complex. The color persists with minimal fading when protected from exposure to light.

Principle Of The Protein Detector Western Blot Kit

The Protein Detector Western Blot Kit is designed for the detection and visualization of proteins immobilized on membranes through either electrophoresis or dot blottting. The combination of a highly specific, stable liquid conjugate with a sensitive chromogenic substrate allows rapid and accurate identification of samples. All solutions required for blocking and washing the membrane and for diluting antibodies are provided.

Kits include affinity purified antibodies specific for mouse and rabbit immunoglobulins, conjugated to horseradish peroxidase and stabilized in liquid form for quick dilution. The TMB substrate is provided as a convenient ready-to-use solution which forms an insoluble blue precipitate when combined with the HRP labeled antibody. Color development occurs rapidly with high resolution of positive reaction sites. Results are easily read, and when properly stored, the developed membrane provides a stable record of results.

MATERIALS AND EQUIPMENT

Kit Components	Prod. Code	Volume
Peroxidase-Labeled Secondary Antibody, 0.1 mg/mL		
Goat Anti-Rabbit IgG(H+L)	374-1506	750 µl
Goat Anti-Mouse IgG(H+L)	374-1806	750 µl
5X Detector Block Solution	71-83-01	120 mL
	71-83-03	50 mL
Detector Block Powder	72-01-01	10 g
Wash Solution Concentrate (20X)	50-63-03	3 x 100 mL
TMB Membrane Peroxidase Substrate	50-77-02	2 x 100 mL

Reagents are stable for a minimum of one year when stored at 2-8°C. Sufficient reagents are provided to test approximately 2500 cm^2 of membrane (approximately 44, 8 cm x 7 cm, mini-blots) when recommended minimal volumes are used.

GUIDELINES FOR KIT USE

The Protein Detector Western Blot Kit includes enzyme-labeled affinity purified antibodies to detect mouse or rabbit antibody or serum samples. Where appropriate, the enzyme labeled secondary antibody provided in this kit may also be used to directly detect mouse or rabbit proteins on a membrane without the use of an intermediate antibody. No additional buffers or solutions are required for use with this kit.

Prior to application of the kit reagents, the protein of interest must be immobilized onto the test membrane. Nitrocellulose, polyvinylidene difluoride (PVDF), and nylon membrane have all been determined to be suitable for use with this kit. For dot blots, proteins are spotted and allowed to adhere to the membrane (4,5). For a Western blot, proteins are separated by SDS-polyacrylamide gel electrophoresis (or a comparable method) and transferred to the membrane through either electrophoretic or passive transfer (6-11).

Materials Not Provided

- Mouse or rabbit primary antibodies
- Nitrocellulose, PVDF, or Nylon membrane (KPL recommends BioTrace NT, BioTrace PVDF, and BioTrace HP from Pall-Gelman)
- Incubation trays or tubes for reagent incubation
- Platform shaker or rocker
- Gloves
- Coomassie blue for gel staining
- Protein stain such as Ponceau S or Amido black
- Protein standards
- Polyacrylamide gels (KPL recommends pre-cast gels from NOVEX, Hoefer or Bio-Rad)
- Electrophoresis equipment (KPL recommends NOVEX, Hoefer or Bio-Rad)

Remember

- Gloves should always be worn while handling the membrane and all immunoassay reagents to avoid contamination with skin oils or proteins.
- Caution should be used when preparing or handling polyacrylamide gels; monomeric acrylamide is a neurotoxin.
- For proper analysis of results, always include positive and negative controls, blanks, and/or protein standards as appropriate.

PREPARATION OF WORKING SOLUTIONS

A. **Blocking Solution/Conjugate Diluent:** To prepare 1X Detector Block, first dilute the 5X Detector Block Solution 1:5 in reagent quality water (i.e. 1 mL Detector Block Solution + 4 mL reagent quality water). Next add 1% (w/v) Detector Block Powder and microwave on high 1 second/mL of solution. Swirl often during the heating process. Prepare a minimum of 0.36 mL/cm^2 of membrane for both the blocking and diluent steps. **Do not allow solution to boil**. **To avoid non-specific background, be sure that all the blocking powder has dissolved.** Alternatively, Detector Block Powder may be added to the diluted solution and placed in a 50°C waterbath until dissolved. Cool to room temperature before use.

Note: 1X Detector Diluent/Blocking Solution may be prepared and stored in the refrigerator for up to 1 week; however, for optimal performance, it is recommended that the solution be prepared fresh on the day of use.

B. **Wash Solution:** To prepare 1X Wash Solution, dilute Wash Solution Concentrate 1:20 with reagent quality water (i.e. 5 mL Wash Solution Concentrate + 95 mL H_2O).

C. **Conjugate:** A suggested starting dilution of the liquid conjugate is 1:1000 in 1X Detector Block (from step A). This concentration may be adjusted, if desired, to optimize the reaction.

D. **Substrate:** Ready to use, requires no dilution.

PROCEDURES
POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTTING

The following is a recommended protocol for polyacrylamide gel electrophoresis and Western blotting. For more information, follow the instructions provided by the equipment manufacturers or consult the references on p. 12.

1. Prepare samples by diluting to desired concentration with sample diluent. Incubate samples at 100°C for 3 minutes prior to electrophoresis.
2. Electrophorese samples and standards until tracking dye approaches bottom of gel. Typically, gels are run at 100-200V constant voltage. Conditions for electrophoresis can vary depending on the type of gel. Check with gel apparatus manufacturer for recommendations.
3. While the gel is running, soak all fiber pads, filter papers, and transfer membranes in the transfer buffer. Both nitrocellulose and nylon membranes can be treated directly with the transfer buffer. PVDF membranes require prewetting in 100% methanol before soaking in transfer buffer.
4. After electrophoresis, cut off the bottom right corner of the gel. This will ensure that the gel is oriented correctly in the transfer apparatus.
5. Assemble the transfer cassette per manufacturer's instructions. Be sure the gel is oriented so that after transfer, the lanes will appear on the membrane in the desired order.
6. Run transfer according to manufacturer's instructions. Transfer from a 1 mm thick minigel in the range of 8-12% acrylamide is usually complete in about 40-45 minutes. Higher percentage gels and larger proteins will take longer. Optimal transfer time should be determined experimentally.
7. Optional: Stain the gel post-transfer with Coomassie blue to determine transfer efficiency. The presence of stained proteins indicates sub-optimal transfer. Pre-stained standards can be used to reliably monitor the efficiency of the transfer.
8. Optional: Stain proteins on membrane with Ponceau-S for 10 minutes at room temperature with shaking. Use a sufficient volume of stain to cover the membrane. Remove membrane from stain and rinse with reagent quality water to remove excess stain. Protein bands will appear as background diminishes. Do not continue to rinse or specific protein staining will diminish. Alternatively, Amido black may be used to stain proteins. Amido black is considered a permanent stain. When using Amido black, destaining with a methanol/acetic acid solution is required for removal of excess stain.
9. Optional: Cut blot to remove any desired lanes for future reference. Stained protein standard lanes, as well as a lane of each stained unknown sample, should be cut from membrane at this point and allowed to air dry. These lanes provide evidence of protein content to compare to immunodetection.
10. Proceed to Detection on p 8.

WESTERN BLOT DETECTION FLOW CHART

Total time: 4 hours

Immobilize Protein on Membrane

⇓

Block Membrane

1 hour

⇓

Incubate Primary Antibody

1 hour

⇓

Wash Membrane

3 x 5 minutes

⇓

Incubate Conjugate

1 hour

⇓

Wash Membrane

3 x 5 minutes

⇓

Incubate TMB Substrate

5-15 minutes

⇓

Stop Reaction

1-2 minutes

DETECTION

Note: Before beginning the assay, mark the orientation of the protein samples on the transfer membrane. The membrane may be cut into strips at this time if desired, although it may be more convenient to cut strips after the entire membrane is blocked.

1. Block the membrane by immersing in 1X Detector Block (refer to Solution Preparation, p. 5) using a minimum of 0.18 mL/cm^2 of membrane. Block for 1 hour, with gentle rocking or shaking, at room temperature or at 2-8°C, stationary, overnight.
2. Incubate membrane with primary antibody or serum sample. This antibody should be diluted in the 1X Detector Block that was used for blocking (step 1). It may be desirable to perform serial dilutions through a dot blot to determine the optimal working dilution. Incubation of the primary antibody for one hour at room temperature is usually sufficient.
3. Wash the membrane in 1X Wash Solution (refer to Solution Preparation, p. 5) using a minimum, of 0.27 mL/cm^2 of membrane for each wash. Wash membrane 3 x 5 minutes each.
4. Dilute appropriate conjugate 1:1000 in freshly prepared 1X Detector Block (i.e. 1 µl conjugate + 999 µl 1X Detector Block) using a minimum of 0.18 mL/cm^2 of membrane. Incubate blot with diluted conjugate for one hour at room temperature. The optimal dilution may vary for different assay systems, and it may be desirable to test serial dilutions to determine the optimal working dilution.
5. Wash 3 x 5 minutes as described in step 3.
6. Apply TMB substrate, approximately 0.05 mL/cm^2 of membrane. Allow the substrate to react for 5-15 minutes at room temperature.
7. After suitable color intensity is observed, stop the reaction by immersing the membrane in reagent quality water for 1-2 minutes.
8. Allow the membrane to air dry. Store sealed under plastic in the dark to reduce the amount of fading. For a permanent record, immediately take a picture.

TROUBLESHOOTING GUIDE

If no color develops:

1. Verify enzyme activity by mixing 10 µl of diluted conjugate with 1 mL of substrate.
2. Check that the specificity of the conjugate is correct for the primary antibody.
3. Use a protein stain on unblocked membrane to verify attachment of target protein.
4. Check that correct orientation of the membrane was maintained during the assay.
5. Be sure that no buffers containing sodium azide were used; azide will inhibit horseradish peroxidase activity.
6. Be sure all steps of the procedure were followed correctly.

If color development is weak:

1. Optimize antibody concentrations. Affinity of the primary antibody may change after proteins are denatured through SDS-PAGE.
2. Increase incubation times for the substrate or conjugate.
3. Increase the amount of protein loaded onto the gel.
4. Washing in excess of recommended procedures may reduce color intensity. Be sure the procedure was followed correctly.

If too much color or background develops:

1. Optimize antibody concentrations. Primary or secondary antibodies may need to be diluted further.
2. Decrease the substrate or conjugate incubation period.
3. Insufficient blocking or washing may cause non-specific staining. Increase blocking time and extend washing time or increase number of washes.
4. Decrease the amount of protein loaded onto the gel.
5. There may be endogenous peroxidase in the sample. Test by adding TMB directly to the blocked membrane. If color develops, blocking reagents such as 3% H_2O_2 in 100% MeOH may be required to remove the endogenous activity.

If bands or dots are poorly defined or "fuzzy":

1. Transfer may not have been performed correctly. Check with the manufacturer of the apparatus used to blot.
2. Certain membranes require special handling. Check with the membrane vendor for correct procedures.

For further assistance, contact KPL Technical Services at 800-638-3167 (USA) or 301-948-7755 or visit our website at www.kpl.com.

RELATED PRODUCTS

Product	Size	Catalog #
Protein Detector Western Blot Kits:		
BCIP/NBT System	2500 cm^2	55-11-50
LumiGLO® System	2500 cm^2	54-12-50
Protein Detector ELISA Kits:		
AP Anti-Human, pNPP System	25 plates	55-80-10
AP Anti-Rabbit, pNPP System	25 plates	55-80-15
AP Anti-Mouse, pNPP System	25 plates	55-80-18
HRP Anti-Human, ABTS System	20-40 plates	54-62-10
HRP Anti-Rabbit, ABTS System	20-40 plates	54-62-15
HRP Anti-Mouse, ABTS System	20-40 plates	54-62-18

REORDERING INFORMATION

Product	Size	Catalog #
Detector Block (5X)	240 mL	71-83-00
Wash Solution Concentrate	800 mL	50-63-00
HRP Anti-Human IgG (H+L)	0.1 mg/mL	374-1006
HRP Anti-Rabbit IgG (H+L)	0.1 mg/mL	374-1506
HRP Anti-Mouse IgG (H+L)	0.1 mg/mL	374-1806
1 Component TMB Membrane Substrate	100 mL	50-77-18
1 Component TMB Membrane Substrate	200 mL	50-77-03

Note: The recommendations of this bulletin are provided solely for the benefit of users who need practical guidance on immunoassay procedures. Because experimental conditions for the use of the suggested products are beyond the control of Kirkegaard & Perry Laboratories, it is impossible for Kirkegaard & Perry Laboratories to implicitly guarantee the performance of the mentioned products for any and all assay procedures. Users who need additional information are encouraged to call Technical Services at 800/638-3167 or 301/948-7755 for assistance.

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