TABLE OF CONTENTS

5410-0013 (55-11-50)

Section	Page
Introduction	4
Materials and Equipment	4
Guidelines for Kit Use	5

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 alkaline phosphatase (AP), derived from calf intestine, through a modified glutaraldehyde procedure (3). The alkaline phosphatase substrate used is 5-bromo-4-chloro-3-indolyl-phosphate / nitroblue tetrazolium (BCIP/NBT). When the dephosphorylation of BCIP is catalyzed by alkaline phosphatase, an insoluble purple 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 KPL PROTEIN DETECTOR WESTERN BLOT KIT

The KPL Protein Detector Western Blot Kit is designed for the detection and visualization of proteins immobilized on membranes through either electrophoresis or dot blotting. 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 alkaline phosphatase and stabilized in liquid form for quick dilution. The KPL BCIP/NBT substrate is provided as a convenient ready-to-use solution which forms an insoluble purple precipitate when combined with the AP labeled antibody. Color development occurs rapidly with high resolution of positive reaction sites. Results are

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 or PVDF membrane
- 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
- Electrophoresis equipment

REMINDERS

- 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. KPL Blocking Solution/Conjugate Diluent: To prepare 1X KPL Detector Block dilute the 5X KPL Detector Block Solution 1/5 in reagent quality water (i.e. 1 mL Detector Block Solution + 4 mL reagent quality water). Prepare a minimum of 0.36 mL/cm^2 of membrane for both the block and diluent steps.

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

C. Conjugate: A suggested starting dilution of the liquid conjugate is 1/500 in 1X KPL 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

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 will 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 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 instructions. Transfer from a 1 mm thick mini-gel 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 page 7.

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 KPL BCIP/NBT
Substrate 5 - 15 minutes

Stop
Reaction 1 -
2 minute(s)

DETECTION

1. Block the membrane by immersing in 1X KPL Detector Block (refer to Solution Preparation, page 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 KPL 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 KPL Wash Solution (refer to Solution Preparation, page 5) using a minimum of 0.27 mL/cm^2 of membrane for each wash. Wash membrane 3 x 5 minutes each.

4. Dilute conjugate 1/500 in freshly prepared 1X KPL Detector Block (i.e. 1 µL conjugate + 499 µL 1X KPL 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. Do not wash for extended periods of time, 3 x 5 minutes is sufficient.
6. Apply KPL BCIP/NBT substrate, approximately 0.05 $mL/10\ cm^2$ 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 prevent the color from fading.

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 phosphate were used; inorganic phosphate will inhibit alkaline phosphatase 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 may cause non-specific staining. Increase blocking time to reduce background.
4. Decrease the amount of protein loaded onto the gel.
5. There may be endogenous phosphatase in the sample. Test by adding KPL BCIP/NBT directly to the blocked membrane. If color develops, blocking reagents such as levamisole 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.

RELATED PRODUCTS

Product	Size	Cat. No.
I N N B U	**I 8**	
TMB System	2500 cm^2	5410-0008 (54-11-50)
LumiGLO System®	2500 cm^2	5410-0009 (54-12-50)

KPL Detector is a trademark of SeraCare, Inc.

L0 1 36 7(0)re.cze

9. Reinhart, M.P. and Malamud, D. (1982). Protein Transfer From Isoelectric Focusing Gels:The Native Blot. *Anal. Biochem.* 123: 229 -235.
10. Gooderham, K (1983). *Protein Blotting. In* J. Walker and W. Gaastra (eds.), Techniques in Molecular Biology. Croom Helm Ltd. Publishers, London.
11. Southern, E.M. (1975). Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis. *J. Mol. Biol.* 98: 503 - 517.

The product listed herein is for research use only and is not intended for use in human or clinical diagnosis.

SeraCare Life Sciences

508.244.6400 800.676.1881 Toll Free 508.634.3334 Fax
www.seracare.com