For chromogenic detection of membrane-bound proteins

KPL Protein Detector™

Western Blot Kit
TMB System

5410-0008 (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 KPL PROTEIN DETECTOR™ WESTERN BLOT KIT

KPL Protein Detector Western Blot Kits are designed for the detection and visualization of proteins immobilized on membranes through either electrophoresis or dot blotting. 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] Using the KPL Protein Detector TMB Western Blot Kit, 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.

Where appropriate, the enzyme labeled secondary antibodies 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.

MATERIALS AND EQUIPMENT

Kit Components	Product Code	Volume
KPL HRP-Labeled Secondary Antibody:		
• Goat Anti-Rabbit IgG (H+L)	5450-0005 (374-1506)	750 µL
• Goat Anti-Mouse IgG (H+L)	5450-0006 (374-1806)	750 µL
KPL 5X Detector Block	5440-0003 (71-83-01)	2 x 120 mL
KPL 20 X Wash Solution	5150-0010 (50-63-03)	3 x 100 mL
KPL Detector Block Powder	5920-0006 (72-01-03)	10 g
KPL TMB Membrane HRP Substrate	5120-0055 (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.

NOTE ON…Warnings and Precautions

⇒ Read ALL instructions thoroughly before using the kit.

⇒ Always wear protective gloves and a lab coat for personal protection, as well as for protection of the membrane and immunoassay reagents from.contaminants such as 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.

⇒ 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.

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
- 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

1. KPL Blocking Solution/Conjugate Diluent:
   A. Based on the total desired 1X KPL Detector Block volume, weigh out 1% w/v KPL Detector Block Powder (1 g Detector Block Powder per 100 mL of diluted KPL Detector Block Solution).
   B. Place the KPL Detector Block Powder in a flat-bottom, screw cap container and add molecular biology grade water to a volume equivalent to 4/5 of the total desired 1X KPL Detector Block volume. Shake the container vigorously until the powder is fully solubilized. (80 mL of H_20 per 100 mL of 1X KPL Detector Block Solution).
   C. Once the powder is in solution, dilute the solution with 1/5 v/v 5X KPL Detector Block Solution.

   Example for 50 mL of 1X KPL Detector Block:
   KPL Detector Block Powder - 0.5 g
   Reagent Quality H_20 - 40 mL
   5X KPL Detector Block Solution - 10 mL

2. 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).

3. 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.

4. Substrate: Ready to use, requires no dilution.

NOTE ON…Preparation of KPL Detector Block Solution

⇒ If the block solution is not prepared daily, sensitivity could be reduced and background will increase.

⇒ Conical tubes are not recommended in the preparation of 1X KPL Detector Block as the KPL Detector Block Powder may become packed in the bottom, making solubilization more difficult. If used, the solution may be vortexed to remove any packed KPL Detector Block Powder from the bottom of the tube.

⇒ Insure that all Detector Block Powder is in solution to avoid speckling patterns on the. blot or insufficient blocking. The amount of powder used can be increased to decrease background. However, excessive KPL Detector Block Powder may reduce sensitivity.

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 page 12.

a. Prepare samples by diluting to desired concentration with sample diluent. Incubate samples at 100°C for 3 minutes prior to electrophoresis.

b. 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.

c. 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.

d. After electrophoresis, cut off the bottom right corner of the gel. This will ensure that the gel is oriented correctly in the transfer apparatus.

e. 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.

f. Run transfer according to manufacturer's 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.

g. 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.

h. 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.

i. 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.

j. Proceed to Detection on page 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 or overnight

↓

Wash Membrane
3 x 5 minutes

↓

Incubate KPL TMB Membrane Substrate
5 - 15 minutes

↓

Stop Reaction
1 - 2 minute(s)

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 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 for at least one hour. 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 1/1,000 in freshly prepared 1X KPL Detector Block (i.e. 1 µL conjugate + 999 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 or overnight at 4°C. 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 TMB Membrane 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.

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.
KPL Protein Detector™ Western Blot Kits:		
BCIP/NBT System	2500 cm^2	5410-0013 (55-11-50)
LumiGLO System®	2500 cm^2	5410-0009 (54-12-50)

KPL Detector is a trademark of SeraCare, Inc.

LumiGLO is a registered trademark of KPL and is protected by the following patents:

US	459804
Australia	575552
Canada	121711
New Zealand	207095
South Africa	84/099
Finland	76380
Japan	164942

Belgium, Sweden, Germany, France, Netherlands, UK, Switzerland, Italy EPO116454

Coomassie is a registered trademark of ICI PLC

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

Notes:

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