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Catalog No. 54-12-50

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 highly sensitive chemiluminescent substrate provides an excellent method for detection and characterization of samples bound to membranes through Western or dot blotting.[1] Following attachment of protein to a membrane, a primary antibody is used to selectively bind the protein of interest.[2] Alternatively, a known protein is bound to the membrane for screening of specific monoclonal antibodies or serum samples.[3] 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.[4] The chemiluminescent substrate used is a luminol-based solution. In the presence of hydrogen peroxide, HRP converts luminol to an excited intermediate dianion. This dianion emits light on return to its ground state.[5] The resulting signal can be measured using a camera luminometer or X-ray film.

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 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] The combination of a highly specific, stable liquid conjugate and a sensitive, chemiluminescent substrate allows rapid and accurate identification of samples without the hazards or disposal concerns associated with the use of radioactivity. All solutions required for blocking and washing the membrane and for diluting antibodies are provided.

Kits include affinity purified antibody specific for mouse and rabbit immunoglobulins, conjugated to horseradish peroxidase and stabilized in liquid form for quick dilution. The chemiluminescent substrate is provided as a stable two component solution, which emits light when combined with the HRP labeled antibody. Positive reaction sites are rapidly detected with high resolution and low background. Results are recorded on X-ray film to provide a permanent record.

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

	Catalog No. 54-12-50 2500 cm^2	
Kit Components	**Product Code**	**Volume**
Detector Block Solution (5X)	71-83-01	2 x 120 mL
Detector Block Powder	72-01-03	10 g
Peroxidase-labeled Secondary Antibody:		
Goat Anti-Rabbit IgG (H+L)	374-1506	750 uL
Goat Anti-Mouse IgG (H+L)	374-1806	750 uL
Wash Solution Concentrate (20X)	50-63-03	3 x 100 mL
LumiGLO Peroxidase Chemiluminescent Substrate Solution A	50-59-00	120 mL
LumiGLO Peroxidase Chemiluminescent Substrate Solution B	50-60-00	120 mL

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

Required Supplies and Equipment Not Included

- 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
- X-ray film (double emulsion such as Kodak BioMax Light)
- Developing chemicals and equipment

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 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.

PROTEIN DETECTOR WESTERN BLOTTING
AT A GLANCE

Total time: 4 hours

Polyacrylamide Gel Electrophoresis

⇓

Immobilize Protein on Membrane

⇓

Block Membrane

1 hour or overnight

⇓

Incubate Primary Antibody

1 hour

⇓

Wash Membrane

3 x 5 minutes

1 x 10 minutes

⇓

Incubate Conjugate

1 hour

⇓

Wash Membrane

3 x 5 minutes

1 x 10 minutes

⇓

Incubate LumiGLO Substrate

1 minute

⇓

Expose to Film

1 - 30 minute(s)

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 protocols provided by the equipment manufacturer or consult the references on page 15.

STEPS	CRITICAL POINTS
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.	*Conditions for electrophoresis will vary depending on the type of gel used. 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 to aid in the correct orientation of the gel 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.	*Higher percentage gels and larger proteins will take longer. Optimal transfer time should be determined experimentally.*

(PAGE AND WESTERN BLOTTING CONTINUED FROM PAGE 6)

STEPS	CRITICAL POINTS
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 8.	

NOTE ON…Preparing Membrane Prior To Detection

⇒ 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. Alternatively, it may be more convenient to cut strips after the entire membrane has been blocked.

DETECTION OF WESTERN BLOTS

STEPS	CRITICAL POINTS
1. Block the membrane by immersing in 1X Detector Block using a minimum of 0.18 mL/cm^2 of membrane. Block at room temperature for 1 hour with gentle rocking or shaking, or stationary at 2 - 8°C overnight.	*See Solution Preparation on page 14 for instructions on preparing 1X Detector Block.*
2. Incubate membrane with primary antibody or serum sample for at least 1 hour. This antibody should be diluted in the 1X Detector Block that was used for blocking (Step 1).	*It may be desirable to first 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 using 0.27 mL/cm^2 of membrane for each wash. Wash membrane 3 times for 5 minutes each, followed by one 10 minute wash.	*See Solution Preparation on page 15 for instructions on preparing 1X Wash Solution.*
4. Dilute appropriate conjugate 1/10,000 – 1/1,000 in freshly prepared 1X Conjugate Diluent using a minimum of 0.18 mL/cm^2 of membrane.	*Example: 1 uL of conjugate + 999 uL (or 9999 uL) of 1X Conjugate Diluent. The optimal dilution may vary for different assay systems and it may be desirable to titrate the conjugate to determine the optimal working dilution.* ***For the best signal-to-noise, Detector Block Powder should not be used in the conjugate diluent. See Solution Preparation on page 14 for instruction on preparing 1X Conjugate Diluent.***
5. Incubate blot with diluted conjugate for one hour at room temperature.	
6. Wash as described in step 3.	

(DETECTION OF WESTERN BLOTS CONTINUED FROM PAGE 8)

STEPS	CRITICAL POINTS
7. Incubate membrane in LumiGLO Chemiluminescent Substrate, 0.05 mL/cm^2 membrane, for 1 minute at room temperature.	*It is not necessary to protect the reaction from light.*
8. Remove the membrane from substrate and touch the corner to a piece of filter paper to remove excess liquid. Seal the membrane in clear plastic and expose to X-ray film for 1 - 30 minutes. An initial 10 minute film exposure is recommended.	*Be sure that the surface of the membrane to which the assay reagents were applied is facing the film. Do not allow the film to get wet, and be careful not to move the film during exposure.* *Optimal exposure time should then be determined by the signal to noise ratio.*
9. Develop film according to manufacturer's instructions.	

STRIPPING AND REPROBING MEMBRANES

This protocol is adapted from Kaufman, *et. al.*.[12] After performing protein transfer, detection with LumiGLO Chemiluminescent Substrate and film exposure, membranes may be stripped and reprobed with new primary and secondary antibodies.

1. Strip antibodies by incubating blot for 30 - 90 minutes at 70°C in erasure buffer: 2% SDS (w/v), 62.5 mM Tris-HCl (pH 6.8 at 20°C), 100 mM β-mercaptoethanol.
2. Wash 2 times, for 10 minutes each, in TBS: 10 mM Tris-HCl (pH 7.4 at 20°C), 150 mM NaCl.
3. Block for 2.5 hours in 1X Detector Block.
4. Incubate with new primary and secondary antibodies.
5. Wash three times for 5 minutes each, followed by one 10 minute wash, with 1X Wash Solution after each antibody incubation.
6. Incubate with LumiGLO Chemiluminescent Substrate.
7. Expose to X-ray film and develop.

LUMIGLO CHEMILUMINESCENT SUBSTRATE USER'S GUIDE

⇒ LumiGLO can be used with nitrocellulose, nylon and PVDF membranes.

⇒ The LumiGLO reaction does not need to be protected from light.

⇒ For maximum signal, expose membrane to film immediately after incubation with LumiGLO. The reaction and film exposure are performed at room temperature. For most applications, exposures of one hour or less produce sufficient sensitivity.

⇒ LumiGLO is an extremely sensitive substrate. Insufficient washing of membranes or contamination of substrate with HRP will result in non-specific background. Following incubation with HRP-labeled antibodies, it is important to transfer membranes to a clean container and wash thoroughly to remove excess enzyme and prevent background problems. Always use a clean container for the substrate incubation.

⇒ Do not allow LumiGLO to contact film. LumiGLO solution will case dark spots to appear on the film.

⇒ Following detection with LumiGLO, membranes may be developed with chromogenic TMB Membrane Substrate (50-77-03). After film exposure, wash membrane in diluted wash solution for 2 minutes. Immerse membrane in TMB Membrane Substrate until desired endpoint is seen (2 - 10 minutes). Rinse membrane in molecular biology grade water for one minute to stop the reaction, then air dry.

LumiGLO Light Emission Over Time

Light emission begins immediately upon incubation with LumiGLO and declines gradually over a period of 1 - 2 hours. Diminished enzyme activity after one hour is due to exposure of the enzyme to the products of the substrate reaction.

TROUBLESHOOTING GUIDE FOR CHEMILUMINESCENT DETECTION OF WESTERN BLOTS

Problem 1: No Signal

Possible Cause	Corrective Measure
• Inactive horseradish peroxidase	Verify enzyme activity by mixing 10 uL of diluted conjugate with 1 mL of substrate (in a dark room, the substrate should glow).
• No binding of primary antibody to target protein	Check that the specificity of the conjugate is correct for the primary antibody.
• No transfer of target to membrane	Use a protein stain on unblocked membrane to verify attachment of target protein.
• Detection of non-blotted side of membrane	Check that correct orientation of the membrane was maintained during the assay and during film exposure.
• Inhibition of horseradish peroxidase	Be sure that no buffers containing sodium azide were used; azide will inhibit horseradish peroxidase activity.
• Missed step in procedure	Be sure all steps of the procedure were followed correctly.

Problem 2: Weak Signal

Possible Cause	Corrective Measure
• Insufficient amount of antibody	Optimize antibody concentrations. Affinity of the primary antibody may change after proteins are denatured through SDS-PAGE.
• Amount of protein loaded/blotted is too low	Increase the amount of protein loaded onto the gel.
• Insufficient incubation of antibodies to targets	Increase the incubation times for primary antibody or conjugate.
• Insufficient exposure time	Increase the time of exposure to film.
• Excessive washing beyond recommended procedure	Be sure the procedure was followed as written.
• Reagents impacted by improper temperatures.	Be sure all reagents were at room temperature.

Problem 3: Excessive Signal or Background

Possible Cause	Corrective Measure
• Overexposure of film to signal	Expose the membrane to film for a shorter period of time.
• Excessive antibody used for detection	Reduce antibody concentrations; optimal conjugate dilution should be 1/1000 – 1/10,000. OR Decrease the primary antibody or conjugate incubation period.
• Excessive protein loaded on the gel	Decrease the amount of protein loaded onto the gel.
• Insufficient blocking or washing, causing non-specific reaction	Increase blocking time and extend washing time or increase number of washes.
• Detector Block Powder in Conjugate Diluent	Prepare fresh Conjugate Diluent without Detector Block Powder; use this component in the preparation of 1X Detector Block Solution for membrane blocking only.
• Endogenous peroxidase in the sample	Test by incubating the blocked membrane in LumiGLO (without antibodies). After film exposure, if signal is obtained, blocking reagents such as 3% H_2O_2 in 100% MeOH may be required to remove the endogenous activity.

Problem 4: Poorly Defined or "Fuzzy" Bands or Dots

Possible Cause	Corrective Measure
• Poor transfer of protein to membrane	Follow manufacturer's recommended procedure or contact the manufacturer for additional support regarding the blotting apparatus.
• Excessive substrate	Remove excess substrate before exposure of the membrane to film.
• Ghost images from shifted position of film during development	Avoid movement of film over membrane during exposure period.
• Inadequate handling of membranes	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.

BUFFER PREPARATION

Sufficient reagents are provided in the Protein Detector™ LumiGLO® Western Blot Kit when volumes are used as indicated. If desired, increased working volumes may be used; however, additional reagents will be necessary. Instructions for preparation of working solutions are provided below.

1X Blocking Solution – to be prepared fresh daily

STEPS	CRITICAL POINTS
1. Based on the total desired 1X Detector Block volume, weigh out 1% w/v Detector Block Powder (1 g Detector Block Powder per 100 mL of diluted Detector Block Solution).	*If the block solution is not prepared daily, sensitivity could be reduced and background will increase.*
2. Place the 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 Detector Block volume. Shake the container vigorously until the powder is fully solubilized. (80 mL of H_20 per 100 mL of 1X Block Solution).	*Conical tubes are not recommended in the preparation of 1X Detector Block as the Detector Block Powder may become packed in the bottom, making solubilization more difficult. If used, the solution may be vortexed to remove any packed Detector Block Powder from the bottom of the tube.*
3. Once the powder is in solution, dilute the solution with 1/5 v/v 5X Detector Block Solution. Example for 50 mL of 1X Detector Block: Detector Block Powder 0.5 g Reagent Quality H_20 40 mL 5X Detector Block Solution 10 mL	*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 Detector Block Powder may reduce sensitivity.*

1X Conjugate Diluent

STEPS	CRITICAL POINTS
1. Dilute 5X Detector Block Solution 1/5 in reagent quality water (*i.e.*, 10 mL 5X Detector Block Solution + 40 mL H_20).	*5X Detector Block Solution Concentrate as a 1X Solution is also suitable as a conjugate diluent when prepared without Detector Block Powder.* *For optimal performance, the diluent solution should also be prepared fresh on the day of use.*

1X Wash Solution	LumiGLO Working Solution
STEPS	**STEPS**
1. Dilute 20X Wash Solution Concentrate 1/20 with reagent quality water (*i.e.*, 5 mL Wash Solution Concentration + 95 mL H_2O).	1. Mix equal parts of LumiGLO Solution A and LumiGLO Solution B (*i.e.*, 5 mL Solution A + 5 mL Solution B).

REFERENCES

1. Kricka, L. J. (1991). Chemiluminescent and Bioluminescent Techniques. *Clin. Chem*. 37(9): 1472-1481.
2. Knect, D.A., and R.L. Dimond (1984). Visualization of Antigenic Proteins on Western Blots. *Anal. Biochem.* 136: 180-184.
3. Blake, M.S., et al (1984). A Rapid, Sensitive Method For Detection of Alkaline Phosphatase Conjugated Antibody on Western Blots. *Anal. Biochem.* 136: 175-178.
4. Nakane, P.K., and A. Kawaoi (1974). Peroxidase Labeled Antibody. A New Method of Conjugation. *J. Histochem. Cytochem*. 22 (12): 1084-1091.
5. Isacsson, V. and G. Wettermark (1974). Chemiluminescence in Analytical Chemistry. *Anal. Chim. Acta*. 68: 339-362.
6. Towbin, H., T. Staehelin, and J. Gordon (1979). Electrophoretic Transfer of Proteins From Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications. *Proc. Natl. Acad. Sci. USA.* 76: 4350-4354.
7. Bittner, M., P. Kupferer, and C. F. Morris (1980). Electrophoretic Transfer of Proteins and Nucleic Acids From Slab Gels to Diazobenzyloxymethyl Cellulose or Nitrocellulose Sheets. *Anal. Biochem.* 102: 459-471.
8. Burnette, W.N. (1980). "Western Blotting": Electrophoretic Transfer of Proteins From Sodium Dodecyl Sulfate-Polyacrylamide Gels to Unmodified Nitrocellulose or Nitrocellulose Sheets. *Anal. Biochem.* 112: 195-203.
9. Reinhart, M.P. and D. Malamud (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.
12. Kaufmann, Ewing, and Shaper (1987). The Erasable Western Blot. *Anal. Biochem*. 161, 89-95.

RELATED PRODUCTS

Product	Size	Catalog No.
Protein Detector Western Blot Kits:		
TMB System	2500 cm^2	54-11-50
BCIP/NBT System	2500 cm^2	55-11-50
Protein Detector ELISA Kits:		
AP Anti-Human, BluePhos™ System	20 plates	55-81-10
AP Anti-Rabbit and Anti-Mouse, BluePhos System	20 plates	55-81-50
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
Detector Block (5X)	240 mL	71-83-00
Wash Solution Concentrate	4 x 200 mL	50-63-00
HRP Anti-Human IgG (H+L)	1.0 mL	474-1006
HRP Anti-Rabbit IgG (H+L)	1.0 mL	474-1506
HRP Anti-Mouse IgG (H+L)	1.0 mL	474-1806
LumiGLO Chemiluminescent Substrate	240 mL	54-61-00
LumiGLO Chemiluminescent Substrate	720 mL	54-61-01

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 KPL, Inc., it is impossible for KPL 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.

L-217-09