PerkinElmer Life Sciences, Inc.

Western Lightning™ Chemiluminescence Reagent *Plus*

NEL103: Reagents for 1000 cm^2 membrane
NEL104: Reagents for 2500 cm^2 membrane
NEL105: Reagents for 5000 cm^2 membrane

For Laboratory Use

CAUTION: A research chemical for research purposes only

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I. INTENDED USE

The Western Lightning™ Chemiluminescence Reagent *Plus* is a non-radioactive light-emitting system designed to detect proteins immobilized on a membrane. The method provides a sensitivity of 1 - 10 pg of protein and yields fast, permanent, hardcopy results on Kodak X-Omat Blue Autoradiography Film or on the KODAK Image Station. Membranes can be stripped and reprobed if stored wet between uses.

II. PRINCIPLE OF THE PROCEDURE

The Western Lightning™ Chemiluminescence Reagent *Plus* is based on an enhanced version of a chemiluminescence reaction in which the enzyme horseradish peroxidase (HRP) catalyzes light emission from the oxidation of luminol. Use of an enhancer increases the emission approximately 1000-fold (1).

In Western blotting, complex mixtures of proteins are separated by electrophoresis and transferred to a membrane (such as PolyScreen® PVDF Transfer Membrane, Catalog Nos. NEF1000, 1002) for immunological detection. Antibodies labeled with horseradish peroxidase (HRP) are reacted directly or indirectly with the immobilized protein antigen. Following addition of the Western Lightning™ Chemiluminescence Reagent *Plus* to the membrane, oxidative degradation of luminol occurs, resulting in light emission at a wavelength of 428 nm. This light is captured on Kodak X-OMAT Blue Autoradiography Film (Catalog Nos. NEF595, 596) or the KODAK Image Station.

III. REAGENTS

Brown Bottle: Enhanced Luminol Reagent.

White Bottle: Oxidizing Reagent.

NEL103 contains 65 ml of each reagent
NEL104 contains 170 ml of each reagent
NEL105 contains 2 x 170 ml of each reagent.

IV. STORAGE RECOMMENDATIONS

Upon arrival both reagents should be stored at 2° - 8°C.

V. PRECAUTIONS

A. Safety Considerations

Wear disposable gloves and safety glasses while working with reagents, and thoroughly wash hands after handling. Do not eat, smoke or drink in areas in which reagents are handles. Refer to product labels and Material Safety Data Sheet (s) for additional information as appropriate.

B. Performance Considerations

1. **These reagents have been formulated and are quality-controlled specifically to detect proteins in Western blots. FOR LABORATORY USE.**

2. **The Western Lightning™ Chemiluminescence Reagent *Plus* has been formulated for use on PolyScreen® and nitrocellulose membranes.**

3. **Do not use kit components beyond the expiration date. This date is printed on the kit label.**

4. **Do not substitute reagents from other kits. Reagents have been optimized for performance with each kit lot. Dilution or other alteration of reagents may result in undesirable modifications of performance, such as loss of sensitivity.**

5. **Do not allow the membrane(s) to dry out after the primary antibody is added.**

6. **Proper blocking and washing of membranes is critical for optimum results. The recommended blocking and washing conditions should be tried first and adjusted as necessary for a particular application.**

7. **Prepare the Chemiluminescence Reagent immediately before use. Prepare only enough for the membranes being processed. Discard any excess.**

8. **Do not interchange bottle caps; this will lead to cross-contamination of reagents. Designate specific containers for specific reagents, and use clean pipettes or pipet tips for each reagent.**

9. **Developing a first film or KODAK Image Station after 30 seconds of exposure allows an estimation of the optimum exposure time to use. (Exposure time can vary from 30 seconds to 2 hours.)**

10. **Except for film exposure and development, all steps can be performed outside the darkroom. With the KODAK Image Station, all steps, including exposure, can be performed without a dark room. With the KODAK Image Station, all steps, including exposure, can be performed without a dark room.**

11. **A method of stripping the antibodies and reprobing the membrane has been reported (2). The stripping procedure can be found in Appendix C. Chemiluminescence is ideally suited for this application because unlike chromogenic formats you need not remove the substrate from the membrane.**

VI. PROCEDURES

A. Membrane Preparation

1. **Western blots are prepared following the researcher's current protocol (see Appendix A). Blocking agents appropriate for the solid phase must be employed. The researcher can use a number of methods of introducing the HRP reporter enzyme. For example, an anti-analyte-HRP conjugate, or a secondary reporter such as an anti-rabbit- or anti-mouse IgG-HRP conjugate can be used. The system will also work with biotinylated antibody/streptavidin-HRP or hapten/anti-hapten-HRP systems.**

2. **Careful rinsing and washing are required to reduce background. See the Reagent Formulation section for a suggested Wash Buffer. After the HRP reporter incubation step, the membranes should be rinsed twice in Wash Buffer followed by one 15-minute and four 5-minute washes with Wash Buffer. All steps are performed at room temperature.**

B. Chemiluminescence Reagent Preparation

1. **Prepare the Chemiluminescence Reagent by mixing equal volumes from Bottle 1 and Bottle 2 immediately before use. For optimum results, the Chemiluminescence Reagent should be used as soon as possible after mixing. Mix no more than is needed at one time.**

2. **Incubate the Chemiluminescence Reagent with the membrane for 1 minute at room temperature. Use at least 0.125 ml per cm^2 membrane and incubate with shaking.**

C. Protein Visualization

1. **It is important to keep the film dry. To drain excess Chemiluminescence Reagent, hold the blot vertically and touch it against tissue paper, or blot it between two sheets of Whatman® 3MM filter paper.**

2. **Place the membrane between the covers of a polypropylene sheet protector with the black interleaf removed (e.g., Boise Cascade Catalog Number L2-A8112). Gently smooth out any air pockets.**

3. **Place the membrane, protein side up, in the film cassette or protein side down on the Image Station plate.**

4. **Switch off the lights and carefully place the film on top of the membrane. If using the KODAK Image Station, just close the lid and you are ready to expose.**

5. **Expose the film for 30 seconds, then develop or take the appropriate exposure using the KODAK Image Station..**

6. **Repeat the film or Image Station exposure, varying the time as needed for optimal detection.**

VII. REAGENT PREPARATION

10X Phosphate Buffered Saline (10X PBS)

For 1 liter:

$NaH_2PO_4 \cdot H_2O$	**2.03 g**
Na_2HPO_4	**11.49 g**
NaCl	**85 g**

The pH of the 10X solution is 6.7 to 6.9. The pH of the 1X solution should be 7.3 to 7.5 (if not, adjust the 1X).
Storage: Room Temperature.

Alternately, Dulbecco's Phosphate Buffered Saline without calcium chloride or magnesium chloride (available from commercial sources), or Tris-based buffers may be used.

10X PBS-TWEEN® 20 (10X PBS-T)

For 1 liter:

10X PBS	**995 ml**
TWEEN ® 20	**5 ml**

A preservative such as thimerosal (1 g/L) may be added to prolong the life of the reagent. Do not use sodium azide because it inhibits HRP activity.
Storage: Room Temperature.

PBST

For 1 liter:

10X PBS-T	**100 ml**
dH_2O	**900 ml**
Storage:	**Room Temperature**

Membrane Blocking Reagent (5% Non-Fat Dry Milk)

For 100 ml:

Carnation™ Instant	
Non-Fat Dry Milk	**5 g**
PBST	**100 ml**

If additional blocking capability is desired, this reagent may be supplemented with normal serum of the same type as the antibody. Casein or BSA may be substituted for the non-fat dry milk. This reagent should be made up fresh for every use.

Antibody Diluent (1% BSA/PBST)

For 1 liter:

10X PBST	**100 ml**
H_2O	**800 ml**
BSA	**10 g**

Adjust the pH to 7.4, add H_2O to 1 liter, and filter through a 0.22 µm membrane.

Storage: 4°C

VIII. TROUBLESHOOTING GUIDE

Problem	Possible Cause	Remedy
No signal or weak signal	Poor transfer of proteins	Repeat transfer
	Membrane preparation inadequate	Check proper membrane hydration
	Primary or secondary antibody concentration too low	Titrate antibody conjugates for optimum concentrations
	Chemiluminescence reagent improperly prepared	Add HRP conjugate to reagent and look for visible light
Excess signal "Black Bands"	Antigen or antibody excess	Adjust concentrations by optimization experiments
High Background	Antigen or antibody excess	Adjust concentrations by optimization experiments
	Inadequate blocking	Increase blocking buffer concentration
	Overexposure to film	Shorter film exposure or let signal decay for 10-15 minutes and repeat exposure
White Bands "Antibands"	Blank bands on film caused by depletion of chemiluminescence substrate at sites of excess antigen and/or antibody	Reduce antigen loading and/or titer antibody concentrations.

IX. REFERENCES

1. **Thorpe, G.H.G., Kricka, L.J., Mosely, S.B. and Whitehead, T.P. Phenols as enhancers of the chemiluminescent horseradish peroxidase-luminol-hydrogen peroxide reaction: Application in luminescence-monitored enzyme immunoassays. Clin. Chem. 31:1335-1341 (1985).**

2. **Kaufmann, S.H., Ewing, C.M. and Shaper, J.H. The erasable Western blot. Analyt. Biochem. 161:89-95 (1987).**

3. **Towbin, H., Staehelin, T. and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. PNAS 76:4340-4354 (1979).**

X. ORDERING INFORMATION

PerkinElmer Life Sciences, Inc.Lightning™ Products for Chemiluminescence

Western Blot Chemiluminescence Reagent *Plus* Kits:

Catalog Number NEL103:	**Reagents for 1000 cm^2 membrane.**
Catalog Number NEL104:	**Reagents for 2500 cm^2 membrane.**
Catalog Number NEL105:	**Reagents for 5000 cm^2 membrane.**

PolyScreen® PVDF Membranes:

Catalog Number NEF1000:	**10 sheets 20 cm x 20 cm**
Catalog Number NEF1002:	**1 roll 30 cm x 3 m**

Kodak X-OMAT Blue Autoradiography Film:

Catalog Number NEF595:	**14" x 17"**
Catalog Number NEF596:	**8" x 10"**

KODAK Image Station

Model 440CF:	**1674373 (110 v)** **1811017 (220 v)** **1215508 (100 v, Japan model)**
Model 1000:	**1189026 (100 v, 110 v, 220 v)**

XI. APPENDIX A

SIMPLIFIED WESTERN BLOTTING PROTOCOL

1. **It is recommended that the transfer buffer [Towbin transfer buffer, pH 8.3 (3)] be made up ahead of time and pre-cooled to 4°C. In this way, it will have a chance to degas before use. Bubbles in the transfer buffer will increase the chance of trapping air between the membrane and the gel. Air bubbles create points of high resistance, resulting in "bald spots" (i.e., areas of low-efficiency transfer and band distortion).**

2. **Cut the membrane slightly larger than the gel. If using a PolyScreen® membrane, pre-wet with ethanol, then rinse with water. For nitrocellulose, just rinse with water. Be sure to wear gloves at all times when handling the membranes. Mark one side of the membrane for future reference.**

3. **Equilibrate both the membrane and the gel in transfer buffer for 15 - 20 minutes.**

4. **Wet two Scotch-Brite® pads and two pieces of filter paper (Whatman® 3MM cut to the size of the gel) in transfer buffer.**

5. **Prepare the "sandwich" as follows:**

- **Put one piece of wet filter paper on a Scotch-Brite® pad.**
- **Place the equilibrated gel on top of the filter paper.**
- **Place the membrane on top of the gel.**
- **Place the second piece of wet filter paper over the membrane.**
- **Be sure to remove any air bubbles trapped between the gel, membrane, and filter paper layers. This is easily done by rolling a clean pipet over the sandwich.**
- **Complete the sandwich with the second Scotch-Brite® pad.**

6. **Insert the sandwich into the transfer apparatus with the membrane positioned between the gel and the appropriate electrode. Most polypeptides are eluted from SDS-polyacrylamide gels as anions and therefore the membrane should usually be placed between the gel and the anode.**

7. **Fill the transfer apparatus with buffer. Pour the transfer buffer slowly to prevent bubble formation. Cool to 4°C and transfer at a constant current or voltage.**

8. **When the transfer is complete, remove the membranes and allow them to air dry at room temperature. Since dehydrated proteins bind more strongly to the membrane, this helps to prevent loss of target during subsequent washes.**

XII. APPENDIX B

SIMPLIFIED CHEMILUMINESCENCE PROTOCOL

This protocol has been used successfully in our laboratories. It may be adapted as necessary to suit individual needs.

Note: All incubations require rocking or shaking. All steps are performed at room temperature.

1. **Membrane Preparation**

   a. **Separate proteins by electrophoresis and transfer to PolyScreen® or nitrocellulose membrane. See Appendix A.**

   b. **Block non-specific binding sites by incubating the membrane in 5% non-fat dry milk in PBS-T for one hour.**

   c. **Wash the membrane twice for 5 minutes with PBS-T.**

   d. **Dilute the primary antibody in 1% BSA/PBS-T and incubate with the membrane for one hour.**

   e. **Wash the membrane with PBS-T once for 15 minutes, and then four times for 5 minutes each.**

   f. **Dilute the HRP-labeled second antibody in 1% BSA/PBS-T and incubate with the membrane for one hour.**

   g. **Wash the membrane with PBS-T once for 15 minutes and then four times for 5 minutes each.**

2. **Chemiluminescence Reagent Protocol**

   a. **Prepare the chemiluminescence reagent (0.125 ml of Chemiluminescence Reagent per cm^2 of membrane) by mixing equal volumes of the Enhanced Luminol Reagent and the Oxidizing Reagent.**

   b. **Incubate the membrane in the chemiluminescence reagent for one minute while shaking.**

3. **Protein Visualization**

   a. **Remove excess chemiluminescence reagent by draining or blotting and place the membrane in a plastic sheet protector.**

   b. **Expose to Kodak X-OMAT Blue Autoradiography Film or KODAK Image Station for 30 seconds. Develop the film and, if necessary, use the result to determine an optimum exposure.**

XIII. APPENDIX C

REPROBING WESTERN BLOTS ON POLYSCREEN® AND NITROCELLULOSE MEMBRANES

The Western Lightning™ Chemiluminescence Reagent *Plus* allows researchers the ability to effectively reprobe their Western Blots. Stripping of antibodies from membranes has been described for ^{125}I labeled proteins (1) and the extension of this technique to chemiluminescence substrates has been found to be feasible. Stripping the antibodies from the membrane allows one to reuse Western Blots thereby saving significant time and money.

The protocol to do the reprobing is as follows:

1. **The Western blot is carried out as usual and after the film exposures are complete the membrane is subjected to the stripping procedure. Best results are obtained if the membrane is not allowed to dry.**

2. **After the film exposure wash the membrane for 4 X 5 minutes in PBST.**

3. **Incubate the membrane for 30 minutes at 50°C in the stripping buffer:**

   62.5 mM Tris-HCl pH 6.8
   2% SDS
   100 mM 2-mercaptoethanol

4. **Wash the membrane for 6 X 5 minutes in PBST.**

5. **Incubate the membrane for 1 minute in the Western Lightning™ Chemiluminescence Reagent *Plus*. Expose to film for 1 minute to 1 hour to make sure that the original signal is removed.**

6. **Wash the membrane again for 4 X 5 minutes in PBST.**

7. **The membrane is now ready for reuse. Start at the blocking step in the Western Lightning™ Chemiluminescence Reagent Plus Manual (Appendix B- Step 1b).**

This protocol has been used on PolyScreen® and nitrocellulose membranes. Four successful reprobings have been carried out on both types of membranes.

XIV. APPENDIX D

POLYSCREEN® MEMBRANE WETTING PROTOCOL

PolyScreen® Membrane is extremely hydrophobic and will not wet in an aqueous solution unless membrane is pre-wet with alcohol.

1. **Wet the membrane in 95% ethanol for at least one minute. Soak the membrane until it changes from an opaque white to a uniform translucent gray.**

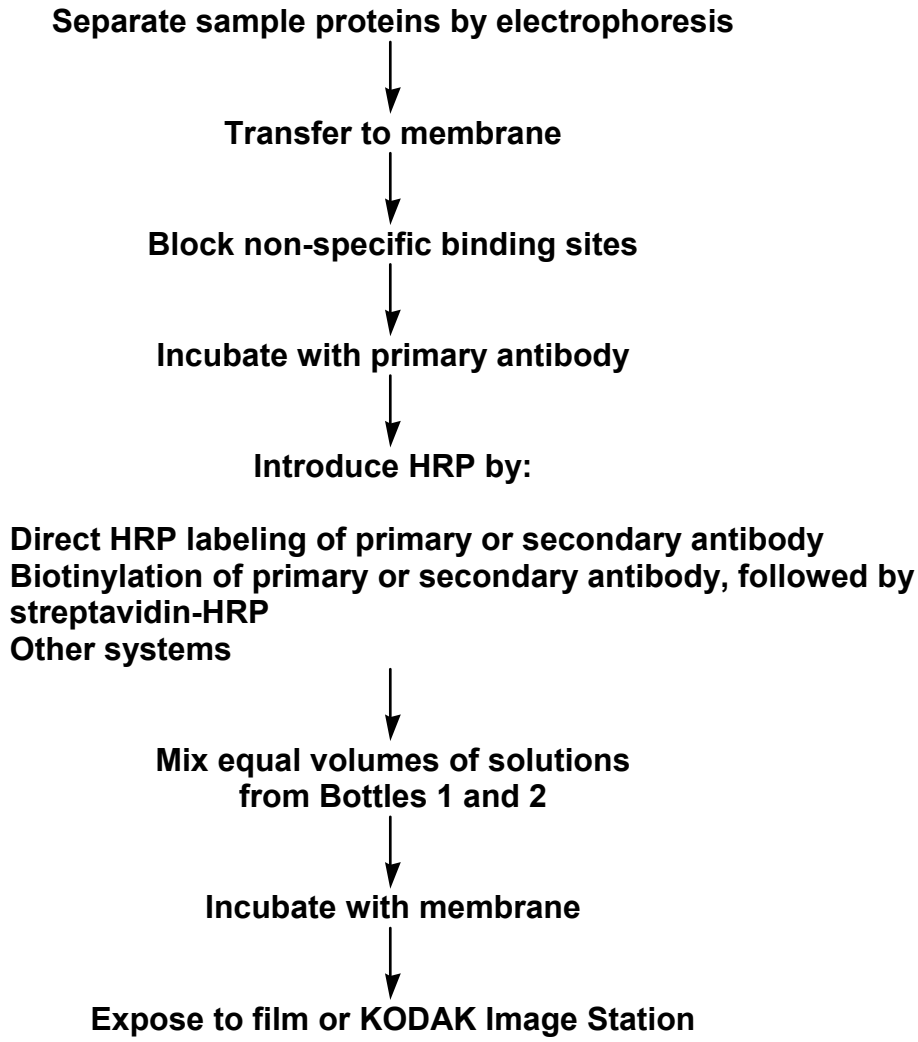
2. **Rinse the membrane in distilled water to wash off the alcohol for 2-3 minutes. If the membrane floats, gently push it into the water with plastic forceps until it wets.**

3. **Equilibrate the membrane in transfer buffer. Soak the membrane in the buffer for 10-15 minutes to displace the water and any bubbles which may form.**

   NOTE: If the membranes dries (even partially) at any time during the experiment, you must wet it with alcohol and rinse with distilled water before proceeding.

XV. APPENDIX E

FLOW CHART FOR WESTERN BLOT CHEMILUMINESCENCE DETECTION



XVI. APPENDIX F

TITRATION OF ANTIBODIES

The high sensitivity achieved with chemiluminescence detection sometimes requires the researcher to use less primary and/or secondary antibodies than that required for chromogenic detection. The excess use of antibodies in chemiluminescence detection can lead to short exposure times (seconds) and/or high background.

To achieve the maximum signal to noise ratio the primary and secondary antibodies should be optimized in a titration experiment. The following table gives an example of a typical titration experiment. The starting primary antibody dilution is 1:1000 and the starting secondary antibody dilution is 1:1000. The membrane samples are shown as #1 through #9.

	Primary Antibody Conc.		
Secondary Antibody Conc.	1:1000	1:2000	1:4000
1:1000	#1	#2	#3
1:2000	#4	#5	#6
1:4000	#7	#8	#9

The above titration allows the determination of the optimum concentration of the primary and secondary antibodies for chemiluminescence detection.

XVII. APPENDIX G

TOTAL PROTEIN STAINING

The detection of proteins transferred to PolyScreen® and nitrocellulose membranes by the total protein stains Ponceau S and Coomassie Brilliant Blue is compatible with subsequent chemiluminescence detection using the Western Lightning™ Chemiluminescence Reagent *Plus*. The Ponceau S stain has the advantage of requiring no destaining procedure as the stain is removed during the subsequent blocking procedure. The Coomassie Brilliant Blue stain is not removed from the membrane during the detection procedure but the color doesn't interfere with the chemiluminescence signal.

The following protocols have been used successfully in our laboratories:

<u>Ponceau S</u>

Prepare a 2% (2 mg/ml) stock solution of Ponceau S dye in 30% (wt/v) trichloroacetic acid. Dilute the stock ten-fold in 1% acetic acid (final concentration of Ponceau S is 0.2%).

Incubate the membrane in Ponceau S stain for 2 minutes. Rinse off the residual stain with distilled water and record the results. Proceed to the chemiluminescence detection procedure.

<u>Coomassie Brilliant Blue</u>

Prepare a 0.1% (1 mg/ml) working solution of Coomassie Brilliant Blue in 50% methanol and 10% acetic acid solution.

Incubate the membrane in Coomassie Brilliant Blue stain for 2 minutes. Pour off the excess stain and if desired destain the membrane in 50% methanol/10% acetic acid for 10 minutes. Rinse the membrane in 1X PBS and proceed to the chemiluminescence detection procedure.

If desired, the stain can be removed from the membrane by destaining in 50% methanol, 10% acetic acid.

XVIII. TRADEMARKS

Scotch-Brite® is a trademark of the 3M Company.
Whatman® is a trademark of Whatman Paper Ltd.
Tween® is a trademark of I.C.I. Ltd.
Carnation™ is a trademark of the Carnation Co.

XIX. CUSTOMER SUPPORT SERVICES

For further technical information or to place an order contact:

In the US: **PerkinElmer Life Sciences Technical Support Department at 800-551-2121.**

Outside the US: **Contact your local PerkinElmer Sales Office or distributor.**

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PC2647-1001