Lumigen® ECL Plus (PS-3)

Chemiluminescent Reagent

Product Application Instructions

Catalog Number **PS3-100**

Contents

Solution A – 100 mL
Solution B - 2.5 mL
For working solution, mix solutions A and B in the ratio of 40:1

Description

Lumigen ECL Plus is recommended primarily for Western blot detection of proteins bound with horseradish peroxidase (HRP) conjugated antibodies. However, it can be used for the detection of any HRP conjugated molecules, proteins or nucleic acids.

Note: Lumigen ECL Plus is invented, developed and manufactured by Lumigen. It is also marketed as Pierce® ECL Plus Western Blotting Substrate by Thermo Scientific.

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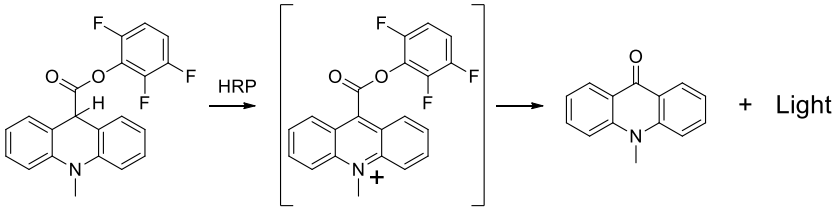
Product Overview

Lumigen ECL Plus utilizes a unique technology for the chemiluminescent and fluorescent detection of HRP conjugated molecules such as proteins and nucleic acids. The detection process, which provides superior sensitivity and ease of use, involves the enzymatic generation of acridinium esters. Reaction of the acridan substrate with an HRP label generates thousands of acridinium ester intermediates per minute which react with peroxide at alkaline pH to produce sustained high-intensity chemiluminescence. Additionally, the reaction end-product exhibits a long-lasting fluorescent signal which can be detected on a CCD imaging device.

Product Structure and Generation of Chemiluminescent Signal

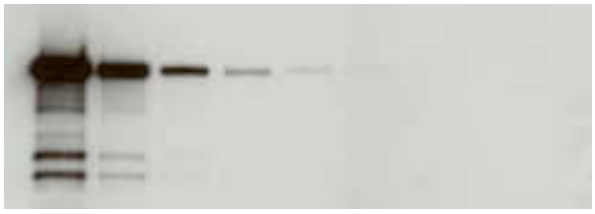


Product Characteristics and Applications

Lumigen ECL Plus produces both a chemiluminescent and fluorescent signal in the presence of HRP with attomole detection sensitivity. This dual detection capability also allows for classic chemiluminescent blot detection on an x-ray film or fluorescence detection in a digital imaging system.

For the detection of proteins on a Western blot, Lumigen ECL Plus has the following characteristics:

- Highly sensitive - about 5 pg per band of an 80 kDa protein can be detected.
- Sustained signal for over several hours - bands down to 5 pg are detectable with less than 30 second exposures of x-ray film.
- Dual signal functionality - Chemiluminescent and Chemifluorescent signals are generated simultaneously which can be captured respectively on an x-ray film and with a digital imager.



1ng 250pg 62pg 16pg 4pg 1pg

Western blot of ß-galactosidase protein detected with Lumigen ECL Plus

Product Handling Instructions

Storage:

Store bottled product at 2 - 8ºC.
Protect from light contamination.
Do not freeze.

Use in subdued light. Indirect incandescent lighting is preferred. Exposure to direct light will cause elevated background.

1) Allow solutions A and B to come to room temperature (Approximately 1 hour for 100 mL)
2) Gently invert (4-5 times) solutions A and B in their packaged containers to assure homogeneity prior to dispensing. **Avoid vigorous agitation of reagent.**
3) Dispense the amount needed of solutions A and B into separate containers. Containers should be opaque or covered with aluminum foil to protect from direct light (daylight or artificial).
4) For working solution, mix solutions A and B in the ratio of 40:1 in a new container.
5) In subdued light, add the working solution onto membrane with HRP bound protein or nucleic acids, and let stand for 2 minutes.
6) Place the membrane sandwiched between two clear plastic sheets. Wipe away excess solution with a tissue leaving a thin layer of solution on the membrane.
7) Capture chemiluminescent signal on an x-ray film or using an imager.

Repackaging:

Repackaging of Lumigen ECL Plus is discouraged as reliability can be compromised by contamination. Bottling is available from Lumigen to suit the volume demands for your specific work process.
If you choose to repackage, new opaque HDPE or PP plastic containers are required. The high temperatures in the container molding process destroy potential contaminants.
Reusing and washing of containers can lead to contamination and subsequent high background.

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Western Blotting - Equipment and Materials Required

- Vertical slab gel equipment.
- Acrylamide gels, freshly cast or precast.
- Power supply for electrophoresis.
- Nitrocellulose or PVDF membranes.
- Methanol (for PVDF membrane)
- Protein transfer equipment.
- Transfer buffer (Tris/Glycine/Methanol).
- Buffers such as Tris buffered saline (TBS) or phosphate buffered saline (PBS).
- Wash buffers (TBS or PBS) containing 0.05% to 0.1% Tween -20.
- Blocking reagent such as 5% non-fat dry milk in wash buffer or any other commercially available blocking reagents.
- Primary antibody (target specific), and HRP labeled secondary antibody.
- Platform shaker for antibody incubation and washing the membrane.
- X-ray film.
- X-ray film processing equipment with developing and fixing reagents.

Important Notes and Precautions

Any time a new chemiluminescent reagent is used for the detection of proteins on Western blots, it is essential that the concentrations of target specific primary antibody and the HRP labeled detection antibody are titrated first to determine the optimal dilution combination of these antibodies. The antibody stocks from commercial vendors vary in their binding specificity and protein concentration. As a general rule, 1mg/mL antibody stocks may be diluted and used in the range of 1 to 0.1 µg/mL (1:1000 to 1:10,000) for the primary antibody and 0.1 to 0.01 µg/mL (1:10,000 to 1:100,000) for the detection antibody. Other variables that influence the detection sensitivity of the chemiluminescent reagent are the type of membrane used for blotting, the efficiency of protein transfer on to the membrane, and the blocking agent used to minimize non-specific background. For consistent results, perform the antibody incubation and washing steps at ambient temperature (22-25ºC) and always make sure the membranes are completely covered with antibody and washing buffers and not let the membranes dry at any stage prior to chemiluminescent detection. To reduce cross-contamination, use fresh plastic ware for incubating the membranes with primary and secondary antibodies, and when treating the membrane with the chemiluminescent reagent. Wear gloves when handling membrane blots and X-ray films.

Western Blotting Procedure and Chemiluminescent Detection

Steps:

1. Electrophorese the proteins of interest on a polyacrylamide gel. Follow the standard protocols to prepare protein samples including measurement of protein concentration, adding the loading buffer, and loading an appropriate amount of sample to the wells. For the gel, choose a concentration of acrylamide based on the size of proteins to be resolved (see the procedures for electrophoresis, transfer and detection of proteins in Volume 2 and Section 10 of Current Protocols in Molecular Biology, eds. FM Ausubel et al.,)

2. Transfer the proteins from the gel onto a nitrocellulose or PVDF membrane using standard gel transfer apparatus and buffers. Before the transfer, prepare the membranes following protocols appropriate to the type of membrane such as immersing PVDF membrane first in methanol and then equilibrating in transfer buffer.

3. Remove the membrane blot from the transfer apparatus and immediately immerse in blocking reagent such as 5% non-fat dry milk prepared in wash buffer or any commercially available blocking reagent. Blocking can be done for 1 hour at ambient temperature (22-25°C) with moderate shaking on a platform or overnight at 2-8°C without shaking.

4. Remove the blocking reagent and incubate the membrane with target specific primary antibody diluted in blocking reagent for 1 hour with gentle shaking.

5. Remove the primary antibody and rinse the membrane 2-3 times with fresh wash buffer followed by 2-3 longer washes (about 10 minutes) with moderate shaking. Use generous amounts of wash buffer to keep the membranes completely immersed while washing.

6. Remove the wash buffer and incubate the membrane with HRP conjugated secondary antibody diluted in blocking reagent for 1 hour with gentle shaking.

7. Remove the secondary antibody and rinse the membrane 2-3 times with fresh wash buffer followed by 3-4 longer washes (about 10 minutes) with moderate shaking. Use generous amounts of wash buffer to keep the membranes completely immersed while washing.

   Note the following steps 8, 9 and 10 are performed under subdued light conditions.

8. While washing the membrane in step 7 above, prepare working solution of Lumigen ECL Plus substrate by mixing solutions A and B in the ratio of 40:1.

9. Place the membrane from step 7 in a fresh plastic dish and add the working solution of Lumigen ECL Plus onto the membrane. Let the membrane stand for 2-3 minutes in the substrate.

10. For chemiluminescent detection, remove the membrane from the plastic dish and place it sandwiched between two clear plastic sheets. Wipe away excess solution with an absorbent tissue leaving a thin layer of solution on the membrane without any air bubbles. Seal the edges of plastic sheets with a sticky tape.

11. Turn off the subdued lights and turn on the red safe light. Expose the protein side of the membrane to an X-ray film in a film cassette or sandwiched between two glass plates.

12. For chemifluorescent detection, capture the image using either Kodak Imaging system with epifluorescent UV setting or on a Typhoon™ Imager using blue chemifluorescence mode.

Troubleshooting Tips

There are mainly two types of problems associated with the detection of proteins on Western blots.

1. **Signal problems:** Weak or distorted signal bands
2. **Background problems:** High non-specific background and spots

Signal Problems	Possible Causes	Troubleshooting Tips
1. Hollow protein bands on the X- ray film	High amount of HRP antibody depleted the substrate at the band	Use more diluted HRP antibody
2. Brown or yellow protein bands on the membrane	High amount of HRP antibody	Use more diluted HRP antibody
3. Protein signal bands fade away quickly	High amount of HRP antibody with low amount of protein	Use more diluted HRP antibody and/or increase amount of protein loaded
4. Weak protein signal bands	• Low amount of protein loaded • Low amount of antibodies used • Inefficient protein transfer	• Load higher amounts of protein into wells • Use higher amount of antibodies • Check protein transfer apparatus, buffers and power supply
5. No signal	• Protein not transferred to membrane • Antibodies did not bind to the target protein band • Inactive HRP enzyme • Expired substrate	• Check protein transfer components • Replace antibodies, both primary and secondary with newer lots • Replace substrate with newer lot

Signal Problems	Possible Causes	Troubleshooting Tips
6. Hollow spots in protein bands	• Inefficient protein transfer due to air bubbles between the gel and membrane • Insufficient/uneven wetting of membrane • Air bubbles on the protein bands when exposed to X-ray film	• Make sure to remove air bubbles while making the gel-membrane sandwich • Wet the membrane in adequate amount of water for at least 5 minutes • Prior to X-ray film exposure wipe away air bubbles on the membrane

Background Problems	Possible Causes	Troubleshooting Tips
1. Dark membrane background on the X-ray film	• Use of high amount of HRP antibody • Insufficient washing of the membrane • Inadequate blocking duration • Inappropriate blocking reagent • Membrane dried during any of the steps • Long X-ray film exposure time	• Use more diluted HRP antibody • Increase wash buffer volume, wash duration, number of washes, and shaking intensity • Block longer period of time • Switch to another blocking reagent • Keep the membrane wet until exposed to X-ray film • Take shorter exposures
2. Membrane visibly glows in the dark room	• Use of high amount of HRP antibody • Inappropriate blocking reagent • Membrane dried during any of the steps • Inappropriate membrane	• Use more diluted HRP antibody • Switch to another blocking reagent • Keep the membrane wet until exposed to X-ray film • Switch to a different membrane
3. Dark spots on the membrane X-ray film exposure	• Use of HRP antibody that aggregated during storage	• Spin down the antibody aggregates in a microcentrifuge

Background Problems	Possible Causes	Troubleshooting Tips
	• Gel or membrane over heated during electrophoresis and/or transfer	for a few seconds and use the supernatant antibody for dilution • Lower voltage during electrophoresis and/or keep the transfer apparatus cool with frozen ice

Lumigen Product Matrix

	Lumigen ECL Plus	Lumigen ECL Extra	Lumigen ECL Ultra	Lumi-Phos Plus	Lumi-Phos 530	Lumigen APS-5	Lumi-Phos HRP	Lumigen SPARCL	Lumigen HyPerBlu
Application	Western Blotting	Western Blotting	Western Blotting	Southern Northern Blotting	ELISA	ELISA	ELISA	ELISA, Other Proximity Assays	H_2O_2 Detection
Enzyme	HRP	HRP	HRP	AP	AP	AP	HRP	HRP	Direct, Non-enzymatic
Sensitivity	Low pg to mid fg	Low pg to mid fg	Mid to low fg	Single Copy Gene Detection	Low pg to fg	Low pg to fg	Low pg to fg	Low pg	2.6 nM
Signal Duration	Up to 5 Hours	Up to 6 Hours	Up to 8 Hours	Up to 24 Hours	Up to 24 Hours	Up to 4 Hours	Up to 5 Hours	Instantaneous Flash	Up to 8 Hours
Shelf Life	2 Years	1 Year	1 Year	2 Years	2 Years	1 Year	1 Year	1 Year	2 Years
Storage Conditions	2 - 8° C	2 - 8° C	2 - 8° C	2 - 8° C	2 - 8° C	2 - 8° C	2 - 8° C	2 - 8° C	2 - 8° C

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