For life science research only.
Not for use in diagnostic procedures.

Lumi-Light^{PLUS} Western Blotting Substrate

Version: 10
Content Version: November 2021

Chemiluminescent POD-substrate for western blots.

Cat. No. 12 015 196 001

1 kit
100 ml (2 x 50 ml)
10 or 100 blots with 10 cm x 10 cm

Store the product at +2 to +8°C.

1. General Information

1.1. Contents

Vial / bottle	Label	Function / description	Content
1	Lumi-Light^{PLUS} Western Blotting Substrate, Lumi-Light^{PLUS} Luminol/Enhancer Solution	▪ Clear solution ▪ Component of substrate solution.	1 bottle, 50 ml
2	Lumi-Light^{PLUS} Western Blotting Substrate, Lumi-Light^{PLUS} Stable Peroxide Solution	▪ Clear solution ▪ Component of substrate solution.	1 bottle, 50 ml

1.2. Storage and Stability

Storage Conditions (Product)

When stored at +2 to +8°C, the product is stable through the expiry date printed on the label.

Vial / bottle	Label	Storage
1	Lumi-Light^{PLUS} Luminol/Enhancer Solution	Store at +2 to +8°C.
2	Lumi-Light^{PLUS} Stable Peroxide Solution	

1.3. Additional Equipment and Reagent required

Standard laboratory equipment

- Standard electrophoresis apparatus
- Imaging system to quantify chemiluminescence signals, or
- X-ray film or Lumi-Film Chemiluminescent Detection Film*
- X-ray film cassette
- Centrifuge
- Powder-free gloves
- Transparency film
- Reciprocal shaker
- Filter paper
- Blunt-ended forceps with non serrated tips
- Incubation trays or Petri dish

⚠ ***The volumes for the washing and incubation solutions recommended in the procedure are only applicable when the size of the incubation trays fits the size of the membrane. Only use disposable trays, such as Petri dishes, or glass trays, which are carefully cleaned.***

For preparation of solutions

See section, **Working Solution** *for information on preparing solutions.*

- Double-distilled water
- Tris-HCl*
- NaCl
- 2 M NaOH
- Tween 20*
- Western Blocking Solution*, or
- Blocking Reagent*
- Alternative Blocking reagents: skim milk powder or BSA

For detection protocol

- PVDF Western Blotting Membranes*, or
- Nitrocellulose membranes
- Tween 20*
- Methanol, isopropanol, or ethanol (wetting the PVDF membranes)
- Ponceau S (optional)
- Primary antibody, antigen specific
- Secondary antibody-POD conjugates

For stripping and reprobing of blots

See section, **Working Solution** *for information on preparing solutions.*

- Tris-HCl*
- SDS*
- 2-mercaptoethanol

1.4. Application

The Lumi-Light^{PLUS} Western Blotting Substrate is used for the detection of antigen blotted onto PVDF or nitrocellulose membranes.

1.5. Preparation Time

Assay Time

3 hours

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Use any antigen which can be immobilized onto PVDF Western Blotting Membranes* or nitrocellulose membranes.

General Considerations

Time response curve

Figure 1 shows the time response curve for the Lumi-Light^{PLUS} signal.

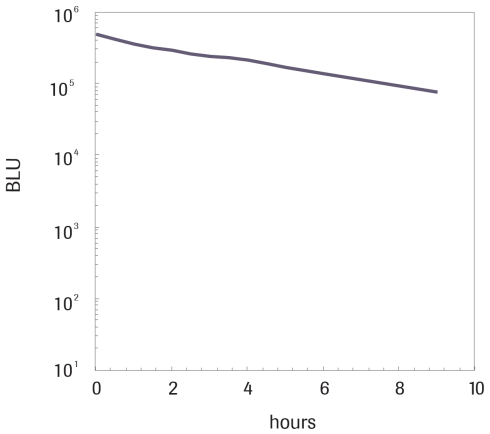

Fig. 1: Lumi- Light^{PLUS} signal in relative light units from a typical western blot, analyzed on the Lumi-Imager Workstation.

Electrophoresis and electrotransfer

- Carry out electrophoresis using either non-denaturing gels, SDS-PAGE, or two-dimensional gels according to standard protocols.
- Perform electrotransfer according to standard protocols.
- After transfer, check blotting efficiency by reversibly staining the transferred proteins with Ponceau S solution.

Membrane handling requirements

Follow good laboratory practice when handling membranes.
- Handle membrane only on the edges and with clean blunt-ended forceps.
- Clean scissors with an ethanol moistened towel before cutting the membrane.
- Wear powder-free gloves to avoid damage or contamination.
- Make sure that there is sufficient solution to entirely cover the membrane.

Special handling of PVDF membranes

- Wet hydrophobic PVDF membranes with a brief rinse in methanol; isopropanol, or ethanol; the membrane changes color from white to gray translucent.
- Wet the membrane in transfer buffer for 3 minutes.

  ⚠ ***Do not use the membrane if parts of the membrane remain white.***
- PVDF membranes must not dry out at any step. If drying occurs, re-wet in 5% Tween 20 (v/v). This may, however, influence antibody binding.

Washing requirements

- Use large volumes of washing solution for all washing steps; at least 0.4 ml/cm^2 of membrane area.
- Rinse briefly with washing solution prior to the washing steps to further increase the efficiency.

Optimization of antibody concentration

ℹ *See section,* **Protocols** *for additional information on optimization.*

Since Lumi-Light^{PLUS} gives very strong signals compared to conventional POD substrates, optimize the antibody concentration in your experimental system.
- First optimize the concentration of the primary antibody using a constant amount of secondary antibody-POD conjugate, such as 50 mU/ml.
- Then, using the optimized primary antibody concentration, adjust the concentration of secondary antibody-POD conjugate.

Safety Information

Precautions

The Lumi-Light^{PLUS} Luminol/Enhancer Solution and Lumi-Light^{PLUS} Stable Peroxide Solution have not been tested to determine their hazards.
- For contact with eyes or skin, flush with water (at least 15 minutes for eyes).
- Remove contaminated clothing.
- If ingested, seek immediate medical attention.

Working Solution

ℹ *This procedure is designed for a membrane of 10 cm × 10 cm; if larger membranes are used, scale up the volumes.*

⚠ ***Do not use azide to stabilize the solutions against microbial growth since azide irreversibly inhibits horseradish peroxidase.***

Preparation of working solutions			
Solution	**Preparation/Composition**	**Storage and Stability**	**For use in...**
Lumi-Light^{PLUS} substrate solution	▪ Depending on the size of the membrane, mix equal amounts of Lumi-Light^{PLUS} Luminol/Enhancer (Bottle 1) and Lumi-Light^{PLUS} Stable Peroxide Solution (Bottle 2). ▪ Mix 100 µl of each solution per cm^2 blot surface (conventional tank incubation) or 10 µl of each solution per cm^2 blot surface (transparency technique).	Store 24 hours at +15 to +25°C.	Detection protocol, Step 7.
TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20 (v/v))	▪ Dissolve 7.9 g Tris-HCl* and 8.8 g NaCl in 900 ml double-distilled water. ▪ Adjust pH to 7.5 with 2 M NaOH. ▪ Add 500 µl Tween 20*. ▪ Fill up with double-distilled water to 1 l total volume.	Store 4 weeks at +2 to +8°C.	Blocking and washing solutions.
1% Western blocking solution (1% casein)	▪ Dilute 10 ml of 10x Western Blocking Reagent* with 90 ml of TBST. ⚠ ***Do not filter. The solution remains turbid.*** ▪ Alternatively, dissolve 1 g Blocking Reagent* in 100 ml TBST with stirring for approximately 30 minutes. ⚠ ***Do not filter. The solution remains turbid.***	Store 1 week at +2 to +8°C.	▪ Membrane blocking ▪ Dilution of antibodies
BSA (alternative blocking solution)	▪ Dissolve 5 g of BSA in 100 ml of 1x TBST with stirring. ▪ Filter through a 0.45 µm filter. ⓘ *Higher concentrations of BSA may be necessary for certain antibodies.*		
Skim milk (alternative blocking solution)	Dissolve 2 g of skim milk powder in 200 ml of TBST with stirring for at least 30 minutes.		
Primary antibody	▪ Centrifuge primary antibody for 2 minutes at 5,000 to 10,000 × *g*. ▪ Dilute an aliquot of the supernatant in 10 ml of 1% Western blocking solution. ⓘ *The optimal antibody concentration should be evaluated, see section,* **Optimization of primary antibody concentration**.	Store 1 day at +2 to +8°C.	Detection protocol, Step 3.
Secondary antibody-POD conjugate	Dissolve secondary antibody-POD conjugate, for example, Anti-rabbit IgG-POD or Anti-mouse IgG-POD, in 1 ml of double-distilled water. ▪ Centrifuge the antibody-POD conjugate solution for 2 minutes at 5,000 to 10,000 × *g* to pellet possible aggregates. ▪ Dilute the necessary amount of secondary antibody-POD conjugate in 10 ml of 1% Western blocking solution, see section, **Optimization of primary antibody concentration**.	Store 3 months at +2 to +8°C. Store 1 day at +2 to +8°C.	Detection protocol, Step 5.
Stripping solution	62.5 mM Tris-HCl, pH 6.8 (1 g), 2% SDS (2 g), 100 mM 2-mercaptoethanol.	–	Stripping of blots.

2.2. Protocols

Optimization of primary antibody concentrations

See section, **Working Solution** *for additional information on preparing solutions.*
Use the following protocol to optimize antibody concentrations for the Lumi-Light^{PLUS} substrate solution.

1. Cut a piece of nitrocellulose or PVDF membrane for each antibody concentration to be tested.
   – Spot an appropriate amount of antigen onto individual pieces of membrane.
   Air dry the samples if nitrocellulose membranes are used.

2. Block the membranes in 1% Western blocking solution (0.4 ml/cm^2) for 1 hour under constant shaking.

3. Prepare primary antibody dilutions with 1% Western blocking solution,
   for example, 1:100, 1:300, 1:1,000, 1:3,000, 1:10,000.
   – Incubate one piece of membrane in each dilution for 1 hour under constant shaking.

4. First rinse and then wash membranes 4 times, 2 minutes each with 40 ml TBST.

5. Depending on which primary antibody was used, add secondary antibody-POD, for example, Anti-rabbit IgG-POD at a concentration of 50 mU/ ml or Anti-mouse IgG-POD at a concentration of 100 mU/ml in 1% Western blocking solution (0.1 ml/cm^2).
   – Incubate membranes 30 minutes under constant shaking.

6. First rinse and then wash membranes, 4 times, 2 minutes each with 40 ml of TBST.
   – Drain TBST from the membranes by holding it with forceps and briefly blotting the lower edge onto absorbent filter paper.

7. Incubate for 5 minutes with 10 ml Lumi-Light^{PLUS} substrate solution in a glass tray or disposable Petri dish.
   – The membranes must be soaked entirely with substrate solution and must not dry out during this step.
   – Remove the membrane from the Lumi-Light^{PLUS} substrate solution and place it protein side up on transparency film.
   – Cover the membranes with a second transparency film and remove air bubbles.

8. Gently squeeze out excess liquid onto an adsorbent filter paper.
   – Seal the membranes.

9. Expose on X-ray film for 1 minute or use an imaging system to quantify chemiluminescence signals.
   – Place another sheet of X-ray film on the membrane while developing the first film.
   – Choose the most suitable dilution of antibodies yielding the highest signal and minimal background.

Optimization of secondary antibody concentrations

See section, **Working Solution** *for additional information on preparing solutions.*
Use the following protocol to optimize secondary antibody concentrations.

1. Cut a piece of nitrocellulose or PVDF membrane for each antibody concentration to be tested.
   – Spot an appropriate amount of antigen onto individual pieces of membrane.
   Air dry the samples if nitrocellulose membranes are used.

2. Block the membranes in 1% Western blocking solution (0.4 ml/cm^2) for 1 hour under constant shaking.

3. Incubate the membranes in the optimized primary antibody concentration from the previous protocol for 1 hour under constant shaking.

4. First rinse and then wash membranes 4 times, 2 minutes each with 1x TBST (0.4 ml/cm^2).

5. Prepare suggested secondary antibody dilutions with 1% Western blocking solution, for example, 200 mU/ml, 100 mU/ml, 50 mU/ml, 25 mU/ ml, 10 mU/ml and 5 mU/ml.
   – Incubate one piece of membrane in each dilution for 1 hour under constant shaking.
   For antibodies of other origin, other dilutions, such as 1:1,000 to 1:50,000 may be necessary.

6. First rinse and then wash membranes 4 times, 2 minutes each with TBST (0.4 ml/cm^2).
   – Drain TBST from the membranes by holding it with forceps and briefly blotting the lower edge onto absorbent filter paper.

7. Incubate for 5 minutes with 10 ml Lumi-Light^{PLUS} substrate solution in a glass tray or disposable Petri dish.
   – The membranes must be soaked entirely with substrate solution and must not dry out during this step.
   – Remove the membrane from the Lumi-Light^{PLUS} substrate solution and place it protein side up on transparency film.
   – Cover the membranes with a second transparency film and remove air bubbles.

8. Gently squeeze out excess liquid onto an adsorbent filter paper.
   – Seal the membranes.

9. Expose on X-ray film for 1 minute or use an imaging system to quantify chemiluminescence signals.
   – Place another sheet of X-ray film on the membrane while developing the first film.
   – Choose the most suitable dilution of antibodies yielding the highest signal and minimal background.

Detection protocol

⚠ ***This procedure is designed for a membrane of 10 cm × 10 cm; if larger membranes are used, scale up the volumes.***

Perform all steps at +15 to +25°C and with gentle agitation on a reciprocal shaker. For reproducible results, equilibrate all solutions to +15 to +25°C before use.

See section, **Working Solution** *for additional information on preparing solutions.*

1. Perform the following optional steps:

If...	Then...
nitrocellulose membrane was stored at +2 to +8°C,	wet membrane with a brief rinse in water.
PVDF membrane was stored at +2 to +8°C,	wet membrane with a brief rinse in methanol, isopropanol, or ethanol, then wash with double-distilled water before proceeding to the next step.
blotting was performed in a buffer system containing methanol,	briefly wash the membrane three times for 2 minutes each with 40 ml TBST (0.4 ml/cm^2) to avoid background staining.

2. Add 40 ml 1% Western blocking solution, to an appropriate incubation tray and incubate the membrane under constant shaking for 1 hour or overnight at +2 to +8°C without shaking.

3. Incubate membrane with 10 ml of primary antibody solution under constant shaking for 1 hour or overnight at +2 to +8°C without shaking.
   Extend incubation time to overnight, if either the affinity of the antibody to the antigen, or if the concentration of specific antibody is low.

4. First rinse and then wash 4 times, 2 minutes each with 40 ml of TBST.

5. Add 10 ml of appropriate secondary antibody-POD solution and incubate membrane 30 minutes under constant shaking.

6 First rinse and then wash 4 times, 2 minutes each with 40 ml of TBST.
– Drain TBST from the membrane by holding it with forceps and briefly blotting the lower edge onto absorbent filter paper.

7 Perform one of the following steps:

If you use a...	Then...
conventional tank blot,	incubate for 5 minutes with 10 ml Lumi-Light^{PLUS} substrate solution in a glass tray or disposable Petri dish. The blot must be soaked entirely with substrate solution and must not dry out during this step. – Remove the membrane from the Lumi-Light^{PLUS} substrate solution and place it protein side up on transparency film. – Cover the membrane with a second transparency film and remove air bubbles. *For increased sensitivity, the substrate incubation time can be increased up to 30 minutes.*
transparency technique,	place the membrane protein side up on a sheet of transparency film. – Add 1 ml Lumi-Light^{PLUS} substrate solution onto the membrane. – Immediately cover the membrane with a second transparency film, remove air bubbles, and incubate for 5 minutes. *For increased sensitivity, the substrate incubation time can be increased up to 30 minutes.*

8 Gently squeeze out excess liquid onto an adsorbent filter paper.
– Seal the membrane.

9 Expose on X-ray film for 1 minute or use an imaging system to quantify chemiluminescence signals.
– Place another sheet of X-ray film on the membrane, while developing the first film.
– Adjust the exposure time between 10 seconds and up to 1 hour, according to the result with the first film.
⚠ ***The X-ray film must not become wet.***

For comparison of blots, it is necessary to use the same substrate incubation time, since the signal intensity may increase. The luminescent reaction reaches its maximum after approximately 10 minutes.

Stripping and reprobing of blots

See section, **Working Solution** *for additional information on preparing solutions.*

1 Incubate blot for 30 minutes at +70°C in Stripping solution.

2 Wash 3 times, 5 minutes each with TBST.

3 Proceed to the **Detection protocol**.

Membrane storage

After blotting, store the membrane as shown in the table:

If...	Then...
you want to stop,	store dry membrane at +2 to +8°C for up to 3 months. After storage, proceed to **Detection protocol, Step 1**.
you want to go ahead,	Proceed to **Detection protocol, Step 2**.

2.3. Parameters

Emission

Emission wavelength: 425 nm.

Sensitivity

Depending on the affinity of the primary antibody, 1 to 5 pg amounts of antigen can be detected.
Long lasting luminescence (>12 hours).

3. Troubleshooting

Observation	Possible cause	Recommendation
No or weak signal present.	Inefficient protein transfer.	Check protein transfer efficiency with Ponceau S solution or by silver staining of the gel after blotting.
		Change transfer conditions if efficiency is low.
	Primary antibody does not detect denatured (in denaturing gels containing SDS or Urea) proteins on blots.	Perform a dot blot with denatured protein and native protein in parallel. If the primary antibody only binds to native protein, use non-denaturing gel systems.
	Affinity of primary antibody is low.	Optimize concentration of the first antibody, see section, **Protocols, Optimization of primary antibody concentrations**.
		Prolong incubation with primary antibody to overnight at +2 to +8°C.
		Shorten washing times and use Washing buffer without Tween 20.
		Incubate primary and secondary antibodies in buffer without Western Blocking Reagent (background may be increased).
	Peroxidase activity of the secondary antibody has decreased.	Dot different dilutions of the POD-conjugate onto a blotting membrane and detect directly, see section, **Protocols, Optimization of secondary antibody concentrations**. ▪ If no signal appears, use fresh POD-conjugate and test in the same way. ▪ If still no signal appears, use fresh Lumi-Light^{PLUS} substrate solution.
	Detection reagent gives no signal.	Check if the Lumi-Light^{PLUS} substrate solution was equilibrated to +15 to +25°C.
		Use non-expired and non-contaminated Lumi-Light^{PLUS} substrate solution.
	Concentrations of secondary antibody too low.	Prolong the incubation time with secondary antibody to 3 hours.
		Prolong detection time.
	Insufficient amount of protein loaded.	Increase amount of protein applied onto the gel.

High background observed on blots.	Inadequate washing.	Prolong washing times and increase number of washes.
		Increase concentration of washing reagent.
	Inadequate blocking.	Block overnight.
		Increase concentration of Western blocking solution up to 10%.
		Use fresh Western blocking solution.
	Concentration of primary and secondary antibodies too high.	As Lumi-Light^{PLUS} gives very high signals, it is absolutely essential to optimize antibody concentrations, see sections, **Optimization of primary and secondary antibody concentrations**. *In general, antibody concentrations must be diluted tenfold in comparison to conventional chemiluminescence substrates.*
	Overexposure of film.	Use shorter exposure time.
Spotted or uneven background present.	Membrane dried partially during the procedure.	Avoid drying of the membrane at any time during the procedure.
	Membranes were not entirely submerged during blocking or washing steps.	Carefully check that the membrane is always covered with buffer during the incubations and moves freely in the working solutions.
	Primary antibody and/or POD conjugate aggregates.	Always centrifuge the primary antibody and POD conjugate and use supernatant before diluting in Western blocking solution.
		Alternatively, filter through a 0.2 µm filter with low protein adsorption.
	Contamination of equipment or solutions.	Use clean equipment.
		Prepare fresh buffers.
	Contamination of membranes.	Use new membranes.
		Follow the membrane handling instructions, see section, **General Considerations**.

4. Additional Information on this Product

4.1. Test Principle

The following steps and Figure 2 show the basic steps of the detection principle.

① Antigen is blotted onto the membrane.

② Primary antibody binds to immobilized antigen.

③ A secondary anti-mouse/rabbit-antibody-POD conjugate binds to the primary antibody.

④ Horseradish peroxidase converts Lumi-Light^{PLUS} substrate, resulting in light emission.

⑤ Light is detected by exposition to X-ray film or an imager.

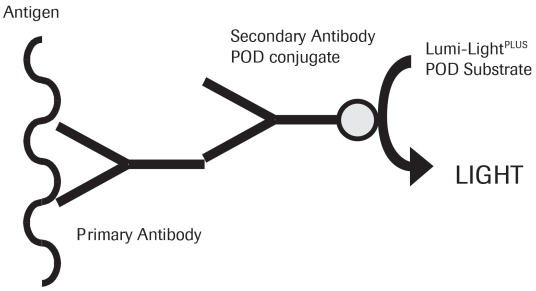

Fig. 2: Chemiluminescent detection of blotted antigens using primary and secondary antibodies and Lumi-Light^{PLUS} Substrate.

Background information

Lumi-Light^{PLUS} Western Blotting Substrate represents a next generation of chemiluminescent POD- substrates for western blotting applications. With its long lasting signal (>12 h) and high sensitivity (>1 pg of protein can be detected) it clearly has enormous advantages over other conventional chemiluminescent western substrates. Lumi-Light^{PLUS} consists of Luminol, a special enhancer, and a stable peroxide solution. It is suited for high sensitivity routine western blotting, especially when quantification is required. The chemiluminescence signal of Lumi-Light^{PLUS} substrate persists for more than nine hours, and since exposures of only a few minutes are necessary, multiple exposures can be taken. Additionally, the ability to take extended exposures allows the detection of rare proteins, or higher dilutions of antigen or antibody to be used.

4.2. Quality Control

For lot-specific certificates of analysis, see section **Contact and Support**.

5. Supplementary Information

5.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

5.2. Changes to previous version

Layout changes.
Editorial changes.

5.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents, kits		
Lumi-Film Chemiluminescent Detection Film	100 films, 7.1 x 9.4 inches, 18 x 24 cm, *Not available in US*	11 666 916 001
	100 films, 8 x 10 inches, 20.3 x 25.4 cm	11 666 657 001
PVDF Western Blotting Membranes	1 roll, 30 cm x 3.00 m	03 010 040 001
Tween 20	50 ml, 5 x 10 ml	11 332 465 001
Tris hydrochloride	500 g	10 812 846 001
Sodium Dodecyl Sulfate (SDS)	1 kg	11 667 289 001
Blocking Reagent	27 g, for one liter blocking solution, *Not available in US*	11 112 589 001
Tris base	1 kg, *Not available in US*	10 708 976 001
	1 kg	03 118 142 001
	5 kg	11 814 273 001
Western Blocking Reagent, Solution	100 ml, 10 blots, 100 cm^2	11 921 673 001
	6 x 100 ml, 60 blots, 100 cm^2	11 921 681 001

5.4. Trademarks

All product names and trademarks are the property of their respective owners.

5.5. License Disclaimer

For patent license limitations for individual products please refer to:
List of biochemical reagent products.

5.6. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

5.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

5.8. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site**.

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

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