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

BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit)

Version: 09
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For detection of proteins with peroxidase-labeled secondary antibodies and the chemiluminescent substrate luminol on western blots.

Cat. No. 11 520 709 001 1 kit
2,000 cm^2 membrane (trays), 12,500 cm^2 membrane (transparent plastic bags)

Store the kit at +2 to +8°C.

1. General Information

1.1. Contents

Vial / bottle	Label	Function / description	Content
1	BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit), Luminescence substrate solution A	Detection reagent	2 bottles, 125 ml each
2	BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit), Starting solution B	To prepare Detection reagent.	2 bottles, 2 ml each
3	BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit), Blocking reagent	▪ 10% (w/v) stock solution ▪ For blocking of nonspecific binding sites.	1 bottle, 100 ml
4	BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit), Anti-mouse IgG-POD/anti-rabbit IgG-POD	Lyophilized secondary antibody.	1 vial, 50 U

1.2. Storage and Stability

Storage Conditions (Product)

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

Vial / bottle	Label	Storage
1	Luminescence substrate solution A	Store at +2 to +8°C.
2	Starting solution B	
3	Blocking reagent	
4	Anti-mouse IgG-POD/anti-rabbit IgG-POD	

1.3. Additional Equipment and Reagent required

Standard laboratory equipment

- Standard electrophoresis apparatus
- Centrifuge
- Powder-free gloves
- Saran wrap
- Transparency film
- Reciprocal shaker
- Blunt-ended forceps with non serrated tips
- Incubation trays or transparent plastic bags

  ⚠ ***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, or trays, which are carefully cleaned.***
- X-ray film

For preparation of solutions

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

- TBS (Tris buffered saline)
- TBST (TBS-Tween 20)
- Tris base*
- Tween 20*
- Double-distilled water

For detection protocol

- PVDF Western Blotting Membranes*, or
- Nitrocellulose membranes
- Primary antibody, antigen specific
- Methanol (wetting the PVDF membranes)
- Ponceau S (optional)

For stripping and reprobing of blots

- 2-mercaptoethanol
- 2% SDS*

1.4. Application

The BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) is used for the detection of any antigen isolated from rabbit or mice, blotted onto PVDF or nitrocellulose membranes.

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Use any antigen isolated from rabbit or mice, which can be immobilized on PVDF Western Blotting Membranes* or nitrocellulose membranes.

General Considerations

Electrophoresis and electrotransfer

- Carry out electrophoresis using either non-denaturing gels, SDS-PAGE, or two-dimensional gels according to standard protocols.
- Blot 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; 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.

Safety Information

Laboratory procedures

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material varies, the operator must optimize pathogen inactivation by the Lysis / Binding Buffer or take appropriate measures, according to local safety regulations.
- Do not eat, drink or smoke in the laboratory work area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

Waste handling

- Discard unused reagents and waste in accordance with country, federal, state, and local regulations.
- Safety Data Sheets (SDS) are available online on dialog.roche.com, or upon request from the local Roche office.

Working Solution

For reproducible results, equilibrate all solutions to +20 to +25°C before use.

Preparation of kit working solutions			
Solution	**Preparation/Composition**	**Storage and Stability**	**For use in...**
1% Blocking solution	Add 10 ml of Blocking reagent, 10% stock solution (w/v) (Bottle 3) to 90 ml TBS.	Store at −15 to −25°C.	Membrane blocking
0.5% Blocking solution	Add 5 ml of Blocking reagent, 10% stock solution (w/v) (Bottle 3) to 95 ml TBS.		Antibody dilution
POD-labeled secondary antibody stock solution	Reconstitute lyophilizate in 100 µl double-distilled water	Store 12 months at +2 to +8°C.	Stock solution
POD-labeled secondary antibody working solution	Dilute stock solution in 0.5% Blocking solution. *40 mU/ml of POD-labeled secondary antibody are usually sufficient for sensitive detection.*	***Always prepare fresh.***	Detection protocol, Step 5.
Detection solution	Mix substrate solution A (Bottle 1) and Starting solution B (Bottle 2) in a ratio of 100:1; allow to equilibrate at +15 to+25°C.	Store 1 week at +2 to +8°C. ***Keep protected from light.***	Detection protocol, Step 8.
Preparation of additional solutions			
Solution	**Preparation/Composition**	**Storage and Stability**	**For use in...**
TBS (50 mM Tris, 150 mM NaCl)	▪ Dissolve 6.05 g Tris base* and 8.76 g NaCl in 800 ml double distilled water. ▪ Adjust pH to 7.5 with approximately 9.5 ml 1 M HCl ▪ Dilute up to 1,000 ml total volume with double-distilled water. ***Do not use sodium azide as an antimicrobial agent as it inhibits POD.***	Store 3 months at +2 to +8°C.	Blocking and washing solutions.
TBST	Dilute 1 ml Tween 20* to 0.1% (v/v) final concentration in 1 l TBS.		Wash solution
Primary antibody	▪ Centrifuge primary antibody for 2 minutes at 5,000 to 10,000 × *g*. ▪ Dilute an aliquot of the supernatant in 0.5% Blocking solution. *4 µg/ml of primary antibody are usually sufficient for sensitive detection.*	***Always prepare fresh.***	Detection protocol, Step 3.

2.2. Protocols

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, 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 twice with TBS (0.4 ml/cm^2) to avoid background staining.

2. Add 12.5 ml 1% Blocking solution to an appropriate incubation tray and incubate the membrane under constant shaking for 60 minutes or overnight at +2 to +8°C without shaking.

3. Incubate membrane with Primary antibody solution under constant shaking for 60 minutes 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 µg/ml of specific primary or polyclonal purified antibodies is usually sufficient.

4. Wash twice in TBST for 10 minutes each.
   – Wash twice with 0.5% Blocking solution.
   – For efficient washing, always use 30 ml of TBST.

5. Add 12.5 ml of POD-labeled secondary antibody solution and incubate membrane 30 minutes under constant shaking.

6. First rinse and then wash 4 times for 15 minutes with 30 ml of TBST.

7. Perform one of the following steps:

If you use a...	Then...
tray for development,	drain excess buffer from the washed blot and place it, sample side up, in a fresh tray which is the same size as the blot.
transparent plastic bag,	use a transparent plastic bag which is the same size as the blot. – Fill the bag with Washing buffer to prevent drying of the membrane. – Transfer the membrane to the bag. – Put the open bag onto sheets of filter paper and press off excess fluid by rolling a 10 ml pipette over the bag towards the open end.

8 For detection, follow these steps:

If you use a...	Then...
tray for development,	add premixed Detection solution, 125 µl/cm^2 are sufficient, and incubate for 60 seconds. – Drain off excess Detection solution and wrap the blot in saran wrap or place the blot between two transparent films; make sure that no air bubbles are trapped.
transparent plastic bag,	add premixed Detection solution, 20 µl/cm^2 are sufficient, immediately seal the bag after eliminating any air bubbles, and distribute the fluid over the entire filter surface using a 10 ml pipette. – Incubate for 60 seconds.

⚠ ***After exposing the blot to the detection solutions, it must be processed very quickly to avoid fading of the chemiluminescent reaction. Work in a dark room.***

9 Insert the membrane protein side up into a film cassette.
– Switch off the light.
– Place a sheet of film onto the blot and close the cassette.
– Expose for 10 to 60 seconds.

10 Immediately replace the exposed film with a new one, reclose the cassette, and immediately develop the exposed film.
– Expose the second film for a suitable time (up to 1 hour), estimated from the signal intensity on the first film.

The luminescent reaction reaches its maximum after 1 to 2 minutes and is relatively constant for 20 to 30 minutes. After 1 hour, the signal intensity decreases to about 60 to 70% of maximum. If signal intensity was too high, wait for 10 minutes before re-exposing the film.

Stripping and reprobing of blots

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

1 Incubate membrane in TBS containing 100 mM 2-mercaptoethanol and 2% SDS with gentle agitation for 30 minutes at +50°C.

2 Wash the membrane 2 times for 15 minutes each in a large volume of TBST at +15 to +25°C.

3 Block the membrane in 1% Blocking solution (in TBS) for 1 hour at +15 to +25°C; proceed as described in the **Detection protocol**.

2.3. Parameters

Sensitivity

Depending on the affinity of the primary antibody, low nanogram amounts of antigen can be detected.

3. Troubleshooting

Observation	Possible cause	Recommendation
No or weak signal present.	Inefficient protein transfer.	Check protein transfer efficiency with Ponceau S solution or 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 antibody concentration.
		Prolong incubation with primary antibody to overnight at +2 to +8°C.
		Shorten washing times and use Washing buffer without Tween 20.
	Peroxidase activity of the secondary antibody has decreased.	Dot different dilutions of POD-conjugate onto a blotting membrane and detect directly. If no signal appears, use fresh AP-conjugate and test in the same way.
	Low activity of Detection reagent.	Check if the Luminescence substrate solution A and Starting reagent B were stored properly at +2 to +8°C.
		Make sure that the premixed Detection solution was not older than 1 week, and protected from light.
	Insufficient amount of protein loaded.	Increase amount of protein applied onto the gel.
Background observed on blots.	High concentration of POD-conjugate.	Lower the concentration of POD-conjugate.
	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**.
	Long exposure time.	Shorten exposure time.

4. Additional Information on this Product

4.1. Test Principle

The BM Chemiluminescence Western blotting System is designed for peroxidase-labeled secondary antibodies and the substrate luminol.

- In the presence of hydrogen peroxide (H_20_2), horseradish peroxidase (POD) catalyzes the oxidation of diacylhydrazides, such as luminol.
- An activated intermediate reaction product is formed, which decays to the ground state by emitting light.
- Strong enhancement of the light emission is produced by 4-iodophenol. This acts as a radical transmitter between the formed oxygen radical and luminol, see Figure 1.

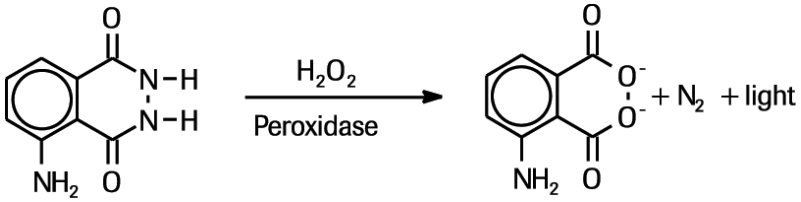

Fig. 1: Reaction mechanism.

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

1. Electrophoretical separation of proteins by gel electrophoresis.
2. Transfer of the proteins from the gel to a suitable membrane.
3. Blocking of nonspecific binding sites with blocking reagent.
4. Primary antibody binding to immobilized antigen.
5. Binding of a secondary anti-mouse/rabbit-antibody-POD conjugate to primary antibody.
6. Chemiluminescent detection of the antibody-POD conjugate.

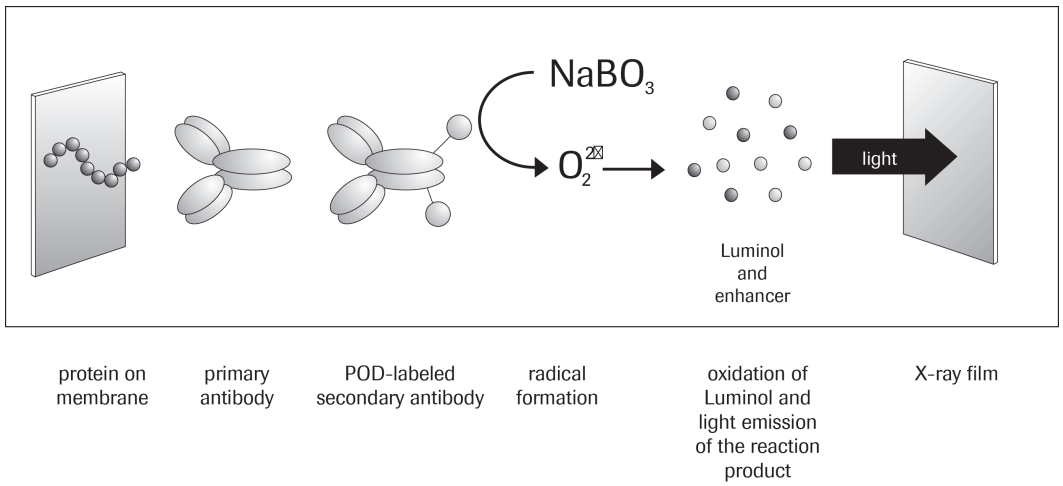

Fig. 2: Test principle.

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.
Update to include new safety Information to ensure handling according controlled conditions.

5.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents, kits		
Tris base	1 kg, *Not available in US*	10 708 976 001
	1 kg	03 118 142 001
	5 kg	11 814 273 001
Tween 20	50 ml, 5 x 10 ml	11 332 465 001
PVDF Western Blotting Membranes	1 roll, 30 cm x 3.00 m	03 010 040 001
Sodium Dodecyl Sulfate (SDS)	1 kg	11 667 289 001

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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