

BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit)

For detection of proteins with peroxidase-labeled secondary antibodies and the chemiluminescent substrate luminol on Western blots.

Cat. No. 11 520 709 001

Version March 2005

Kit for 2 000 cm² membrane (trays) or for 12 500 cm² membrane (transparent plastic bags)

Store this kit at 2–8°C

1. Preface

1.1 Contents

Contents

Bottle/Cap	Label	Content including function
1	Luminescence substrate solution A	2 × 125 ml
2	Starting solution B	2 × 2 ml
3	Blocking reagent	<ul style="list-style-type: none"> 1 × 100 ml 10% (w/v), stock solution for blocking of unspecific binding sites
4	Anti-mouse IgG-POD/ anti-rabbit-IgG-POD	<ul style="list-style-type: none"> 50U lyophilized secondary antibody

2. Introduction

2.1 Product overview

Test principle

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

In the presence of hydrogen peroxide (H₂O₂), horseradish peroxidase (HRP) catalyzes the oxidation of diacylhydrazides like luminol (see figure 1). An activated intermediate reaction product is formed, which decays to the ground state by emitting light (1).

Strong enhancement of the light emission is produced by 4-iodophenol. This acts as a radical transmitter between the formed oxygen radical and luminol (2).

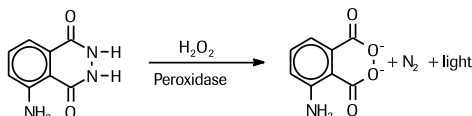


Fig. 1: Reaction scheme

Test principle basic steps

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

Step	Action
1	Electrophoretical separation of proteins by gelelectrophoresis.
2	Transfer of the proteins from the gel to a suitable membrane.
3	Blocking of non-specific 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.

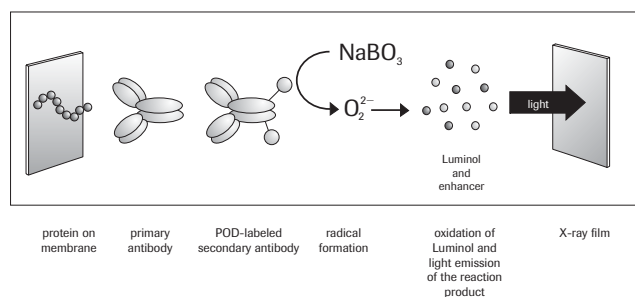


Fig. 2: Test principle

Application

Detection of any antigen isolated from rabbit or mice, blotted on PVDF or Nitrocellulose membrane.

Sample material

Any antigen isolated from rabbit or mice, which can be immobilized on PVDF or Nitrocellulose membranes.

Number of tests

The reagents are sufficient for 2,000 cm² size.

Storage / stability

The kit components are stable at 2–8°C until the expiration date printed on the label.

Sensitivity

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

3. Procedures and required material

3.1 Before you begin

Additional equipment required

- Transparency film
- Reciprocal shaker
- Blunt-ended forceps with non serrated tips
- Incubation trays

Note: 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.

Additional reagents required

- PVDF* membranes
- Primary antibody, antigen specific
- TBS
- TBST
- Double distilled water for reconstitution and dilution purposes
- Methanol for wetting PVDF membranes

Preparation of additional reagents and solutions

The table describes the preparation of working solutions.
For reproducible results equilibrate all solutions to 20-25°C before use.

Solution	Preparation of working solution	Storage and stability	Use
TBS	[50 mM Tris, 150 mM NaCl] Dissolve 6.05 g Tris/HCl and 8.76 g NaCl in 800 ml double distilled water. Adjust pH to 7.5 with approx. 9.5 ml 1 M hydrochloric acid. Fill up with double distilled water to 1000 ml total volume. Note: Do not use sodium azide as an antimicrobial agent as it inhibits POD.	3 months at 2 - 8°C	Blocking and washing solutions
TBST	Dilute 1 ml Tween 20 to 0.1% (v/v) final concentration in 1 l TBS.	3 months at 2 - 8°C	Wash solution
Primary antibody	Centrifuge primary antibody for 2 min at 5000 - 10 000 × g. Dilute an aliquot of the supernatant in 0.5% Blocking Solution. Note: 4 µg/ml of primary antibody are usually sufficient for sensitive detection.	Prepare freshly	Detection protocol step 2

Preparation of kit working solutions Please refer to the following table.

Solution	Preparation of working solution	Storage and stability	Use
1% Blocking solution	Add 10 ml of Blocking reagent, 10% stock solution (w/v) (bottle 3) to 90 ml TBS.	Stable at -15 to -25°C	Blocking of membrane
0.5% Blocking solution	Add 5 ml of Blocking reagent, 10% stock solution (w/v) (bottle 3) to 95 ml TBS.	Stable at -15 to -25°C	Dilution of antibody
POD-labeled secondary antibody stock solution	Reconstitute lyophilizate in 100 µl double dist. water.	12 months at 2-8°C	stock solution
POD-labeled secondary antibody working solution	Dilute stock solution in 0.5 % Blocking solution. Note: 40 mU/ml of POD-labeled secondary antibody are usually sufficient for sensitive detection.	Prepare always fresh	Detection protocol step 4
Detection solution	Mix substrate solution A (bottle 1) and starting solution B (cup 2) in a ratio of 100:1 and allow to equilibrate at 15-25°C.	1 week at 2-8°C protected from light	Detection protocol step 7

3.2 Preparation of membrane

Electrophoresis and electrotransfer

- Carry out electrophoresis either use non-denaturing gels, SDS-PAGE or two-dimensional gels according to usual protocols (3).
- Blot according to usual protocols (4,5,6).
- After transfer blotting efficiency can be checked by reversibly staining the transferred proteins with Pon-ceau S solution.

General handling recommendations

The table describes general hints on good laboratory practice for the preparation of membranes.

Recommendation	Guideline
Membrane handling requirements	<ul style="list-style-type: none"> • Handle membrane only on the edges and with clean blunt-ended forceps • Clean scissors before cutting the membrane with an ethanol moistened towel • Wear powder free gloves • Make sure that always enough solution is present to cover the membrane entirely
Special handling of PVDF membrane	<ul style="list-style-type: none"> • Wet hydrophobic PVDF membranes by a short rinse in methanol, whereby the membrane changes color from white to grey translucent. Then wet the membrane in transfer buffer for 3 min. Note: 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.

3.3 Detection protocol

Procedure

This procedure is designed for a membrane of 10 cm × 10 cm, if larger membranes are used volumes have to be scaled up.

Note: All steps are performed at 15 to 25°C and with gentle agitation on a reciprocal shaker. For reproducible results, equilibrate all solutions to 15-25°C before use.

Step	Action								
1a	optional <table border="1"> <thead> <tr> <th>IF...</th> <th>THEN...</th> </tr> </thead> <tbody> <tr> <td>NC membrane was stored at +4°C.</td> <td>wet NC membranes by a short rinse in water</td> </tr> <tr> <td>PVDF membrane was stored at +4°C.</td> <td>wet PVDF membrane by a short rinse in methanol, then wash with double dist. water before proceeding to the next step.</td> </tr> <tr> <td>blotting was performed in a buffer system containing methanol</td> <td>briefly wash the membrane twice with TBS (0.4 ml/cm²) to avoid background staining.</td> </tr> </tbody> </table>	IF...	THEN...	NC membrane was stored at +4°C.	wet NC membranes by a short rinse in water	PVDF membrane was stored at +4°C.	wet PVDF membrane by a short rinse in methanol, then wash with double dist. 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.
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1b	Add 12.5 ml 1% Blocking Reagent to an appropriate incubation tray and incubate the membrane under constant shaking for 60 min or O/N at 2 - 8°C without shaking.								
2	Incubate membrane with primary antibody solution under constant shaking for 60 min or O/N at 2 - 8°C without shaking. Note: Extend incubation time to O/N, if either the affinity of the antibody to the antigen, or if the concentration of specific antibody is low. 4 µg per ml of specific primary or polyclonal purified antibodies is usually sufficient.								
3	<ul style="list-style-type: none"> • Wash twice in TBST for 10 min each, • Wash twice with 0.5% Blocking solution. For efficient washing, always use 30 ml of TBST.								

continued on next page

3.3 Detection protocol, continued

Procedure

Step	Action						
4	Add 12.5 ml of POD-labeled secondary antibody solution and incubate membrane 30 min under constant shaking.						
5	First rinse and then wash 4× for 15 min with 30 ml of TBST .						
6	<table border="1"> <thead> <tr> <th>If you use a ...</th> <th>Then...</th> </tr> </thead> <tbody> <tr> <td>tray for development</td> <td>drain excess buffer from the washed blot and place it, sample side up, in a fresh tray which should be the same size of the blot.</td> </tr> <tr> <td>transparent plastic bag</td> <td>use a transparent plastic bag which should be the same size of the blot. <ul style="list-style-type: none"> Fill the bag with washing buffer to prevent drying of the membrane. Transfer the membrane to the bag. Put the open bag onto some sheets of filter paper and press off excess fluid by rolling a 10 ml pipette over the bag towards the open end. </td> </tr> </tbody> </table>	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 should be the same size of the blot.	transparent plastic bag	use a transparent plastic bag which should be the same size of the blot. <ul style="list-style-type: none"> Fill the bag with washing buffer to prevent drying of the membrane. Transfer the membrane to the bag. Put the open bag onto some sheets of filter paper and press off excess fluid by rolling a 10 ml pipette over the bag towards the open end.
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8	<ul style="list-style-type: none"> 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 s. 						
9	<ul style="list-style-type: none"> Immediately replace the exposed film with a new one, reclose the cassette and develop the exposed film at once. Expose the second film for a suitable time (up to 1 h) estimated from the signal intensity on the first film. <p>Note: The luminescent reaction reaches its maximum after 1-2 min and is relatively constant for 20 - 30 min. After 1 h the signal intensity decreases to about 60 - 70 % of maximum. If signal intensity was too high, wait for 10 min before re-exposing.</p>						

3.4 Stripping and reprobing of blots

Procedure

Please refer to the following table.

Step	Action
1	Incubate membrane in TBS containing 100 mM 2-mercaptoethanol and 2% SDS with gentle agitation for 30 min at 50°C.
2	Wash the membrane 2× 15 min in a copious volume of TBST at 15-25°C.
3	Block the membrane in 1% Blocking reagent (in TBST) for 1 h at 15 to 25°C and proceed as described above.

4. Appendix

4.1 Troubleshooting

Problem	Possible Cause	Recommendation
No or weak signal	Inefficient protein transfer	<ul style="list-style-type: none"> 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, try to use non-denaturing gel systems.
	Affinity of primary antibody is low	<ul style="list-style-type: none"> 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
	AP-activity of the secondary antibody has decreased	Dot different dilutions of AP-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	<ul style="list-style-type: none"> Check if the substrate solution A and starting reagent B been stored correctly at 2-8°C. Make sure that the premixed Detection solution was not older than 1 week, protected from light.
	Insufficient amount of protein loaded	Increase amount of protein applied onto the gel.
Background problems	High concentration of POD-conjugate	Lower the concentration of POD-conjugate
	Contamination of equipment or solutions	<ul style="list-style-type: none"> Use clean equipment Prepare fresh buffers
	Contamination of membranes	<ul style="list-style-type: none"> Use new membranes Follow general handling instructions of membranes in section 3.3.1.
	Long exposure time	Shorten exposure time.

4.2 References

- 1 Thorpe, G. H. et al. (1985) *Clin. Chem.* **31**, 1335.
- 2 Rosell, D. F. & White, E. H. (1978) *Methods Enzymol.* **57**, 409.
- 3 Dunn, M. M. (1989) *Advances in electrophoresis*, Radola, B. J., Dumm, M.J. & Chrambach, A. (eds.) VCH Verlagsgesellschaft, Weinheim, Vol. 1.
- 4 Burnette, W.N. (1981) "Western Blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112**, 195.
- 5 Towbin, H.T. et al (1979) Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci.* **76**, 4350.
- 6 Gershoni, J. M. & Palade, C. E. (1983) *Anal. Biochem.* **131**, 1.

4.3 Ordering Information

Kits

Product	Pack size	Cat. No.
BM Chemiluminescence Blotting Kit (Biotin/Streptavidin)	Reagents for 1000 cm ² of membrane surface area	11 559 460 001
BM Chromogenic Western Blotting Kit (AP, Mouse/Rabbit)	Reagents for 2000 cm ² of membrane surface area for 2000 cm ² membrane	11 647 644 001
BM Western Blotting Kit (Mouse/Rabbit)	Reagents for 2000 cm ² of membrane surface area	11 520 707 001
Lumi-Light Plus Western Blotting Kit (Mouse/Rabbit)	Reagents for 1000 cm ² of membrane surface area	12 015 218 001

Single reagents

Product	Pack size	Cat. No.
BM blue POD substrate, precipitating (TMB solution, ready-to-use)	100 ml	11 442 066 001
BM Chemiluminescent Blotting substrate (POD)	for 1000 cm ² membrane for 4000 cm ² membrane	11 500 708 001 11 500 694 001
BM purple POD substrate, precipitating (BCIP/NBT solution, ready-to-use)	100 ml	11 442 074 001
PVDF Western blotting membranes	10 sheets 15 × 15 cm each 1 roll 26.5 cm × 3.75 m	11 722 026 001 11 722 034 001
Tris-HCl	1 kg	10 812 854 001
Western Blotting reagent	100 ml (10×) 6 × 100 ml (10×)	11 921 673 001 11 921 681 001

Tween is a trademark of ICI Americas Inc., USA.

5. Quick reference procedure

Step	Action	Volume	Time	Temp.
1	Add Blocking solution 1%, and incubate the membrane under constant shaking.	0.125 ml/cm ²	60 min or O/N	15 - 25°C 2 - 8°C
2	Incubate membrane in primary antibody solution shake carefully.	0.125 ml/cm ²	60 min or O/N	15 - 25°C 2 - 8°C
3	Wash 2× in TBST and 2× in 0.5% Blocking solution.	at least 0.125 ml/cm ²	4 × 10 min	15 - 25°C
4	Add Secondary antibody-AP conjugate and incubate membrane shake carefully	0.125 ml/cm ²	30 min	15 - 25°C
5	Rinse in TBST then wash 4× in TBST	at least 0.125 ml/cm ²	4 × 10 min	15 - 25°C
6	Incubate with Detection reagent and expose to film	0.125 ml/cm ² for trays and 20 µl/cm ² for plastic bags	10 - 60 s up to 1h	15 - 25°C

Contact and Support

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

www.roche-applied-science.com/support

To call, write, fax, or email us, visit the Roche Applied Science home page, www.roche-applied-science.com, and select your home country. Country-specific contact information will be displayed. Use the Product Search function to find Pack Inserts and Material Safety Data Sheets.

Inspiring Discovery



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