Technical Manual

ECL Western Blotting Substrate

INSTRUCTIONS FOR USE OF PRODUCTS W1001 AND W1015.

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ECL Western Blotting Substrate

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1. Description

The ECL Western Blotting Substrate is a highly sensitive non-radioactive, enhanced luminol-based chemiluminescent substrate for the detection of horseradish peroxidase (HRP) on immunoblots. The ECL Western Blotting Substrate detects picogram amounts of antigen, and with the use of photographic or other imaging methods, visualizes the presence of HRP. Blots can be repeatedly exposed to X-ray film to obtain optimal results or stripped of the immunodetection reagents and reprobed.

2. Product Components and Storage Conditions

Product	Size	Cat.#
ECL Western Blotting Substrate	250ml	W1001

Includes:

- 125ml Peroxide Solution
- 125ml Luminol Enhancer Solution

Product	Size	Cat.#
ECL Western Blotting Substrate	500ml	W1015

Includes:

- 250ml Peroxide Solution
- 250ml Luminol Enhancer Solution

Storage Conditions: Store at 2–8°C.

3. General Considerations

- Ensure the membrane never becomes dry throughout the procedure.
- Always wear gloves or use clean forceps when handling the membrane.
- Use a shaker or platform rocker when incubating the membrane.
- Do **not** use sodium azide as a preservative for antibodies or buffers because it inhibits HRP.
- Add 0.05–0.1% Tween®-20 to blocking buffer and diluted antibodies to minimize background.
- Substrate working solution is light sensitive. Avoid exposure to intense light. Short-term exposure to laboratory lighting will not harm the substrate.

4. Protocol

Materials to be Supplied by the User

- blotted membrane
- blocking buffer
- wash buffer
- primary antibody
- HRP-conjugated secondary antibody
- tray for incubating and washing membrane
- rotary or rocking platform shaker
- X-ray cassette and film

1. After protein transfer, remove membrane from the transfer apparatus, and block nonspecific sites with either Tris-buffered saline (TBS), 0.05–0.1% Tween® 20, 2–5% bovine serum albumin (BSA) or phosphate-buffered saline (PBS), 0.05–0.1% Tween® 20, 2–5% BSA. Incubate for 1 hour at room temperature with shaking or at 4°C overnight without shaking.

   Note: Milk may be substituted for BSA, depending on the primary antibody used.

2. Remove blocking solution, and add diluted primary antibody solution. Incubate for 1 hour at room temperature with shaking or 4°C overnight without shaking. Overnight incubation may, however, increase background. Optimal conditions depend on the primary antibody used.

3. Wash 3 times, five minutes each wash, using TBS and 0.05–0.1% Tween® 20 (TBST).

4. Incubate membrane with diluted secondary antibody solution (conjugated to HRP) for 1 hour at room temperature with shaking.

5. Wash three times with TBST, 5 minutes each wash. Additional washes may help minimize background.

6. Prepare the substrate working solution by mixing equal parts of the Peroxide Solution and the Luminol Enhancer Solution. Mix just enough substrate to cover the membrane (e.g., 6–7ml per 10cm × 5cm membrane).

   Note: For best results, use the prepared substrate working solution immediately after mixing. The solution is stable for up to 1 hour at room temperature.

7. Incubate the membrane for 1 minute at room temperature.

8. Remove the membrane from solution, blot excess liquid with an absorbent towel, and place in a plastic sheet protector or clear plastic wrap.

9. Working in a dark room with a safe light, place covered membrane in a film cassette with protein side facing up. Place X-ray film on top of membrane, and expose for 1 minute. Exposure time can be increased to achieve optimal results, with light emission being most intense immediately after substrate incubation and significantly decreasing within 1 hour.

5. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Weak or no signal	Insufficient quantities of antigen or antibody. Strip and reprobe blot using increased amount of antibody.
	Inefficient protein transfer. Optimize transfer conditions.
	Low HRP or substrate activity. Ensure HRP has been stored properly and did not expire. Use the substrate working solution within 1 hour of mixing. Expose blot to film immediately after treatment with substrate working solution.
High background	Excessive HRP conjugate used. Dilute conjugate.
	Inadequate blocking and washing conditions. Verify that the correct blocking buffer, incubation time and number of washes were used. Increase the volume, number and time of washes.
	Overexposed film. Decrease exposure time.
	Excessive antigen or primary antibody or both. Decrease the concentration.

5. Troubleshooting (continued)

Symptoms	Causes and Comments
Spots on membrane	Insufficient protein transfer. Optimize transfer procedure.
	Inadequate hydration of membrane. Hydrate membrane according to manufacturer's instructions.
	Bubble between X-ray film and membrane. Remove all bubbles before exposing blot to film.
	Inadequate volume of buffers used for membrane. Increase volume to ensure adequate coverage during incubations and washes.
Nonspecific bands	Excessive HRP conjugate. Decrease conjugate concentration.
	Insufficient washing. Increase the volume, number and time of washes.
	Incorrect blocking conditions. Increase the concentration of blocking agent. Do **not** use milk with phosphopeptide-specific antibodies or streptavidin-biotin blotting.
	SDS caused nonspecific binding to protein. Do **not** use SDS in any buffers used for the Western blotting procedure.
White bands with black centers	Too much HRP in the system. Dilute the HRP conjugate further.
Blot glows in the darkroom	Too much HRP or excess antigen or both in the system. Decrease the concentration of HRP, primary antibody or antigen or any combination of these reagents.

6. Appendix

6.A. Composition of Buffers and Solutions

blocking buffer

Tris-buffered saline with Tween®-20 (TBST) or phosphate-buffered saline with Tween®-20 (PBST) containing 2–5% dried milk or bovine serum albumin (BSA).

TBST

20mM	Tris-HCl (pH 7.5)
150mM	NaCl
0.05–0.1%	Tween® 20

PBST (pH 7.4)

137mM	NaCl
2.68mM	KCl
1.47mM	KH_2PO_4
8.1mM	Na_2HPO_4
0.05–0.1%	Tween® 20

6.B. Related Products

Horseradish Peroxidase-Conjugated Antibodies

Product	Size	Cat.#
Anti-Rabbit IgG (H+L), HRP Conjugate*	300µl	W4011
Anti-Mouse IgG (H+L), HRP Conjugate*	300µl	W4021
Anti-Human IgG (H+L), HRP Conjugate*	300µl	W4031
Anti-Chicken IgY, HRP Conjugate	300µl	G1351
Donkey Anti-Goat IgG, HRP*	60µl	V8051

*For Laboratory Use.

TMB Stabilized Substrate for Horseradish Peroxidase

Product	Size	Cat.#
TMB Stabilized Substrate for Horseradish Peroxidase	200ml	W4121

ProtoBlot® II AP Systems with Stabilized Substrate and *Western Express*® Fast Blotting Protocol

Product	Size	Cat.#
ProtoBlot® II AP System with Stabilized Substrate, Human	each	W3940
ProtoBlot® II AP System with Stabilized Substrate, Mouse	each	W3950
ProtoBlot® II AP System with Stabilized Substrate, Rabbit	each	W3960

For Laboratory Use.

6.B. Related Products (continued)

Alkaline Phosphatase-Conjugated Antibodies

Product	Size	Cat.#
Anti-Mouse IgG (H+L), AP Conjugate	100µl	S3721
Anti-Rabbit IgG (Fc), AP Conjugate	100µl	S3731
Anti-Human IgG (H+L), AP Conjugate	100µl	S3821
Anti-Rat IgG (H+L), AP Conjugate	100µl	S3831
Anti-Chicken IgY, AP Conjugate	100µl	G1151
Donkey Anti-Goat IgG, AP	60µl	V1151

For Laboratory Use.

AttoPhos® AP Fluorescent Substrate System

Product	Size	Cat.#
AttoPhos® AP Fluorescent Substrate System	3 x 36mg	S1000
AttoPhos® AP Fluorescent Substrate System Trial Size	1 x 36mg	S1001

AttoPhos® Products Available Separately

Product	Size	Cat.#
AttoPhos® Substrate	36mg	S1011
	100mg	S1012
	1g	S1013
AttoPhos® Buffer	60ml	S1021
	240ml	S1022

Western Blue® Stabilized Substrate for Alkaline Phosphatase

Product	Size	Cat.#
Western Blue® Stabilized Substrate for Alkaline Phophatase	100ml	S3841

BCIP/NBT Color Development Substrate

Product	Size	Cat.#
BCIP/NBT Color Development Substrate	1.25/2.5ml	S3771

For Laboratory Use.

Blocking Agents

Product	Size	Cat.#
Tween® 20	2.5ml	W3831
Blot-Qualified BSA	10g	W3841

For Laboratory Use.

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