Pierce™ ECL Western Blotting Substrate

USER GUIDE Pub. No. MAN0011536 Rev. B.0

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Contents and storage

Component	Cat. No. 32109 (for 400 cm²)	Cat. No. 32209 (for 2,500 cm²)	Cat. No. 32106 (for 5,000 cm²)
Detection Reagent 1, Peroxide Solution	25 mL	125 mL	250 mL
Detection Reagent 2, Luminol Enhancer Solution	25 mL	125 mL	250 mL
Storage: Store at 2-8°C.			

Product description

Thermo Scientific™ Pierce™ ECL Western Blotting Substrate is an, enhanced luminol-based chemiluminescent substrate for the detection of horseradish peroxidase (HRP) on immunoblots. Pierce™ ECL Western Blotting Substrate enables the detection of picogram amounts of antigen. This reaction produces a prolonged chemiluminescence which can be visualized on X-ray film or an imaging system. Optimal signal intensity and duration can be attained with appropriate primary and secondary antibody dilutions (see Table 1).

Required materials

- Western blot membrane
- X-ray film or CCD imaging system (e.g., Invitrogen™ iBright™ western blot imaging systems)
- Rotary or rocking platform shaker

Online resources

- Visit thermofisher.com/chemisubstrates for additional information and protocols.
- For support, visit thermofisher.com/support.

Table 1. Recommended antibody concentration

Primary antibody dilution (from a 1 mg/mL stock)	Secondary antibody dilution (from a 1 mg/mL stock)
1:100–1:5,000 or 0.2–10 µg/mL	1:1,000–1:15,000 or 0.07–1.0 µg/mL

Important guidelines

- Optimize your western blot procedure for best results. Variables include sample amount, gel type, transfer method, membrane type, blocking reagent, wash buffer, primary and secondary antibody concentrations, and incubation times.
- Use a sufficient volume of all solutions to ensure that membranes do not dry out.
- Use a shaking or rocking platform during incubation steps for optimal results.
- Do not use sodium azide in buffers, because it inhibits HRP.
- Always wear gloves or use clean, plastic forceps. Metallic devices (e.g., scissors) must have no visible signs of rust, which may cause speckling and/or high background.
- The substrate Working Solution is stable for 1 hour at room temperature. Avoid exposure to the sun or other intense light. Short-term exposure to lab lighting is okay.

Manufacturer: Life Technologies | Carlsbad, CA 92008 USA | Toll Free in USA 1.800.955.6288

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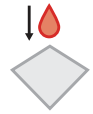
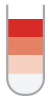
Perform western detection using Pierce™ ECL Western Blotting Substrate

Step		Action
1 	**Wash membrane**	After protein transfer, remove the blot from the transfer apparatus and wash the membrane in deionized water for 5 minutes using agitation to remove all transfer buffer.
2 	**Block membrane**	Block nonspecific sites with Blocking Reagent (e.g., StartingBlock™ (PBS) Blocking Buffer, Cat. No. 37538) for 30–60 minutes at room temperature with shaking. Alternatively, block overnight at 2–8°C without shaking.
3 	**Add primary antibody**	Incubate the membrane with primary antibody solution (0.2–10 µg/mL or follow manufacturer's recommended dilution) containing 10% blocking solution with continuous rocking for 1 hour. If desired, incubate the blot overnight at 2–8°C.
4 	**Wash membrane**	a. Wash the membrane for 10 minutes using agitation with Tris-buffered saline (TBS), phosphate-buffered saline (PBS), or other physiological wash buffer containing 0.05% Tween 20 detergent. b. Repeat wash step 2 more times. c. Proceed to next step, or if using an enzyme-conjugated HRP primary antibody, proceed to Step 6.
5 	**Add secondary antibody**	Incubate blot with the secondary antibody HRP-conjugate working dilution (0.07–1.0 µg/mL or 1:1,000–1:15,000, from a 1 mg/mL stock solution) for 30 minutes to 1 hour at room temperature using shaking.
6 	**Wash membrane**	Wash the membrane 6 times for 5 minutes each in wash buffer to remove any unbound secondary antibody conjugate. It is crucial to thoroughly wash the membrane after incubation with the HRP enzyme conjugate.
7 	**Prepare substrate**	Prepare the substrate working solution by mixing equal parts of Detection Reagent 1 and Detection Reagent 2 (e.g., for a mini blot mix 5 mL Detection Reagent 1 with 5 mL Detection Reagent 2). Use a sufficient volume to ensure that the blot is completely wetted with the substrate and the blot does not become dry (0.1 mL/cm^2). **Note:** The working solution is stable for up to 1 hour at room temperature.
8 	**Develop substrate**	Incubate the membrane with the substrate working solution for 1 minute.
9 	**Image membrane**	a. Remove blot from working solution and place it in a plastic sheet protector or clear plastic wrap. b. Use an absorbent tissue to remove excess liquid and carefully press out any bubbles from between the blot and the membrane protector. c. Image the blot using an imaging system or X-ray film.

Troubleshooting

Observation	Cause	Solution
Reverse image on film (i.e., white bands with a black background)	Too much HRP in the system.	Further dilute the HRP-conjugate (see guidelines in Table 1).
Membrane has brown or yellow bands		
Blot glows in the darkroom		
Signal duration is less than 8 hours		
Weak or no signal	Too much HRP in the system depleted the substrate and caused the signal to fade quickly.	Further dilute the HRP-conjugate (see guidelines in Table 1).
	Insufficient quantities of antigen or antibody.	Increase amount of antibody or antigen.
		Use SuperSignal™ Western Blot Enhancer (Cat. No. 46640).
	Inefficient protein transfer.	Optimize transfer conditions.
	Reduction of HRP or substrate activity.	Test the activity of the system.[1]
High background	Too much HRP in the system.	Further dilute the HRP-conjugate (see guidelines in Table 1).
	Inadequate blocking.	Optimize blocking conditions.
	Inappropriate blocking reagent.	Try a different blocking reagent.
	Inadequate washing.	Increase length, number or volume of washes.
	Film has been overexposed.	Decrease exposure time or use Pierce Background Eliminator (Cat No. 21065).
	Concentration of antigen or antibody is too high.	Decrease amount of antigen or antibody.
	Poor antibody specificity.	Use SuperSignal™ Western Blot Enhancer (Cat. No. 46640).
Spots within the protein bands	Inefficient protein transfer.	Optimize transfer conditions.
	Unevenly hydrated membrane.	Perform manufacturer's recommendations for hydrating membrane properly.
	Bubble between the film and the membrane.	Remove bubbles before exposing blot to film.

[1] To test the activity of the system in the darkroom, prepare 1–2 mL of the SuperSignal™ Substrate Working Solution in a clear test tube. With the lights turned off, add 1 µL undiluted HRP-conjugate to the Working Solution. The solution should immediately emit a blue light that will fade over the next several minutes. If no light emission is evident, test another source of HRP to determine the root cause.

Troubleshooting

Observation	Cause	Solution
Speckled background on film	Aggregate formation in the HRP-conjugate.	Filter conjugate through a 0.2 µm filter.
Nonspecific bands	Too much HRP in the system.	Further dilute the HRP-conjugate (see guidelines in Table 1).
	SDS caused nonspecific binding to protein bands.	Do not use SDS during the Western blot procedure.
	Poor antibody specificity.	Use SuperSignal™ Western Blot Enhancer (Cat. No. 46640).
	Insufficient blocking.	Increase blocking time or use different blocking reagent.

Choosing the right substrate for your application

Thermo Scientific™ Substrate	Choose when...	Sensitivity	Signal Duration (hours)	Recommended Antibody Dilutions (From 1 mg/mL stock)
Pierce™ ECL	Detecting protein targets that are in high abundance and the sample is abundant.	Low picogram	1 to 2	1°: 1:1,000 2°: 1:1,000-1:15,000
SuperSignal™ West Pico PLUS	Performing routine western blot or setting up new procedures that need to be optimized.	Low picogram to high femtogram	Up to 24	1°: 1:1,000 2°: 1:20,000-1:100,000
SuperSignal™ West Dura	Performing quantitative western blots or if extended signal duration is necessary.	Mid-femtogram	24	1°: 1:5,000 2°: 1:50,000-1:250,000
SuperSignal™ West Atto	Detecting very low abundance protein targets, or if antibodies or sample volume is limited.	Low femtogram to high attogram	6	1°: 1:1,00-1:5,000 2°: 1:100,000-1:250,000

Related products

Product	URL
Western blot transfer devices and membranes	thermofisher.com/transfer
Blocking buffers, wash buffers and stripping buffers	thermofisher.com/westernbuffers
Enhanced chemiluminescence (ECL) kits	thermofisher.com/chemisubstrates
Western blot antibodies	thermofisher.com/antibodies
Western blot imaging and analysis	thermofisher.com/westernimaging