INSTRUCTIONS

Pierce ECL Plus Western Blotting Substrate

32132 32124

2412.2

Number	Description
32132	**Pierce ECL Plus Western Blotting Substrate,** sufficient reagents for 1000cm^2 of membrane **Contents:** **Substrate A,** 100mL **Substrate B,** 2.5mL
32134	**Pierce ECL Plus Western Blotting Substrate,** sufficient reagents for 250cm^2 of membrane **Contents:** **Substrate A,** 25mL **Substrate B,** 0.625mL

Storage: Upon receipt store at 4°C. Product shipped on ice.

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IMPORTANT NOTE: Use the same blotting conditions when switching from Amersham™ ECL Plus Western Blotting Detection Reagent to Thermo Scientific™ Pierce™ ECL Plus Western Blotting Substrate.

If you are currently using a Thermo Scientific™ SuperSignal™ Chemiluminescent Substrate, switching to Pierce ECL Plus Western Blotting Substrate requires increasing the antigen and antibody concentrations.

Introduction

The Thermo Scientific™ Pierce™ ECL Plus Western Blotting Substrate is a highly sensitive, nonradioactive, enhanced acridan-based chemiluminescent/chemifluorescent substrate for the detection of horseradish peroxidase (HRP) on immunoblots. Pierce ECL Plus Western Blotting Substrate enables picogram detection of antigen by converting the substrate to acridinium ester intermediates by HRP and peroxide. The reaction of the acridinium ester intermediates with peroxide produces a prolonged chemiluminescence, which can be visualized on X-ray film or an imaging system. Because Pierce ECL Plus Western Blotting Substrate is the same as Amersham ECL Plus Western Blotting Detection Reagent, there is no need for additional optimization of assay conditions when switching substrates.

Important Product Information

- If you are currently using a SuperSignal™ Chemiluminescent Substrate, switching to Pierce ECL Plus Western Blotting Substrate requires changing antigen and antibody concentrations.
- Pierce ECL Plus Western Blotting Substrate requires more dilute antibody concentrations than those used with precipitating colorimetric HRP substrates.
- Empirical testing is essential to determine the appropriate blocking reagent for each Western blot system, as cross-reactivity of the blocking reagent with antibodies can cause nonspecific signal and varying system sensitivity.
- When using avidin/biotin systems, avoid using milk as a blocking reagent as milk contains variable amounts of endogenous biotin, which causes high background signal.
- Use sufficient volumes of wash buffer, blocking buffer, antibody solution and substrate working solution to cover the blot and ensure that it never becomes dry. Using large blocking and wash buffer volumes minimizes nonspecific signal.
- For optimal results, use a shaking platform during incubation steps.
- Add Tween™ 20 Detergent (final concentration of 0.05-0.1%) to the blocking buffer and all diluted antibody solutions to minimize nonspecific signal. Use only high-quality products such as Thermo Scientific Surfact-Amps-20 (Product No. 28320), which is an ampule packaged, purified detergent guaranteed to be low in peroxides and other contaminants.
- Do not use sodium azide as a preservative for buffers. Sodium azide is an inhibitor of HRP and may interfere with this system.
- Do not handle the membrane with bare hands. Always wear gloves or use clean forceps.
- All equipment must be clean and free of foreign material. Metallic devices (e.g., scissors) must have no visible signs of rust. Rust may cause speckling and high background.
- Do not use polystyrene vessels to mix and prepare the substrate working solution; this type of plastic causes the solution to become cloudy and produce a precipitate.
- Exposure to the sun or any other intense light can harm the substrate. For best results keep the substrate working solution in an amber bottle and avoid prolonged exposure to any intense light. Short-term exposure to typical laboratory lighting will not harm the working solution.
- We offer a variety of protein transfer membranes, blocking buffers, primary antibodies, enzyme-labeled secondary antibodies, buffers and detergents. Please consult the website or catalog for product and ordering information.

Additional Materials Required

- **Dilution Buffer:** Tris buffered saline (TBS, Product No. 28376) or phosphate buffered saline (PBS, Product No. 28374)
- **Wash Buffer:** Add 5-10mL of 10% Tween-20 (Product No. 28320) to 1000mL of Dilution Buffer for a final Tween-20 concentration of 0.05-0.1%.
- **Blocking Reagent:** Add 0.5-1mL of 10% Tween-20 to 100mL of a blocking buffer such as Thermo Scientific StartingBlock (PBS) Blocking Buffer (Product No. 37538) or StartingBlock™ (TBS) Blocking Buffer (Product No. 37542). Choose a blocking buffer with the same base component as the Dilution Buffer.
- **Primary Antibody:** Choose an antibody specific to the target protein(s). Prepare the antibody stock solution in Dilution Buffer or Wash Buffer. Use Blocking Reagent or Wash Buffer to prepare a working dilution of **0.05-1µg/mL** or a **1:1000-1:20,000** dilution from a 1mg/mL stock. The optimal dilution to use depends on the specific Western blotting system, including the target antigen, the specific primary and secondary antibodies, and the type of membrane.
- **HRP-conjugated Secondary Antibody:** Choose an HRP-conjugate that specifically binds to the primary antibody. Prepare the antibody stock solution in Dilution Buffer or Wash Buffer. Use the Blocking Reagent or Wash Buffer to prepare a working dilution of **0.005-0.04µg/mL** or a **1:25,000-1:200,000** dilution from a 1mg/mL stock. This concentration range also applies when using either Thermo Scientific Streptavidin-HRP (Product No. 21124) or Thermo Scientific NeutrAvidin-HRP (Product No. 31001). The optimal antibody dilution depends on the specific Western blotting system, including the target antigen, the specific primary and secondary antibodies, and the type of membrane.
- Film cassette and developing and fixing reagents for processing autoradiographic film
- Rotary platform shaker for agitation of membrane during incubations

Procedure Summary

Note: Antigen and antibody amounts may require optimization.

1. Dilute the primary antibody to 0.05-1µg/mL.
2. Dilute the secondary antibody to 0.005-0.04µg/mL.
3. Mix Substrate A and Substrate B at a 40:1 ratio (i.e., 12mL Substrate A + 300µL Substrate B for 8 × 12cm^2 membrane) and add it to the blot. Incubate blot for 5 minutes.
4. Drain excess reagent from the membrane by tapping the membrane's edge with forceps against an absorbent tissue. Cover blot with a clear plastic sheet protector or clear plastic wrap. Remove any air bubbles.
5. Expose blot to X-ray film or imaging system.

Detailed Western Blotting Procedure

1. Remove blot from the transfer apparatus and block nonspecific sites with Blocking Reagent for 60 minutes at room temperature (RT) with shaking. If desired, block overnight at 2-8ºC without shaking.
2. Remove the Blocking Reagent and add the primary antibody working dilution. Incubate blot for 1 hour at RT with shaking or overnight at 2-8°C without shaking.
3. Briefly rinse membrane in Wash Buffer two times.
4. Wash membrane by suspending it in Wash Buffer and agitating for ≥ 5 minutes. Replace Wash Buffer at least 4-6 times. Increasing the Wash Buffer volume, the number of washes and wash duration may help minimize background signal.
5. Incubate blot with the secondary antibody HRP-conjugate working dilution for 30 minutes to 1 hour at RT with shaking.
6. Repeat Steps 3 and 4 to remove nonbound HRP-conjugate. The membrane MUST be thoroughly washed after incubation with the HRP-conjugate.
7. Prepare the substrate working solution by mixing Substrate A and Substrate B in a 40:1 ratio. Use 0.125mL working solution per cm^2 of membrane.

   Note: The working solution is stable for up to 1 hour at RT.
8. Incubate blot with working solution for 5 minutes at RT.
9. Remove blot from working solution and place it in a plastic sheet protector or clear plastic wrap. Use an absorbent tissue to remove excess liquid and carefully press out any bubbles from between the blot and the membrane protector.

   Note: For chemifluorescent detection, place the protected membrane on a Typhoon™ Imager with the protein side facing down. Scan using the 457nm excitation and 520 BP 40 emission filter in fluorescence/chemifluorescence mode with an appropriate resolution setting. The absorption/emission maxima of the substrate are 430/503nm.
10. Place the protected membrane in a film cassette with the protein side facing up. Turn off all lights except those appropriate for X-ray film exposure (e.g., a red safelight).

    Note: Film must remain dry during exposure. For optimal results, perform the following precautions:
    - Make sure excess substrate is removed from the membrane and the membrane protector.
    - Use gloves during the entire film-handling process.
    - Never place a blot on developed film, as chemicals on the film may reduce signal.
11. Carefully place X-ray film on top of the membrane. Perform a first-time exposure of 60 seconds. Vary the exposure time to achieve optimal results.

    Light emission is most intense during the first 5-30 minutes after substrate incubation. Light emission continues for several hours but decreases with time. Longer exposure times may be necessary as time elapses.

    If using a storage phosphor imaging device (e.g., Molecular Imager™ System from Bio-Rad) or a CCD Camera (e.g., ChemiImager™ System from Cell Biosciences), longer exposure times may be necessary.

    Caution: Any movement between the film and membrane can cause artifacts on the film.

12. Develop film using appropriate developing solution and fixative. If the signal is too intense, reduce exposure time or strip and re-probe the blot with decreased antibody concentrations. For best results, use Thermo Scientific™ Restore™ Western Blot Stripping Buffer (Product No. 21059) or Restore Plus Western Blot Stripping Buffer (Product No. 46430).

    Note: Thermo Scientific Pierce Background Eliminator (Product No. 21065) can be used to reduce background on X-ray film.

Troubleshooting

Problem	Possible Cause	Solution
White bands with a black background	Too much HRP in the system	Dilute HRP-conjugate further
Membrane has brown or yellow bands		
Blot glows in the darkroom		
Weak or no signal or signal fades quickly	Too much HRP exhausted the substrate	Dilute HRP-conjugate further
	Used insufficient quantities of antigen or antibodies	Strip and re-probe blot using increased amount of antibodies
	Inefficient protein transfer	Optimize transfer conditions
	Low HRP or substrate activity	See note below*
High background	Too much HRP in the system	Dilute HRP-conjugate further
	Inadequate blocking or used inappropriate blocking reagent	Optimize blocking conditions
	Washed inadequately	Increase duration, number and volume of washes
	Overexposed film	Decrease exposure time or use Pierce Background Eliminator (Product No. 21065)
	Used too much antigen and/or antibody	Strip and re-probe blot using decreased amount of antibodies
Spots within the protein bands	Inefficient protein transfer	Optimize transfer procedure
	Unevenly hydrated membrane	Hydrate membrane according to manufacturer's instructions
	Bubble between X-ray film and membrane	Remove all bubbles before exposing blot to film
Speckled background	Aggregate formed in the HRP-conjugate	Filter HRP-conjugate through a 0.2µm filter before use
	Over-heated during electrophoresis or transfer	Control temperature during electrophoresis and transfer
Nonspecific bands	Too much HRP-conjugate	Strip and re-probe blot using a more dilute HRP-conjugate
	SDS caused nonspecific binding to protein	Do not use SDS during immunoassay procedure

*To test system activity, in a darkroom, prepare 1-2mL of the substrate working solution in a clear test tube. With the lights turned off, add 1µL of undiluted HRP-conjugate to the working solution. The solution should immediately emit a blue light that fades during the next several minutes.

Related Thermo Scientific Products

34090	**CL-XPosure™ Film, 5" × 7" sheets**, 100 sheets/pkg
34080	**SuperSignal West Pico Chemiluminescent Substrate**, 500mL
34075	**SuperSignal West Dura Extended Duration Substrate**, 100mL
34095	**SuperSignal West Femto Maximum Sensitivity Substrate**, 100mL
46640	**SuperSignal Western Blot Enhancer**, 500mL kit
21059	**Restore Western Blot Stripping Buffer**, 500mL
21065	**Pierce Background Eliminator Kit**, for eliminating background from X-ray film
37515	**SuperBlock (PBS) Blocking Buffer**, 1L
37535	**SuperBlock (TBS) Blocking Buffer**, 1L
32110	**Pierce Antibody Extender NC**, 500mL, for using three times less primary antibody on nitrocellulose membranes while maintaining signal intensity
88018	**Nitrocellulose Membrane**, 0.45µm, 33cm × 3m, 1 roll
77010	**Nitrocellulose Membrane**, 0.45µm, 8 × 12cm, 25/pkg.
88025	**Nitrocellulose Membrane**, 0.45µm, 8 × 8cm, 15/pkg.
88600	**Western Blotting Filter Paper**, 8cm × 10.5cm, 100 sheets

General References

CRC Handbook of Immunoblotting of Proteins: Volume 1 Technical Description. Eds Ole J. Bjerrum, Ph.D., M.D. and Niels H.H. Heegaard, M.D. CRC Press, Inc.: Boca Raton, FL, 1988.

Kaufmann, S.H., *et al.* (1987). The erasable Western blot. *Anal Biochem* **161:**89-95.

Mattson, D.L. and Bellehumeur, T.G. (1996). Comparison of three chemiluminescent horseradish peroxidase substrates for immunoblotting. *Anal Biochem* **240:**306-8.

Walker, G.R., *et al.* (1995). SuperSignal CL-HRP: A new enhanced chemiluminescent substrate for the development of the horseradish peroxide label in Western blotting applications. *J of NIH Research* **7**:76.