

Cat. # T7104A

For Research Use

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**TAKARA**

**Western BLoT Ultra  
Sensitive HRP Substrate**

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Product Manual

v201912

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## I. Description

Western BLoT Ultra Sensitive HRP Substrate allows detection of very small amounts of protein during Western blot analysis. This chemiluminescence substrate offers the highest sensitivity, and can be used to detect horseradish peroxidase (HRP)-labeled antibodies bound to target proteins on immunoblots. It is possible to detect protein levels in the femtogram range using this product.

This substrate maintains a strong emission signal with linear signal across a wide dynamic range, allowing you to take full advantage of CCD camera detection sensitivity. Since background is very low, this system can be used for long exposure times, enabling detection of very small amounts of target protein. This product is useful when detecting minute amounts of target protein or when a limited amount of primary antibody is available.

Western BLoT Ultra Sensitive HRP Substrate can be also used for X-ray film detection. However, since the emission intensity is extremely high, it may be necessary to limit the exposure time and adjust the antibody dilution when using X-ray film.

<Western BLoT HRP Substrate series>

Western BLoT Chemiluminescence HRP Substrate	(Cat. #T7101A)
Western BLoT Quant HRP Substrate	(Cat. #T7102A)
Western BLoT Hyper HRP Substrate	(Cat. #T7103A)
Western BLoT Ultra Sensitive HRP Substrate	(Cat. #T7104A)

## II. Components (100 ml, for 1,000 cm<sup>2</sup> of membrane)

Western BLoT Ultra Sensitive Luminol/Enhancer Solution	50 ml (light-resistant bottle)
Western BLoT Ultra Sensitive Peroxide Reagent	50 ml (clear bottle)

## III. Storage

4°C

## IV. Materials Required but not Provided

1. Protein blotted membrane:

After separating the protein sample(s) by electrophoresis, transfer proteins to a membrane by Western blotting. If the protein-blotted membrane is to be stored, soak in TBS-T or PBS-T (TBS or PBS containing 0.05% Tween 20) and store at 4°C.

2. Dilution buffer and wash buffer:

Use TBS or PBS as dilution buffer, and use TBS-T or PBS-T as wash buffer.

These can be prepared using the following products:

TBS : Tris Buffered Saline (TBS) Tablets, pH 7.6 (Cat. #T9141)  
TBS Powder (Cat. #T903)

PBS : Phosphate Buffered Saline (PBS) Tablets, pH 7.4 (Cat. #T9181)  
PBS Powder (Cat. #T900)

Phosphate Buffered Saline (PBS) Tablets without Potassium, pH 7.4 (Cat. #T9182)

TBS-T : Tris Buffered Saline with Tween 20 (TBS-T) Tablets, pH 7.6 (Cat. #T9142)

PBS-T : Phosphate Buffered Saline with Tween 20 (PBS-T) Tablets, pH 7.4 (Cat. #T9183)

3. Blocking buffer:  
Dissolve the blocking reagent in the same type of buffer used for antibody dilution with Tween 20 (i.e., TBS-T or PBS-T).  
Examples of blocking reagents:
  - Nonfat dry milk : recommended concentration 1 - 5%  
Start with 5% concentration at first and adjust as needed.  
Nonfat dry milk is the most commonly used blocking reagent, but it interferes with the detection of phosphorylated proteins.
  - BSA : 0.3 - 3%
  - Gelatin : 2%  
Gelatin is not a suitable blocking reagent when using biotinylated secondary antibodies.

The products below are conveniently available.

Western BLoT Blocking Buffer (Fish Gelatin) (Cat. #T7131A)  
Western BLoT Blocking Buffer (Protein Free) (Cat. #T7132A)

**Note:** For additional recommendations regarding blocking buffers, refer to V. Precautions.

4. Primary antibody:  
Choose an antibody that is specific to the target protein. Prepare a stock solution of this antibody in dilution buffer (e.g. 1 mg/ml). Use blocking buffer to make all working dilutions. Prepare the primary antibody working dilution. The appropriate dilution depends on the specific primary antibody and the amount of antigen on the membrane and requires optimization for each experimental system.
5. HRP-labeled secondary antibody:  
Prepare a 1 mg/ml stock solution of a HRP-conjugate secondary antibody that specifically binds the primary antibody. Prepare a working dilution by diluting with the blocking buffer. The optimal dilution varies depending on the HRP-conjugate antibody and the amount of antigen on the membrane. It is possible to improve the sensitivity of detection using Western BLoT Immuno Booster (Cat. #T7111A) instead of dilution buffer. For more information, refer to the User Manual for Western BLoT Immuno Booster.
6. CCD camera  
(Or in the case of X-ray film detection: film cassettes, developing solution, fixing solution, etc.)
7. Tray (large enough to hold the membrane)
8. Platform shaker: Use to agitate the membrane during the antibody reaction, washing, etc.
9. Plastic wrap
10. Plastic tweezers

## V. Precautions

These are precautions when using this product. **Please be sure to review prior to use.**

1. It is important to optimize the amount of sample, concentrations of primary antibody and secondary antibody, and membrane type, buffer, and blocking reagent to be used for each experiment. The high sensitivity level provided by Western BLoT Ultra Sensitive HRP Substrate should reduce the amount of primary and secondary antibodies needed in comparison to other common conventional substrates that are commercially available.
2. The concentration of antibody required for HRP detection is much less than is required for the chromogenic method. Please run preliminary tests (e.g., dot blots) and optimize the antibody concentration appropriately.
3. No blocking reagent is optimal for all systems. Therefore, it is essential to determine the appropriate blocking buffer for each Western blot system. Determining the proper blocking buffer can increase sensitivity and prevent non-specific signal caused by cross-reactivity between the antibody and the blocking reagent.
4. Use adequate volumes of wash buffer, blocking buffer, antibody solution, and Western BLoT Ultra Sensitive Working Solution to cover the membrane sufficiently and ensure that it does not dry out. Using adequate quantities of blocking buffer and wash buffer can reduce non-specific signal.
5. Non-fat dry milk can be used as blocking buffer with this system.  
Caution: Since non-fat dry milk contains biotin and phosphorylated proteins, it cannot be used in avidin-biotin detection systems or for detecting phosphorylated proteins.
6. To obtain the best results, please agitate the membrane throughout each incubation step using a shaker.
7. To reduce non-specific signal, add Tween 20 at a final concentration of 0.05% in blocking buffer and all antibody dilution solutions.
8. Do not use sodium azide as a preservative for buffers. Sodium azide is an HRP inhibitor and will inhibit the reaction in this system.
9. Do not handle the membrane with bare hands. Always wear powder-free gloves or use plastic tweezers. All containers and other materials used throughout the procedure should be free of dirt or foreign matter. Metal apparatuses, such as scissors, should be free of rust. Rust may cause signal irregularity or high background.
10. Western BLoT Ultra Sensitive Working Solution is stable for several hours at room temperature, but exposure to direct sunlight or strong light may cause the Working Solution to become unstable. Shield the Working Solution from light by placing it in a light-proof bottle or wrapping it in aluminum foil, and use it as quickly as possible after preparation.

## VI. Protocol

### 1. Blocking:

Prepare blocking buffer in a tray (0.5 ml for every 1 cm<sup>2</sup> of membrane). Remove the membrane from the Western blotting apparatus and soak in blocking buffer. Shake for 1 hour at room temperature. Discard the blocking buffer and wash twice with an adequate quantity of fresh wash buffer.

**2. Primary antibody incubation:**

Dilute primary antibody to an appropriate concentration with blocking buffer. Recommended antibody concentrations for use with this kit are as follows:

Recommended dilution ratios for stock antibody solution (1 mg/ml)

Primary antibody 1 : 3,000 - 1 : 30,000

Secondary antibody 1 : 10,000 - 1 : 300,000

Soak the membrane in primary antibody solution diluted with blocking buffer and shake gently for 1 hour at room temperature or overnight at 4°C. Rinse the membrane briefly twice with an adequate quantity of fresh wash buffer. Then, add fresh wash buffer and shake at room temperature according to the following washing conditions:

5 min x 4 to 6 times (use 4 ml of wash buffer per 1 cm<sup>2</sup> of membrane)

\* If using X-ray film for detection, wash 6 times to reduce background.

**3. HRP-labeled secondary antibody:**

Soak the membrane in secondary antibody diluted with blocking buffer and shake for 1 hour at room temperature. Rinse the membrane briefly twice with an adequate quantity of fresh wash buffer.

Wash with fresh wash buffer at room temperature according to the following washing conditions:

5 min x 4 to 6 times (use 4 ml of wash buffer per 1 cm<sup>2</sup> of membrane)

\* If using X-ray film for detection, wash 6 times to reduce background.

**4. Detection:**

Bring this kit to room temperature prior to use. Prepare Working Solution by mixing equal volumes of Luminol/Enhancer Solution and Peroxide Reagent. Use 0.1 ml of Working Solution for every 1 cm<sup>2</sup> of membrane. For an 8 cm x 10 cm membrane, mix 4 ml of each reagent to prepare a total of 8 ml of Working Solution. Prepare Working Solution immediately prior to use.

Remove excess wash buffer from the membrane.

Place the membrane horizontally on a piece of plastic wrap so that the surface of the blot faces upward. Apply Working Solution so that it is completely covers the membrane surface.



Let stand for 5 min at room temperature.



Pick up the membrane using tweezers and remove excess Working Solution by applying a laboratory tissue to the edge of the membrane.

Wrap the membrane in fresh plastic wrap. Make sure only one layer of plastic wrap covers the blotted surface of the membrane. Avoid introducing air bubbles or forming creases.



Detect chemiluminescent signal.

Detection using a CCD camera:

Set the membrane in the CCD camera imaging system and detect signal. Adjust settings (sensitivity, resolution, exposure time, etc.) according to the instrument manual for the imaging system.

Detection using X-ray film:

Set the membrane on a film cassette so that the surface of the blot faces upward.

Place X-ray film above the blot surface and close the cassette. We recommend an initial trial exposure time of 60 seconds. Adjust the exposure time as needed to obtain the best results. Develop the X-ray film.

## VII. Troubleshooting

Since Western blotting is a multi-step process, it may be necessary to optimize conditions. We recommend preliminary investigations to determine the appropriate quantity of protein, the optimum dilution ratios for primary and secondary antibodies, and other parameters.

Problem	Cause	Solution
High background	The concentration of primary antibody used is too high.	Increase the dilution factor of the primary antibody to decrease the antibody concentration.
	The concentration of HRP used is too high.	Increase dilution factor of the secondary antibody.
	Too much antigen has been used.	Reduce the quantity of antigen.
	Blocking is insufficient.	Optimize blocking conditions.
	The blocking reagent is unsuitable.	Use a different type of blocking reagent
	Exposure time is too long (if detecting with X-ray film).	Shorten the exposure time.
	Washing is insufficient.	Increase the washing time, number of washes, or volume of wash buffer.
No band is visible or signal is weak	The primary antibody is unsuitable.	Confirm that the primary antibody recognizes the target protein, and the protein is not degraded.
	The type of secondary antibody is unsuitable.	Confirm that the secondary antibody recognizes the primary antibody and is not degraded.
	Quantities of antigen or antibody are insufficient.	Increase the quantity of antigen or antibody.
	Transfer of protein is insufficient.	Optimize transfer conditions.
	Concentration of HRP is too high (may cause attenuated signal by premature depletion of substrate).	Increase the dilution factor of the secondary antibody to decrease the HRP concentration.
	The film exposure time is too short (if using X-ray film for detection).	Increase the exposure time.
	Reduced activity of HRP or substrate has occurred.	Use fresh reagents. Prepare Working Solution just prior to detection and use within a few hours.
White dots are present in the band	Efficiency of protein transfer is poor.	Optimize transfer conditions.
	Membrane hydration is uneven.	Hydrate membrane properly.
	Air bubbles have formed between the gel and membrane.	Remove all air bubbles before starting the transfer process.
	With X-ray film: Air bubbles present between the film and membrane	Remove all air bubbles prior to exposure.
Irregular background	Secondary antibody has aggregated.	Filter the secondary antibody.
	Particles (e.g., dust) are contaminating the blocking buffer and/or wash buffer.	Filter the blocking buffer and/or wash buffer. Perform experiments in a clean environment and cover the container during incubation and wash steps.
	Unsuitable glove type.	Use powder-free gloves (nitrile, polyethylene, etc.)
Membrane emits signal visible to the unaided eye in darkness; Brown bands are present on the membrane; An inverse image forms	The concentration of HRP used is too high.	Increase the dilution factor of the secondary antibody to decrease the HRP concentration.
Non-specific bands appear	Signal duration is too long (the concentration of HRP is excessive).	Increase the dilution factor of the secondary antibody to decrease the HRP concentration.
	The concentration of primary antibody used is too high	Increase the dilution factor of the primary antibody to decrease the antibody concentration.

## VIII. Related Products

- < Western BLoT HRP Substrate Series >
  - Western BLoT Chemiluminescence HRP Substrate (Cat. #T7101A/B)
  - Western BLoT Quant HRP Substrate (Cat. #T7102A/B)
  - Western BLoT Hyper HRP Substrate (Cat. #T7103A/B)
  
- < Western Blot Chemiluminescence Enhancer >
  - Western BLoT Immuno Booster (Cat. #T7111A)
  - Western BLoT Immuno Booster PF (Cat. #T7115A)
  
- < In place of a labeled secondary antibody >
  - Western BLoT Rapid Detect v2.0 (Cat. #T7122A)
  
- < Blocking Buffer >
  - Western BLoT Blocking Buffer (Fish Gelatin) (Cat. #T7131A)
  - Western BLoT Blocking Buffer (Protein Free) (Cat. #T7132A)
  
- < Stripping Buffer >
  - Western BLoT Stripping Buffer (Cat. #T7135A)
  
- < Buffer Tablets and Powders >
  - Tris-Glycine-SDS Buffer (TG-SDS) Powder, pH 8.3 (Cat. #T9101)
  - Tris-Glycine Buffer (TG) Powder, pH 8.3 (Cat. #T9102)
  - Tris Buffered Saline (TBS) Tablets, pH 7.6 (Cat. #T9141)
  - Tris Buffered Saline with Tween20 (TBS-T) Tablets, pH 7.6 (Cat. #T9142)
  - Phosphate Buffered Saline (PBS) Tablets, pH 7.4 (Cat. #T9181)
  - Phosphate Buffered Saline (PBS) Tablets without Potassium, pH 7.4 (Cat. #T9182)
  - Phosphate Buffered Saline with Tween20 (PBS-T) Tablets, pH 7.4 (Cat. #T9183)
  
- < Protein Ladder Marker >
  - CLEARLY Protein Ladder (Unstained) (Cat. #3453A/B)
  - CLEARLY Stained Protein Ladder (Cat. #3453A/B)

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**NOTE:** This product is for research use only. It is not intended for use in therapeutic or diagnostic procedures for humans or animals. Also, do not use this product as food, cosmetic, or household item, etc.

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