

Cat. # T7103A

For Research Use

TAKARA

**Western BLoT Hyper
HRP Substrate**

Product Manual

v201211_2_201802

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I. Description

The Western BLoT Hyper HRP Substrate is a horseradish peroxidase (HRP) substrate specially developed for Western blotting detection of very low-abundance proteins. Western BLoT Hyper produces a strong, long-lived signal. These features in combination with very low background levels, allows for long exposure times and enables the detection of low-abundance proteins. Additionally, the signal from this product is linear with respect to protein amount over a broad range of concentrations.

Western BLoT Hyper HRP is more sensitive than Western BLoT Quant HRP Substrate, and is suitable for detection of low-abundance proteins or in situations when the amount of primary antibody is very limited and a high dilution factor is desired, or when the primary antibody has a relatively low binding affinity. Western BLoT Hyper HRP is also compatible with X-ray film detection, though the limited dynamic range of film will make resulting data less amenable to quantification.

<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 cm² of membrane)

Western BLoT Hyper Luminol/Enhancer Solution	50 ml (light-proof bottle)
Western BLoT Hyper Peroxide Reagent	50 ml (clear bottle)

III. Storage

4°C

* Store at the recommended temperature and use within one year.

IV. Materials Required but not Provided

1. Protein blotted membrane:
After separating the protein sample(s) via 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. Either nitrocellulose membranes or PVDF membranes can be used following the manufacturer's protocol.
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, pH7.6 (Cat. #T9141)
 - PBS : Phosphate Buffered Saline (PBS) Tablets, pH7.4 (Cat.#T9181)
Phosphate Buffered Saline (PBS) Tablets without Potassium, pH7.4 (Cat. #T9182)
 - TBS-T : Tris Buffered Saline with Tween® 20 (TBS-T) Tablets, pH7.6 (Cat. #T9142)
 - PBS-T : Phosphate Buffered Saline with Tween® 20 (PBS-T) Tablets, pH7.4 (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% as a default concentration and adjust as needed. Nonfat dry milk is the most commonly used blocking agent, 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.

Note : For additional recommendations regarding blocking buffers, please 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 that specifically binds the primary antibody. Use the blocking buffer for all dilutions. Prepare a working dilution. The optimal dilution varies depending on the HRP-conjugate and the amount of antigen on the membrane.
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 agent to be used for each experiment.
2. The concentration of antibody required for HRP detection is much less than is required for the chromogenic method. Please run preliminary tests 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 Working Solution to sufficiently cover the membrane and ensure that the membrane 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 the Western BLoT Chemiluminescence HRP Substrate.
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 Working Solution is stable for several hours at room temperature, but exposure to direct sunlight or strong light may cause degradation. 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 cm² 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 : 1,000 - 1 : 10,000 (for detection with CCD camera)

Secondary antibody 1 : 5,000 - 1 : 20,000 (for detection with CCD camera)

* When detecting with X-ray film, dilute antibodies 2 to 5-fold more than the concentrations indicated above.

Incubate the membrane in primary antibody solution 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. Then, add fresh wash buffer and shake at room temperature according to the following washing conditions:

15 minutes (use 0.7 ml of wash buffer per 1 cm² of membrane)



5 minutes x 3 times (use 0.3 ml of wash buffer per 1 cm² of membrane)

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 minutes x 3 times (use 0.3 - 0.7 ml of wash buffer per 1 cm² of membrane)

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 cm² 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 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 completely covers the surface.



Let stand for 2 minutes 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. Light emission is maintained for several hours, allowing repeated imaging of the membrane. Approximately 70% of the signal will remain after 1 hour.

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 three initial trial exposure times of 30 seconds, 2 minutes, and 5 minutes. 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 the dilution factor of the secondary antibody to decrease the HRP concentration.
	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.
	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 immediately 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.
	Air bubbles have formed between the film and membrane (if using X-ray film for detection)	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.
	The gloves being used are unsuitable.	Use powder-free gloves or change the type of 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. Reduce the concentration of HRP.
Non-specific bands appear	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)
Western BLoT Quant HRP Substrate	(Cat. #T7102A)
Western BLoT Ultra Sensitive HRP Substrate	(Cat. #T7104A)
<Western blot chemiluminescence enhancer>	
Western BLoT Immuno Booster	(Cat. #T7111A)
<Instead of HRP labeled secondary antibody>	
Western BLoT Rapid Detect	(Cat. #T7121A)
PBS (Phosphate Buffered Salts) Tablets	(Cat. #T900)
TBS (Tris-Buffered Saline) powder	(Cat. #T903)
Tris-Glycine-SDS Buffer (TG-SDS) Powder, pH8.3	(Cat. #T9101)
Tris-Glycine Buffer (TG) Powder, pH8.3	(Cat. #T9102)
Tris Buffered Saline (TBS) Tablets, pH7.6	(Cat. #T9141)
Tris Buffered Saline with Tween®20 (TBS-T) Tablets, pH7.6	(Cat. #T9142)
Phosphate Buffered Saline (PBS) Tablets, pH7.4	(Cat. #T9181)
Phosphate Buffered Saline (PBS) Tablets without Potassium, pH7.4	(Cat. #T9182)
Phosphate Buffered Saline with Tween®20 (PBS-T) Tablets, pH7.4	(Cat. #T9183)

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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