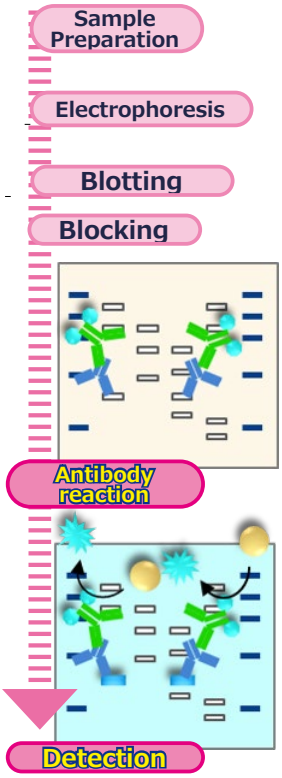
Western Blotting - Antibody reaction and Detection -

Application Note

1. Overview

In Western blotting, target protein bands are probed with antibodies and further detected via enzymes or fluorescence. The sensitivity and signal intensity of the antibody reaction are affected by the dilution ratio of the antibody, reaction time and temperature, and the detection method (luminescence, chromogenesis, fluorescence, etc.) and the type of enzyme substrate, resulting in significantly different experimental results. In this article, we will introduce the antibody reaction and detection using ATTO' s products.

2. Experiment Flow



Proteins are separated by SDS-PAGE. If the molecular weight of the target protein is unknown, a gradient gel is used. Also, use a colored marker to determine the transcription efficiency and molecular weight. If the expression levels are to be compared, a control sample is run together.

After electrophoresis, the separated proteins are transferred from the gel to a PVDF membrane.

Blocking is performed to prevent nonspecific binding to the surface of the membrane where no bands are present.

Gel,Buffer,Molecular weight maker
ePAGEL HR, EzRun, EzProteinLadder
Transfer buffer,Blocking reagent
EzFastBlot HMW, EzBlockChemi,
Electrophoresis,Power supply
PageRun-Ace, MyPower,
Blotter
HorizeBLOT

Reacts with antibodies against the target protein (primary antibodies) and enzyme-labeled antibodies (secondary antibodies).

Antibody,Wash buffer
EzTBS, EzTween,
shaker
SeesawShaker atto

The target protein are detected indirectly by reacting the enzyme labeled on the secondary antibody with a chromogenic or luminescent substrate.

Substrate
EzWestLumiOne,EzWestLumi plus,
EzWestBlue W
Chemiluminescence Imager
LuminoGraph series

3. Protocol

3-1. Electrophoresis, Blotting,Blocking

Electrophoresis

High molecular weight

: Use acrylamide gels with the lowest possible concentration. Acrylamide gels with a concentration of less than 7.5% are difficult to handle, in which case a concentration gradient gel should be used.

Low molecular weight

: Highly concentrated acrylamide gels, which are suitable for separating low molecular weight bands, significantly reduce transcription efficiency. Using the electrophoresis buffer EzRun MOPS, low molecular weight bands can be separated even in acrylamide gels of around 10%, and the uniform concentration of acrylamide prevents gel deformation before and after blotting and enables highly efficient transcription.

Blotting

High molecular weight

: Since the transcription efficiency of high molecular weight proteins above 150 kDa is significantly lower, semi-dry blotting using EzFastBlot HMW, a transfer buffer specifically designed for high molecular weight proteins, or a tank-type blotter should be used. In addition, equilibrating the gel with transfer buffer (about 30 minutes) before transcription may increase the protein transcription efficiency. Note, however, that prolonged equilibration time may result in the loss of low-molecular-weight bands or band diffusion.

Low molecular weight

: When transcribing low molecular weight proteins (~200 kDa), EzFastBlot can be used for highly efficient transcription in a short time of about 10 minutes. Since low molecular weight proteins may escape from the blotting membrane, a PVDF membrane (P plus membrane) with a pore size of 0.2 μ m should be used. Adding methanol to the transfer buffer improves adsorption to the membrane (do not add methanol to the EzFastBlot series).

Blocking

Select the blocking agent according to the target protein. If the blocking reaction time is too long, overblocking will occur, resulting in poor detection sensitivity. Conversely, if the blocking reaction time is too short, the protein will not be blocked sufficiently, causing background to rise and nonspecific bands to appear.

3-2. Preparation for Antibody Reaction and Detection

Prepare antibodies while blocking is being done.

Wash buffer Prepare 500 mL per minigel.

TBS-T: EzTween (or 0.01~0.1%Tween 20) including 1xEzTBS (or 50 mM Tris, 137 mM NaCl, 2.7 mM KCl/pH7.4)

PBS-T: EzTween (or 0.01~0.1%Tween 20) including 1xEzPBS (or 137 mM NaCl, 2.7 mM KCl, 81 mM Na_2HPO_4, 14.7 mM KH_2PO_4

*When detecting phosphorylated proteins, use TBS-T as much as possible.

Antibody

Generally, a primary antibody against the target protein and a secondary antibody labeled with an enzyme or fluorescence for detection are prepared; if the primary antibody is directly labeled, a secondary antibody is not required.

The dilution rate of the antibodies should be determined by referring to the package inserts for the antibodies and detection reagents. If samples and time are available, the dilution rate should be considered to achieve the appropriate antigen detection sensitivity for dot blotting. The antibody should be diluted immediately before the antibody reaction. If the antibody is left in a diluted state for a long time, it may be inactivated.

Detection reagent

If an antibody labeled with a fluorescent dye is used, no detection reagent is required. Use an excitation light suitable for each fluorescent dye, and separate the excited fluorescence at a specific wavelength using a filter or other means to acquire the fluorescence.

When using antibodies labeled with enzymes such as ALP (alkaline phosphatase) or HRP (horseradish peroxidase), the signal is acquired using a detection reagent consisting of the substrate of the respective enzyme. ATTO sells the following three types of detection reagents for HRP.

Antibody selection

primary antibody : Select the appropriate antibody for the purpose of the experiment by referring to the antigen (Antigen), cross reactivity (Cross reactivity), immunized animal (host), application (WB, IP, ICC,,,,), and application data. If you wish to detect without secondary antibody, select a labeled antibody.

(Example) Human transferrin antibody for Western blotting
Anti-HumanTransferrin **Rabbit Poly** etc.

Antigen: Human transferrin Immunized Animal: Rabbit
ntigen: (Human) transferrin (other peptides, expressed proteins, etc.)
Cross species: Human, mouse, rat (must contain human*)
Immunized Animal: Rabbit (and other mice, goats, etc.)
Application: WB, EIA, IP (must contain WB)

secondary antibody : The same as for primary antibodies, but select a labeled antibody (HRP, ALP, Rhodamine,,,,) appropriate for the detection method.

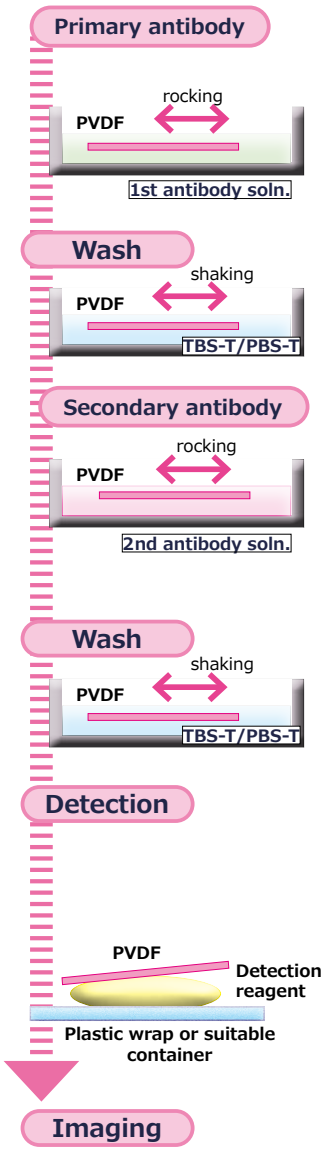
(Example) Secondary antibody against the above primary antibody
Goat **Anti-Rabbit IgG H&L** **(HRP)** など
Immunized Animal: Goat Antigen: Rabbit IgG (heavy chain light chain)
Labeling: HRP
Antigen: Rabbit IgGpr (antibody of the immunizing animal for the primary antibody; if the primary antibody is a monoclonal antibody, note the isotype of the antibody (IgG, IgA, IgM, etc.), and if the antibody is Fab fragmented, select antibodies that recognize only the heavy chain (Fc) to be excluded)
Cross-species: None (as few cross-species as possible)
Immune animals: goat (without rabbit, which is the immune animal for the primary antibody, and human, which is the target protein*)
Uses: WB, EIA, IP (those that always contain WB)
Labeling: HRP (generally HRP or ALP for luminescence detection)

*If the antigen is human

Reagent	EzWestBlue	EzWestLumiOne	EzWestLumi plus
Cat#	AE-1490	WSE-7110	WSE-7120
Detection	coloring 1 liquid	Luminescence 1 liquid	Luminescence mix 2 liquid
Substrate	TMB	Luminol	Luminol
Sensitivity	10 pg/band	low pg/band	fg midrange/band
Duration	ND	1 hour~	3 hour~
amount used	0.05mL/cm^2	0.05mL/cm^2	0.05mL/cm^2

3-3. Antibody Reaction and Detection Methods

Antibody reaction~detection reaction and photography are performed according to the following



After blocking is completed, discard the blocking solution and add the primary antibody against the target protein diluted with TBS-T, etc. The dilution rate of the primary antibody should be determined based on the information provided on the accompanying sheet of the purchased antibody. Generally, dilute the antibody 100~5,000 times with TBS(-T) or PBS(-T). Incubate in a shaker for approximately 1 hour at room temperature or overnight at 4° C in general. The temperature and time should be considered depending on the antibody.

Discard the antibody solution and rinse gently with a small amount of TBS-T/PBS-T. Add approximately 50 mL of new TBS-T/PBS-T per membrane and incubate in a shaker for 5 minutes. Repeat this washing process three times.

Discard the wash solution (TBS-T/PBS-T) and add the enzyme- or fluorescently-labeled secondary antibody. the dilution rate of the secondary antibody should be determined based on the information provided on the accompanying sheets of the purchased antibody and detection reagent. Generally, dilute 5,000~500,000x in TBS-T/PBS-T. If the background is high, use a blocking solution (diluted accordingly) instead of TBS-T/PBS-T. Incubate in a shaker at room temperature for approximately 1 hour or overnight at 4 ° C in general.

Discard the antibody solution and rinse gently with a small amount of TBS-T/PBS-T. Add approximately 50 mL of new TBS-T/PBS-T per membrane and incubate in a shaker for 5 minutes. Repeat this washing process three times.

Weigh, mix, and otherwise adjust the detection reagent immediately prior to detection. Pour all of the adjusted detection solution into a clean plastic wrap or container. Pick up the blotting membrane (PVDF membrane) with tweezers, etc. and remove excess TBS-T/PBS-T completely with paper towels, etc. Immerse the blotting membrane in the detection reagent. Be careful not to allow air to enter the membrane so that the detection reagent is spread quickly and evenly across the membrane. Ensure that the detection reagent is fully absorbed on both the front and back of the membrane. For chromogenic detection, react until sufficient color is obtained, then rinse with distilled water and photograph the signal. For luminescence detection, the membrane is sealed in plastic wrap or film sheet and photographed with a photographic device.

High-sensitivity luminescence/ fluorescence imaging equipment is used to capture luminescence or fluorescence signals.

Luminescence Imaging

Set the aperture to fully open and use no filters or other devices. Place the sealed blotting membrane in the center of the imaging area and bring it into focus. Select a shooting mode (Standard, High, etc.), set the shooting time (5 seconds, 1 minute, 5 minutes, etc.), and shoot. If colored markers are used, a bright field image is also taken to obtain a merged image.

Fluorescence Imaging

Use the appropriate excitation light for the labeled fluorophore and the appropriate filter for fluorescence acquisition. Place the sealed blotting membrane in the center of the imaging area and focus the camera with the excitation light on.

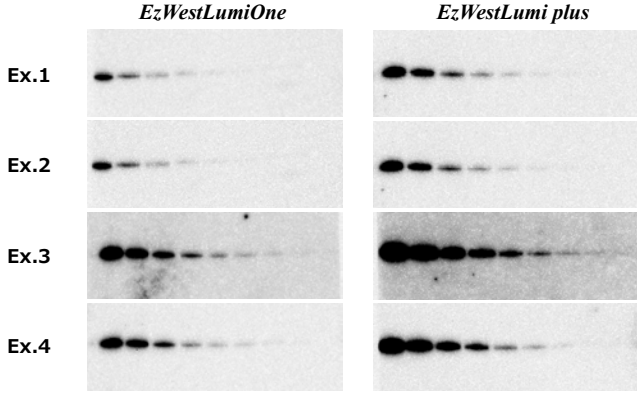
Related products WSE-6170 LuminoGraph I CMOS

The LuminoGraph I CMOS is a chemiluminescence imager with a next-generation cooled CMOS camera. It allows easy luminescence imaging for Western blotting and high-sensitivity detection.

Camera	cooled CMOS camera
Resolution	2688 × 1512 16bit Tiff image
Gain	HQ/STD/High/Ultra 4step
Capture	AutoExposure/Sum (Saturation detection function)
Control	Windows PC

3-4. Reference data

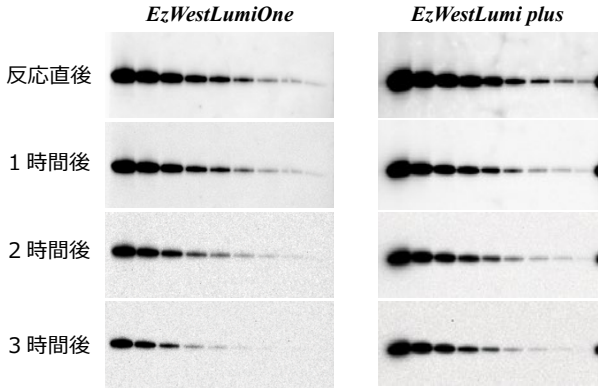
Example 1: Effect of antibody dilution concentration



	primary antibody dilution	secondary antibody dilution
Ex.1	1/10,000	1/50,000
Ex.2	1/4,000	1/50,000
Ex.3	1/4,000	1/10,000
Ex.4	1/2,000	1/50,000
reaction	RT 3 hour	RT 1 hour

The above figure shows the results of electrophoresis of 200 pg/lane to 1/2 dilution of human transferrin and detection with human transferrin antibody and HRP-conjugated secondary antibody. plus. Compared to condition 2, condition 1 has less than half the primary antibody concentration, but the results are almost the same. On the other hand, conditions 2 and 3 have the same primary antibody concentration, but the secondary antibody concentration is five times higher, and comparing these results, condition 3 has much better signal intensity and detection sensitivity. Conditions 1 and 4 have the same secondary antibody concentration, but the primary antibody concentration is five times higher. The detection sensitivity is higher with the higher primary antibody concentration, but the signal remains weak. Thus, even using blotting membranes that were transferred by swimming the same amount of protein, the detection sensitivity and signal intensity differed greatly depending on the concentration of primary and secondary antibodies. Of course, detection sensitivity also differs depending on the detection reagent, but it is clear that it is important to consider not only the detection reagent but also the antibody concentration.

Example 2: Detection Sensitivity and Duration



The above figure shows the results of electrophoresis of 500 pg/lane to 1/2 diluted human transferrin, reaction of the transferred blotting membrane with 1/4,000 diluted human transferrin antibody and 1/10,000 diluted HRP-conjugated secondary antibody, and detection of bands with EzWestLumiOne or EzWestLumi PLUS. As shown in the above results, EzWestLumiOne showed stable signals for more than 1 hour, and EzWestLumi plus showed stable signals for up to 3 hours, although the luminescence intensity was slightly reduced.

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