User Guide for

Immobilon™ Western

Chemiluminescent HRP Substrate

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Introduction

Chemiluminescent detection uses an enzyme to catalyze a reaction that results in the generation of visible light. The horseradish peroxidase (HRP) chemiluminescent reaction is based on the catalyzed oxidation of luminol by peroxide. Oxidized luminol emits light as it decays to its ground state. This technique has the speed and safety of chromogenic detection methods, at higher sensitivity levels.

Immobilon Western HRP Substrate provides high sensitivity in western or dot/slot/spot blotting applications on both PVDF and nitrocellulose transfer membranes, and is compatible with all commonly used buffers and blocking reagents. Blots on PVDF membrane may be reprobed, allowing detection of multiple target proteins on the same blot.

The HRP substrate consists of Luminol Reagent and Peroxide Solution. Working HRP substrate is prepared by combining equal volumes of Luminol Reagent and Peroxide Solution. The HRP substrate produces a high intensity signal with low background for detection of both high and low abundance proteins.

For more detailed immunodetection protocols, troubleshooting and background information on western blotting, see the Millipore Protein Blotting Handbook (pub. no. TP001 available on-line at www.millipore.com/publications.nsf/docs/tp001).

Kit Contents

Kit No. WBKL S00 50
covers 500 cm²
of membrane area
Luminol Reagent, 25 mL
Peroxide Solution, 25 mL

Kit No. WBKL S01 00
covers 1,000 cm²
of membrane area
Luminol Reagent, 50 mL
Peroxide Solution, 50 mL

Kit No. WBKL S05 00
covers 5,000 cm²
of membrane area
Luminol Reagent, 250 mL
Peroxide Solution, 250 mL

Storage conditions: 2–8 °C

Materials Required for Western Blotting

- Immobilon-P or Immobilon P^{SQ} PVDF membrane; or other PVDF or nitrocellulose membrane
- Sheets of filter paper, cut to dimension of the gel
- 100% methanol (to pre-wet PVDF membrane)
- Transfer system and transfer buffer
- Milli-Q® water

CAUTION: Stored water may contain pyrogens that may interfere with immunodetection.

Materials Required for Western Blotting, continued

- Wash buffer: Phosphate-buffered saline (PBS) or Tris-buffered saline (TBS) containing 0.05–0.1% Tween®-20 surfactant;
  PBS: 10 mM sodium phosphate, 150 mM NaCl, pH 7.2
  TBS: 10 mM Tris, 150 mM NaCl, pH 7.4
- Blocking buffer: 1–5% (w/v) blocking agent (e.g., casein, BSA, or nonfat dry milk) in wash buffer

  NOTE: Immobilon Western HRP Substrate is compatible with all blocking buffers. 2% casein solution is recommended for the lowest background and highest signal-to-noise ratio.
- Primary antibody specific for the protein of interest, diluted in wash buffer or blocking buffer
- HRP-conjugated secondary antibody, specific for primary antibody, diluted in wash buffer or blocking buffer
- Shallow trays, large enough to hold the blot
- Plastic wrap, plastic bag, transparency or sheet protector
- X-ray film and developer reagents or chemiluminescence-compatible imaging systems

Usage Guidelines

- Due to the high sensitivity of the Immobilon Western HRP Substrate, lower amounts of antigen and higher dilutions of primary and secondary antibodies are recommended. Typical primary antibody dilutions are 1:1,000–1:20,000 and secondary antibody dilutions typically range from 1:20,000–1:200,000. **IMPORTANT: If switching to Immobilon Western HRP Substrate from a lower sensitivity substrate, previous antibody dilution factors may need to be increased at least five-fold for the primary antibody and two- to five-fold for the secondary antibody to achieve the optimal signal-to-noise ratio.**
- Optimization of blocking reagents and incubation times will improve results and should be determined experimentally.
- The high sensitivity of the Immobilon Western HRP Substrate may result in a significant reduction in required x-ray film exposure time. An initial exposure time of 30 seconds is recommended. Optimum exposure time should be determined for each antibody system.
- Always wear gloves and use blunt tip forceps (such as Millipore cat. no. XX62 000 06) when handling the membrane to avoid contamination.
- Use care when handling the membrane to prevent tearing.
- Do not use sodium azide, which inhibits HRP activity, in any buffers or reagents.
- Use of blocking buffer to dilute antibodies may reduce background and increase sensitivity.

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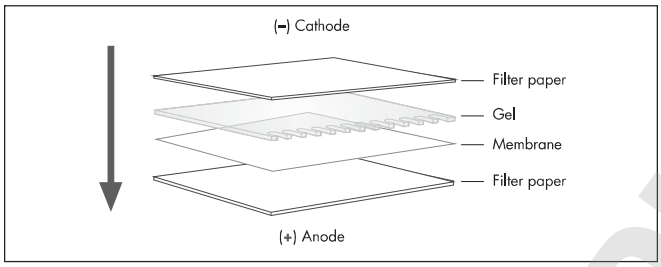
Western Blotting Protocol

Protein Transfer

1. Resolve the protein mixture on a 1-D or 2-D polyacrylamide gel.
2. Immerse the gel in an appropriate transfer buffer and allow it to equilibrate for 10–15 minutes.
3. **If working with a PVDF membrane:** Wet the membrane in 100% methanol for 15 seconds, or until the membrane appearance changes uniformly from opaque to semitransparent.
   If working with a nitrocellulose membrane: Proceed to step 4. Nitrocellulose membranes do not require prewetting.
4. Equilibrate the membrane for at least 5 minutes in the transfer buffer.
5. Soak filter paper in the transfer buffer for at least 30 seconds.
6. Assemble the transfer stack as shown below.

   CAUTION: To ensure an even transfer, remove air bubbles by carefully rolling a clean pipette over the surface of each layer in the stack. Avoid excessive pressure that can damage the gel and membrane.



7. Transfer proteins according to blotting apparatus manufacturer's instructions.
8. Remove the blot from the transfer system and briefly rinse the membrane in Milli-Q water to remove gel debris. Proceed with immunodetection protocol below. If required, the PVDF membrane blot may be air dried and stored refrigerated for several months.

Antibody Incubations

1. If PVDF membranes were dried after transfer, wet the blots in 100% methanol for 15 seconds. The blot will uniformly change from opaque to semitransparent.
   NOTE: Omit this step if using nitrocellulose membrane.
2. Rinse the blot with water and then place the blot in blocking buffer and incubate for 1 hour with gentle agitation at room temperature.
3. Prepare primary antibody solution by diluting the antibody in wash or blocking buffer. See Usage Guidelines for antibody dilutions.
4. Place the blot in the diluted primary antibody solution and incubate for at least 1 hour with gentle agitation. Ensure that the solution moves freely across the entire surface of the membrane.
5. Wash the blot with fresh wash buffer a minimum of three times with gentle agitation for 5–10 minutes. Additional or longer washes may further reduce background. Immobilon-P^{SQ} (0.2 µm) membrane may require additional washing due to its greater surface area.

Antibody Incubations, continued

6. Prepare HRP-conjugated secondary antibody solution by diluting the antibody in wash or blocking buffer. See Usage Guidelines for antibody dilutions.
7. Place the blot in the diluted HRP-conjugated secondary antibody solution, and incubate for 1 hour with gentle agitation. Ensure that the solution moves freely across the entire surface of the membrane.
8. Wash the blot with fresh wash buffer a minimum of three times with gentle agitation for 5–10 minutes. Additional or longer washes may further reduce background.

Chemiluminescent Detection

1. To prepare working HRP substrate, mix equal volumes of Luminol Reagent and Peroxide Solution in a clean container or test tube. Approximately 0.1 mL of working HRP substrate is required per cm^2 membrane area.

   The volumes of working HRP substrate needed for some common membrane sizes are indicated below:

Blot Size	Working HRP Substrate Required	
7 × 8.5 cm	6 mL	(3 mL luminol reagent + 3 mL peroxide solution)
10 × 10 cm	10 mL	(5 mL luminol reagent + 5 mL peroxide solution)
8.5 × 13.5 cm	12 mL	(6 mL luminol reagent + 6 mL peroxide solution)

2. Allow the HRP substrate to reach room temperature (~10 minutes). Protection from light is not required.
3. Place the blot protein side up in a clean container, and add the HRP substrate onto the blot.
4. Incubate the blot for 5 minutes at room temperature.
5. Drain the excess substrate.
6. Cover the blot with a clean plastic wrap or sheet protector and remove any air bubbles. Ensure that the surface of the plastic wrap or sheet protector is dry and unwrinkled.
7. Expose the blot to a suitable X-ray film for an appropriate duration. Because of the high sensitivity of the Immobilon Western HRP Substrate, a shorter exposure time may be required. The recommended initial exposure time is 30 seconds. The chemiluminescent signal on the blot will last at least two hours. If necessary, fresh HRP substrate can be added to the same blot for consecutive exposures.

   NOTE: The working HRP substrate can be stored up to 7 days in the dark at 2–8 °C without any detectable loss of activity.

Membrane Stripping (Only for PVDF Membranes)

A single blot on PVDF membrane can be sequentially probed by stripping the first antibody from the blot, and then incubating with a different primary antibody. This is especially useful for method optimization or when sample amount is limited. The stripping process disrupts the antigen-antibody interaction, usually by a combination of detergent and heat or by exposure to low pH. Please refer to the Millipore Protein Blotting Handbook (TP001) for detailed stripping protocols.

Troubleshooting

This section describes solutions to problems you may encounter with western blotting protocols. Further information on related protocols, troubleshooting and background information on western blotting, can be found in the "Protein Blotting Handbook" (pub. no. TP001) available on-line at www.millipore.com/publications.nsf/docs/tp001.

Problem	Possible Cause	Solution
High background	Insufficient washes	Increase wash buffer volumes and wash cycle repetitions. Pre-filter all solutions including the transfer buffer using 0.45 µm Millex® syringe filter units or Steriflip® filter units.
	Poor quality blotting reagents or buffers	Use high grade reagents and Milli-Q water.
	Cross-reactivity between blocking reagent and antibody	Use Tween-20 surfactant in the washing buffer or use different blocking agent. Do not use milk as blocking agent when using avidin-biotin systems as milk contains endogenous biotin.
	Membrane drying during incubation processes	Use sufficient volumes to cover entire surface of the membrane during incubations.
	Poor quality antibodies	Use high quality affinity-purified antibodies.
	Secondary (enzyme-conjugated) antibody concentration is too high	Decrease the antibody concentration and/or reduce x-ray exposure time. See Usage Guidelines.
	Protein-protein interactions	Use Tween-20 (0.05–0.1%) surfactant in the wash and detection solutions to minimize protein-protein interactions and increase the signal-to-noise ratio.
	Excess detection reagents	Drain blots completely before exposure.
	Film has been overexposed	Shorten exposure time. Initial exposure of 30 seconds is recommended.
	Membrane was not re-blocked after stripping of the initial detection	The membrane has to be blocked with the appropriate blocking agent prior to re-probing of the blot.
on Immobilon-P^{SQ} transfer membrane	Insufficient washing or blocking	Increase the concentration or volume of the blocking agent used to compensate for the greater surface area of the membrane. In addition, incubation times for both the wash and blocking steps may need to be extended.
Weak or no signal	Inefficient protein transfer	Optimize protein transfer. If necessary stain the blot to visualize protein and confirm complete transfer. See Protein Blotting Handbook (TP001) for recommendations.
	Antigen concentration is too low	Load more antigen on the gel.
	Azide inhibits HRP	Do not use azide in the blotting solutions.
	Incorrect blocking reagent	The blocking agent may have an affinity for the protein of interest and thus obscure the protein from detection. Try a different blocking agent and/or reduce either the amount or exposure time of the blocking agent.
	Antigen is being washed off the membrane by Tween-20 surfactant	Reduce or eliminate the use of Tween-20 surfactant except for the wash step following membrane blocking. Tween-20 surfactant should be 0.05–0.1%.
	Primary antibody was raised against native protein	Separate proteins under non-denaturing conditions or use antibody to denatured antigen.
	Antibody concentration is too low	Increase both primary and secondary antibody concentration.
	Antibody is inactive	Multiple freeze-thaw or bacterial contamination of antibody solution can change antibody titer or activity. Prepare fresh antibody working solutions.
	Insufficient antibody reaction time	Increase the incubation time.
	Outdated substrate	Prepare fresh working HRP substrate and store properly. Outdated substrate can reduce sensitivity.
Speckled background	Aggregates in the blocking reagent	Filter blocking reagent solution through 0.45 µm Millex filter unit.
	Aggregates in HRP-conjugated secondary antibody	Filter secondary antibody solution through 0.45 µm Millex filter or Steriflip unit.
Non-specific bands	Primary antibody concentration too high	Increase primary antibody dilution.
	Secondary antibody concentration too high	Increase secondary antibody dilution.
	Antigen concentration too high	Decrease amount of protein loaded on the gel.
Reverse images on film (white bands on dark background)	Too much HRP-conjugated antibody	Reduce concentration of secondary, HRP-conjugated antibody.
Poor detection of small proteins	Small proteins are masked by large blocking molecules such as BSA	Consider using casein, gelatin or a low molecular weight polyvinylpyrrolidone (PVP) as blocking reagents. Surfactants such as Tween-20 and Triton® X-100 may have to be minimized. Avoid excessive incubation times with antibody and wash solutions. Use Immobilon-P^{SQ} membrane for small proteins (< 20 kDa).
	Primary antibody specificity too low	Use a different antibody.
Uneven blot	Fingerprints, fold marks or forceps imprints on the blot	Avoid touching membrane with bare hands or folding membrane; use gloves and blunt end forceps.
	Bubble between the membrane and X-ray film	Check blot for bubbles before exposure to the X-ray film.
	Air bubbles were trapped during transfer	Using a pipette or a stirring rod, gently roll out any trapped air bubbles while assembling the transfer stack.
	Fiber pads in wet tank transfer assembly are not clean	Clean fiber pads thoroughly to remove salts and soak in transfer buffer before assembling the transfer stack.

Ordering Information

Millipore offers Immobilon Western Chemiluminescent Substrates and a variety of PVDF membranes for western blotting applications. See the Technical Assistance section for information about contacting Millipore Corporation.

Description	Qty	Catalogue No.
Immobilon Western Chemiluminescent Substrates for Western Blotting Applications		
HRP Substrate	50 mL	WBKL S00 50
	100 mL	WBKL S01 00
	500 mL	WBKL S05 00
AP Substrate	25 mL	WBKD S00 25
	100 mL	WBKD S01 00

PVDF Membranes

Type	Size	Qty/Pk	Catalogue No.
Immobilon-P Blotting Sandwiches (0.45 µm pore size) for General Western Blotting Applications			
Pre-cut PVDF Sandwiches	8.5 × 13.5 cm	20	IPSN 081 32
	7 × 8.4 cm	20	IPSN 078 52
Each sandwich includes one sheet of Immobilon-P membrane and two sheets of blotting filter paper.			
Immobilon-P Membrane (0.45 µm pore size) for General Western Blotting Applications			
Roll	26.5 × 375 cm	1	IPVH 000 10
Cut Sheet	26 × 26 cm	10	IPVH 304 F0
	20 × 20 cm	10	IPVH 202 00
	15 × 15 cm	10	IPVH 151 50
	10 × 10 cm	10	IPVH 101 00
	9 × 12 cm	10	IPVH 091 20
	8.5 × 13.5 cm	10	IPVH 081 30
	8 × 10 cm	10	IPVH 081 00
	7 × 8.4 cm	50	IPVH 078 50
Immobilon-P^{SQ} Membrane (0.2 µm pore size) for Blotting Proteins < 20 kDa.			
Roll	26.5 × 375 cm	1	ISEQ 000 10
Cut Sheet	26 × 26 cm	10	ISEQ 262 60
	20 × 20 cm	10	ISEQ 202 00
	15 × 15 cm	10	ISEQ 151 50
	10 × 10 cm	10	ISEQ 101 00
	9 × 12 cm	10	ISEQ 091 20
	8.5 × 13.5 cm	10	ISEQ 081 30
	8 × 10 cm	10	ISEQ 081 00
	7 × 8.4 cm	50	ISEQ 078 50
Immobilon-FL Membrane (0.45 µm pore size) for Fluorescence Detection Applications			
Roll	26.5 × 375 cm	1	IPFL 000 10
Cut Sheet	20 × 20 cm	10	IPFL 202 00
	10 × 10 cm	10	IPFL 101 00
Blotting Filter Paper			
Cut Sheet	7 × 8.4 cm	100	IBFP 078 5C
Cut Sheet	8.5 × 13.5 cm	100	IBFP 081 BC

Technical Assistance

For more information, contact the Millipore office nearest you. In the U.S., call **1-800-MILLIPORE** (1-800-645-5476). Outside the U.S., see your Millipore catalogue for the phone number of the office nearest you or go to our web site at www.millipore.com/offices for up-to-date worldwide contact information. You can also visit the tech service page on our web site at www.millipore.com/techservice.

Standard Warranty

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