

# Immobilon® Classico Western HRP Substrate

## Introduction

Immobilon® Classico Western HRP Substrate is a premixed, ready-to-use reagent for chemiluminescent detection in western blotting applications that employ horseradish peroxidase (HRP)-conjugated antibodies. Immobilon® Classico Substrate is ideal for the detection of high to medium abundance proteins. It can be used with traditional immunodetection techniques or the SNAP i.d.® 2.0 Protein Detection System. Visualization of target proteins is achieved through digital imaging with a CCD camera or x-ray film.

## Immobilon® Classico Package Contents

Item number	Volume	Membrane Coverage
WBLUC0020	20 mL	200 cm <sup>2</sup>
WBLUC0100	100 mL	1,000 cm <sup>2</sup>
WBLUC0500	500 mL	5,000 cm <sup>2</sup>

## Storage/Shelf Life

Store at 2–8 °C. Store at room temperature. Protection from light is not required. Refer to bottle label for expiration date.

## Usage Guidelines

Immobilon® Classico Substrate is slightly more sensitive than other chemiluminescent HRP substrates, but it is less sensitive than Immobilon® Crescendo, Immobilon® Forte, and the original Immobilon® Western HRP Substrates. Compared with these three detection reagents, Immobilon® Classico Substrate requires a higher concentration of antigen and/or antibodies. See the table below for general guidelines.

Immobilon® HRP Substrate	Classico	Crescendo	Forte	Western	Ultra
Sensitivity	•	••	•••	••••	•••••
Antibody Concentration	•••	••	•	•	•

Prior to using this product, review the Safety Data Sheet (SDS) to ensure awareness of associated hazards and use of appropriate controls.

## Other Important Considerations

- If switching to Immobilon® Classico Substrate from a colorimetric substrate or other Immobilon® Substrate, the primary and secondary antibody concentrations may need to be decreased to achieve the optimal signal-to-noise ratio.
- If switching to Immobilon® Classico Substrate from Immobilon® Crescendo, Immobilon® Forte or Immobilon® HRP Substrates, the primary and secondary antibody concentrations may need to be increased to achieve the optimal signal-to-noise ratio, or the x-ray film exposure time may need to be increased.
- Optimum exposure time to the x-ray film should be determined for each antibody system.
- Use of blocking buffer to dilute antibodies may reduce background and increase sensitivity.
- To avoid membrane tearing or high background in the blots, always wear gloves and use blunt tip forceps (cat. no. XX6200006P) when handling the membrane.
- Never use objects (scissors or forceps) that show visible signs of rusting, since they may create undesirable artifacts or high background areas.

- Do not use sodium azide in any blocking buffers or wash solutions, since it inhibits HRP activity.

## Chemiluminescent Detection

Approximately 0.1 mL of HRP substrate is required per cm<sup>2</sup> of membrane area. The volumes of HRP substrate needed for some common membrane sizes are indicated below:

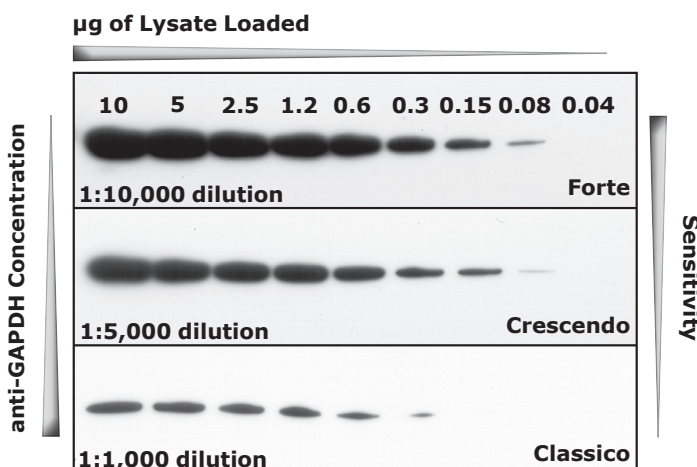
Blot Size (cm)	HRP Substrate Required
7 × 8.4	6 mL
10 × 10	10 mL
8.5 × 13.5	12 mL

1. Place the blot protein-side up in a container or clear plastic sheet protector, and add the HRP substrate onto the blot.
2. Incubate the blot for 2 to 5 minutes at room temperature.
3. Drain the excess substrate, transfer to a clean sheet protector, and cover the blot with plastic wrap or another sheet protector.
4. Expose the blot to a suitable x-ray film for an appropriate amount of time. Typical exposure time for Immobilon® Classico Substrate is 1 to 5 minutes. The chemiluminescent signal on the blot will last for at least 30 minutes. If higher sensitivity is desired, the detection procedure can be repeated on the same blot, using Immobilon® Crescendo, Immobilon® Forte, or Immobilon® HRP Substrate.

## Proof of Performance

### Comparison of Signals from Immobilon® Forte, Crescendo, and Classico HRP Substrates

Blots of A431 cell lysates (a series of two-fold dilutions ranging from 10 µg to 0.04 µg) were prepared using Immobilon®-P membrane and probed with varying concentrations of anti-GAPDH antibody on the SNAP i.d.® 2.0 Protein Detection System. Following incubation with a goat anti-mouse HRP-conjugated secondary antibody, the blots were visualized with Immobilon® Classico, Crescendo, or Forte Substrate.



## Troubleshooting

Symptom	Possible Cause	Solution
High background Negative staining (white bands on black background) Nonspecific bands	Ab concentration is too high.	Reduce exposure time. Decrease Ab concentration. Use a lower sensitivity HRP detection substrate. Decrease antigen concentration.
Signal disappears quickly in a blot that initially had a very high signal	High HRP-Ab concentration has exhausted the substrate prematurely.	Decrease Ab concentration significantly. Use a lower sensitivity HRP detection substrate. Decrease antigen concentration.
Weak or no signal	Ab concentration is too low.	Expose to x-ray for longer period of time. Increase Ab concentration. Increase antigen concentration. Change the blocking solution.

## Ordering Information

This section lists the catalogue numbers for Immobilon® Classico Substrate and related products. You can purchase these products on-line at [www.SigmaAldrich.com/westernblot](http://www.SigmaAldrich.com/westernblot) or see contact information below.

### Immobilon® Western Chemiluminescent Substrates for Western Blotting Applications

Description	Qty	Cat. No.
Immobilon® Classico Western HRP Substrate	20 mL	WBLUC0020
	100 mL	WBLUC0100
	500 mL	WBLUC0500
Immobilon® Crescendo Western HRP Substrate	20 mL	WBLUR0020
	100 mL	WBLUR0100
	500 mL	WBLUR0500
Immobilon® Forte Western HRP Substrate	20 mL	WBLUF0020
	100 mL	WBLUF0100
	500 mL	WBLUF0500
Immobilon® Western HRP Substrate	50 mL	WBKLS0050
	100 mL	WBKLS0100
	500 mL	WBKLS0500
Immobilon® ECL Ultra Western HRP Substrate	100 mL	WBULS0100
	500 mL	WBULS0500

### Other Western Blotting Related Products

Immobilon® Block-CH Chemiluminescent Blocker	500 mL	WBAVDCH01
Immobilon® Block-FL Fluorescent Blocker	500 mL	WBAVDFL01
Immobilon® Block-PO Phosphoprotein Blocker	500 mL	WBAVDP001
ReBlot™ Western Blot Recycling Kit	1	2060
ReBlot™ Plus Kit	1	2500

## Immobilon®-E PVDF Membrane (0.45 µm pore size) for General Western Blotting Applications

Description	Qty/Pk	Cat. No.
26.5 × 187.5 cm roll	1	IEVH00005
7 × 8.4 cm sheet	4	IEVH07804
7 × 8.4 cm sheet	50	IEVH07850
8 × 10 cm sheet	10	IEVH08100
9 × 12 cm sheet	10	IEVH09120
10 × 10 cm sheet	10	IEVH10100
Blotting Sandwich 7 × 8.4 cm	20	IESN07852
Blotting Sandwich 8.5 × 13.5 cm	20	IESN08132

### Related Products for General Western Blotting Applications

Immobilon® blotting filter paper, 7 × 8.4 cm sheet	100	IBFP0785C
Immobilon® blotting filter paper, 8.5 × 13.5 cm sheet	100	IBFP0813C
Ponceau S solution, 0.1% (w/v) in 5% acetic acid	1 L	P7170

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## Contact Information

For the location of the office nearest you, go to [www.millipore.com/offices](http://www.millipore.com/offices).

## Technical Assistance

Visit the tech service page on our web site at [www.millipore.com/techservice](http://www.millipore.com/techservice).

## Standard Warranty

The applicable warranty for the products listed in this publication may be found at [www.millipore.com/terms](http://www.millipore.com/terms) ("Conditions of Sale").

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