Immun-Star™ WesternC™ Chemiluminescent Kit

Instruction Manual

For Use With Nitrocellulose and PVDF Membranes

Catalog #

170-5070

For technical support, call your local Bio-Rad office, or in the US, call 1-800-4BIORAD (1800-424-6723)

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IMPORTANT PRODUCT INFORMATION

- For best results, it is IMPORTANT to optimize all components of this kit, including sample amount, antibody concentration, blocking reagent, and choice of membrane.
- The antibody concentration required will be much more dilute than those used with precipitating colorimetric-HRP systems. Please perform a systematic dot blot analysis for optimization.
- Empirical testing is necessary to determine the optimal blocking reagent for each Western blot system. The proper blocking reagent can increase sensitivity and prevent nonspecific signal due to cross-reactivity between the antibody and the blocking reagent.

- Avoid using milk as a blocking reagent when using avidin/biotin systems because milk contains variable amounts of endogenous biotin.
- Use sufficient volume of wash buffer, blocking reagent, antibody solution and substrate solution to cover blot and ensure that it never becomes dry.
- For optimal results, use a shaking platform during the incubation steps.
- Add Tween 20 (final concentration of 0.05%) to the blocking buffer when preparing all antibody dilutions to reduce nonspecific signal.
- Do not use sodium azide as a preservative for buffers. Sodium azide is an inhibitor of HRP and could interfere with this system.

- Do not handle membrane with bare hands.
- Substrate solution is stable for 24 hours at room temperature. Exposure to sun or other intense light can harm the working solution. For best results, keep the substrate solution in an amber bottle.

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Section 1
Preparation

1.1 Introduction

The Immun-Star™ WesternC™ kit provides a highly sensitive chemiluminescent substrate with the long light emission necessary for optimization of results on CCD imagers. Chemiluminescence is a chemical reaction associated with the production of light, which has become a common detection method for western blotting because of its enhanced sensitivity (mid-femtogram level of detection). In the presence of hydrogen peroxide, the horseradish peroxidase (HRP) that is conjugated to the secondary antibody catalyzes the oxidation of luminol. When oxidized luminol returns to its original state, light is produced. An enhancer is used to increase the longevity

and intensity of the emitted signal. When combined with a Bio-Rad Molecular Imager® ChemiDoc™ XRS or Molecular Imager® VersaDoc™ MP imager, the Immun-Star WesternC kit can produce clear digital images that can be easily enhanced for publication purposes.

1.2 Immun-Star WesternC Kit Products

Catalog #	Immun-Star Product Description
170-5070	Immun-Star WesternC Kit, includes 50 ml luminol/enhancer, 50 ml peroxide solution
170-5070S	Immun-Star WesternC Kit, includes 10 ml luminol/enhancer, 10 ml peroxide solution

1.3 Storage and Stability

Substrates should be stored at room temperature.

Section 2
Assay Instructions

2.1 Working Solutions

Tris-Buffered Saline (1x TBS) – (20 mM Tris, 150 mM NaCl, pH 7.5)

If using Bio-Rad liquid concentrate 10x TBS (catalog # 170-6435), add 100 ml of 10x TBS to 900 ml of dd water.

Alternatively, phosphate buffered saline (PBS) can be used. Add 100 ml of 10x PBS (catalog # 161-0780) to 900 ml of dd water.

Or, to mix your own wash buffer, use the following table.

1x TBS	1x PBS
For TBS (1 L) mix and dissolve: 8 g NaCl 0.2 g of KCl 3 g of Tris base 800 ml of dd water	For PBS (1 L) mix and dissolve: 8 g of NaCl 0.2 g of KCl 1.44 g of Na_2HPO_4 0.24 g KH_2PO_4 800 ml of dd water

Adjust pH to 7.5 and bring volume to 1 L with dd water.

Wash Solution, (TTBS) — (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5)

Add 0.5 ml Tween 20 (catalog # 170-6531) to 1 L TBS solution to make 0.05% TTBS.

Blocking Solution — 5% nonfat dry milk in TTBS

Mix 60 ml of TTBS with 3 g of nonfat dry milk (catalog # 170-6404); this is enough for one 8.5 x 13.5 cm Criterion™ blot. Alternatively, 3% bovine serum albumin (BSA) can also be used as a blocking solution.

Primary Antibody Solution

1:1,000–1:50,000 dilution of primary antibody from a 1 mg/ml stock in TTBS. 60 ml is sufficient for one 8.5 x 13.5 cm Criterion™ blot.

Secondary Antibody Conjugate Solution

The recommended secondary antibody dilution is 1:50,000–1:250,000 (1 mg/ml starting concentration) in TTBS. 60 ml is sufficient for one 8.5 x 13.5 cm Criterion blot.

Substrate Solution

1:1 mixture of luminol/enhancer and peroxide buffer. 12 ml total solution (6 ml of luminol/enhancer and 6 ml of peroxide solution) is sufficient for one 8.5 x 13.5 cm Criterion blot

Note: PBS can be substituted for TBS in the recipes above.

2.2 Detailed Assay Procedure

Note: Before starting, read through the entire procedure.

1. **Electrophoretic Blotting –** The antigens of interest are electrophoretically transferred to the membrane from a gel (i.e., SDS-PAGE gel, IEF gel, or native gel) using the Bio-Rad Trans-Blot® cell (catalog # 170-3939), Mini Trans-Blot® cell (catalog # 170-3930), Criterion blotter (catalog # 170-4070) or Trans-Blot® SD transfer cell (catalog # 170-3940). Please refer to instruction manual provided with this equipment for proper directions.

2. **Blocking –** Block with 5% nonfat dry milk dissolved in TTBS (0.05% Tween 20) or 3% BSA for 60 min at room temperature on a shaker. Decant blocker.

3. **Primary Antibody Incubation –** Incubate the membrane with primary antibody (1:1000–1:50,000 dilution) in TTBS for 60 min at room temperature on a shaker with gentle agitation, or overnight at 4°C.

4. **Wash –** Decant the primary antibody solution and wash with TTBS. A large volume of wash buffer with five changes is recommended. Each wash should last 10 min, with **strong** agitation at room temperature. The large volume of buffer with frequent changes helps to reduce background. Decant final wash solution. Using 200 ml for a 8.5 x 13.5 cm membrane is recommended. Please ensure the membrane is covered by liquid at all times.

5. **Secondary Antibody Incubation –** Incubate the membrane with secondary antibody, 1:50,000–1:250,000 dilution in TTBS, for 60 min at room temperature with gentle agitation. Decant secondary antibody.

Note: When using Bio-Rad Precision Plus Protein™ WesternC™ standards, we recommend diluting the StrepTactin-HRP conjugate to 1:10,000–1:50,000 for chemiluminescent detection.

6. **Wash –** Wash the membrane with TTBS for 10 min with **strong** agitation; repeat six times using a large volume of wash buffer to help reduce background. Decant wash solution.

7. **Blot Development –** Mix luminol/enhancer and peroxide buffer solutions in a 1:1 ratio; 0.125 ml of substrate solution/cm^2 (12 ml is sufficient for one 8.5 cm x 13.5 cm Criterion gel). Incubate the membrane in the substrate mixture for 3 to 5 min. Make sure the surface of the blot is completely covered with substrate (no air bubbles; do not allow blot to dry out).

8. **Drain –** Take out the membrane and drain off excess substrate by touching one

corner of the membrane to a piece of paper towel in order to draw the excess solution off the membrane surface. Do not allow blot to dry out. Membrane can be placed in a membrane protector, such as plastic wrap, to prevent drying. Remove any bubbles between protector and membrane.

9. **Camera Detection –** An imaging system, such as the Bio-Rad Molecular Imager ChemiDoc XRS or Molecular Imager VersaDoc MP should be used to quantitatively and qualitatively analyze protein blots. Please refer to the instruction manual for these systems for a detailed operation procedure.

10. **Film Detection –** Film may be used for chemiluminescent detection, however, higher dilution of the primary and secondary antibodies may be necessary.

Section 3
Troubleshooting Guide

Problem	Probable Cause	Recommended Solution
No signal	a) Exposure time was too short	Increase exposure time
	b) Blot was allowed to dry after incubation with substrate	Use heat-sealable bags to prevent drying of membrane
	c) Substrate is inactive	Store reagent at proper temperature
	d) Primary antibody solution is inactive	Store antibody at temperature, avoid bacterial contamination, heat inactivation, and repeated freeze-thaw cycles
		Antibody titer is too low. Increase concentration of antibody used in assay

Problem	Probable Cause	Recommended Solution
	e) Conjugate is inactive	The concentration of the conjugate was insufficient; increase the the conjugate concentration Conjugate may be contaminated causing inactivation of antibody or enzyme Use only dd water to make solutions
	f) Little or no antigen is bound to the membrane	Tween 20 may wash bound antigen from the membrane. Decrease amount of Tween 20 in the assay Check transfer technique, equipment, and buffer dilutions Stain membrane with protein stain, such as Ponceau S to ensure transfer efficiency

Problem	Probable Cause	Recommended Solution
Weak signal	a) Check all points above	
	b) Insufficient protein loaded	Load more protein on the gel
	c) Azide inhibits HRP	Do NOT use azide to prevent bacterial contamination of solutions
High background	a) Exposure time was too long	Decrease exposure time
	b) Blocking was insufficient	Increase time of blocking step and/or the concentration of blocker used
	c) Wash stringency and number of washes were insufficient	Tween 20 is necessary to help reduce background. The concentration can be increased up to 0.3%; increase number and length of washes

Problem	Probable Cause	Recommended Solution
	d) Secondary antibody was used at an excessive concentration	Use the recommended dilution of conjugate or determine optimal dilution experimentally
	e) Contamination occurred during transfer	Refer to blotting apparatus instruction manual for recommendations
	f) Solutions and buffers are contaminated	Autoclave or sterile filter dd water prior to making solutions
		Use blotting-grade nonfat dry milk for blocking. This product has been QC tested for acceptable background temperatures
		Avoid bacterial contamination of all solutions by storing at the appropriate temperatures

Problem	Probable Cause	Recommended Solution
Signal too strong	a) Protein is overloaded	Load less protein in the gel
	b) Antibody is too concentrated	Optimize antibody solution; see page 4 for recommendations
	c) Incorrect gel running and blotting conditions	Check gel and buffer recipes, optimize conditions
	d) Inappropriate incubation time with substrate	Decrease the incubation time

Section 4
Ordering Information

Catalog #	Description
170-5070	Immun-Star WesternC Kit, includes 50 ml luminol/enhancer, 50 ml peroxide solution
170-5070S	Immun-Star WesternC Kit, includes 10 ml luminol/enhancer, 10 ml peroxide solution

Electrophoresis/Blotting Products

161-0376	Precision Plus Protein WesternC Standards, 250 µl
161-0380	Precision Plus StrepTactin-HRP Conjugate, 0.3 ml
170-5047	GAM-HRP Conjugate, 2 ml
170-5046	GAR-HRP Conjugate, 2 ml
170-6404	Blotting-Grade Blocker, nonfat dry milk, 300 g

Catalog #	Description
170-6435	10x Tris Buffered Saline, 1 L
161-0780	10x Phosphate Buffered Saline, 1 L
170-6531	Tween 20, EIA grade, 100 ml
170-3939	Trans-Blot Cell With Plate Electrodes and Super Cooling Coil
170-3930	Mini Trans-Blot Electrophoretic Transfer Cell
170-4070	Criterion Blotter With Plate Electrodes
170-3940	Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell

Molecular Imager Products

170-8650	Molecular Imager VersaDoc MP 5000 System
170-8070	Molecular Imager ChemiDoc XRS System, PC
170-8071	Molecular Imager ChemiDoc XRS System, Mac

Catalog #	Description
Blotting Membranes	
Nitrocellulose Membranes, (0.45 µm)	
162-0113	Sheets, 20 x 20 cm, 5
162-0114	Sheets,15 x 9.2 cm, 10
162-0115	Roll, 30 cm x 3.5 m, 1
162-0116	Sheets, 15 x 15 cm, 10
162-0117	Sheets, 9 x 12 cm, 10
162-0145	Sheets, 7 x 8.4 cm, 10
162-0148	Sheets, 11.5 x 16 cm, 10
162-0214	Nitrocellulose/Filter Paper Sandwiches, 7 x 8.5 cm, 20
162-0215	Nitrocellulose/Filter Paper Sandwiches, 7 x 8.5 cm, 50
162-0234	Nitrocellulose/Filter Paper Sandwiches, 8.5 x 13.5 cm, 20
162-0235	Nitrocellulose/Filter Paper Sandwiches, 8.5 x 13.5 cm, 50

Catalog #	Description
Nitrocellulose Membranes, (0.2 µm)	
162-0112	Roll, 30 cm x 3.5 m
162-0146	Sheets, 7 x 8.4 cm, 10
162-0147	Sheets, 13.5 x 16.5 cm, 10
162-0150	Sheets, 20 x 20 cm, 5
162-0212	Nitrocellulose/Filter Paper Sandwiches, 7 x 8.5 cm, 20
162-0213	Nitrocellulose/Filter Paper Sandwiches, 7 x 8.5 cm, 50
162-0232	Nitrocellulose/Filter Paper Sandwiches, 8.5 x 13.5 cm, 20
162-0233	Nitrocellulose/Filter Paper Sandwiches, 8.5 x 13.5 cm, 50
Immun-Blot® PVDF Membranes, (0.2 µm)	
162-0174	Sheets, 7 x 8.4 cm, 10
162-0175	Sheets, 10 x 15 cm, 10
162-0176	Sheets, 20 x 20 cm,10
162-0177	Roll, 26 cm x 3.3 m, 1

Catalog #	Description
162-0218	Immun-Blot Membrane/Filter Paper Sandwiches, 7 x 8.5 cm, 20
162-0219	Immun-Blot Membrane/Filter Paper Sandwiches, 7 x 8.5 cm, 50
162-0238	Immun-Blot Membrane/Filter Paper Sandwiches, 8.5 x 13.5 cm, 20
162-0239	Immun-Blot Membrane/Filter Paper Sandwiches, 8.5 x 13.5 cm, 50

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