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4110020 Rev A

Immun-Star™ HRP Chemiluminescent Kit

Instruction Manual

For Use with Nitrocellulose and PVDF Membranes

Catalog #

170-5040, 170-5041, 170-5042, 170-5043,

170-5044, 170-5045, 170-5046, 170-5047

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

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Section 1
Preparation

1.1 Introduction

Chemiluminescence is a chemical reaction associated with the production of light. Chemiluminescence detection has become a common detection method for western blotting because of its enhanced sensitivity (low picogram levels of detection). In the presence of hydrogen peroxide, horseradish peroxidase (HRP), the enzyme that is conjugated to the secondary antibody, catalyzes the oxidation of luminol, the substrate. 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.

1.2 Method Overview

- The first step in western blotting is the transfer of antigen to a solid support membrane, for example, nitrocellulose or PVDF. This can be accomplished by one of several methods: electrophoretically using the Bio-Rad Trans-Blot system or the Bio-Rad Trans-Blot SD cell, passively by directly spotting the antigen onto a membrane,

or by vacuum filtration using the Bio-Rad Bio-Dot microfiltration apparatus.

- Following antigen binding, the remaining protein binding sites on the membrane are blocked with a protein blocking agent such as nonfat dry milk, gelatin, casein, or BSA.

- The membrane with the bound antigen is then incubated with a primary antibody specific for the antigen to be detected.

- The blot is washed to remove any excess antibody and incubated with the secondary antibody conjugated to HRP.

- The membrane is then treated with the chemiluminescent substrate and enhancer, followed by exposure to X-ray film, instant film, or an imager capable of detecting chemiluminescent signals, such as the VersaDoc™ Imaging system.

1.3 Immun-Star HRP Products

Catalog #	Immun-Star Product Description
170-5040	Substrate, 500 ml
170-5041	Substrate, 100 ml
170-5042	GAR-HRP Detection Reagents, (500 ml substrate, 2 ml conjugate)
170-5043	GAM-HRP Detection Reagents, (500 ml substrate, 2 ml conjugate)
170-5044	GAM-HRP Detection Kit, (500 ml substrate, 2 ml conjugate, blotting reagents*)
170-5045	GAR-HRP Detection Kit, (500 ml substrate, 2 ml conjugate, blotting reagents*)
170-5046	GAR-HRP Conjugate, 2 ml, lyophilized
170-5047	GAM-HRP Conjugate, 2 ml, lyophilized

*Blotting reagents included in the detection kits are: 1 L 10x TBS, 75 g nonfat dry milk, 15 ml Tween 20.

1.4 Complementary Products

Catalog #	Description
170-6531	Blotting Grade Blocker, nonfat dry milk
170-6532	Premixed TBS, 10x, 1 L
161-0780	Premixed PBS, 10x, 1 L
170-6531	Tween 20, EIA grade, 100 ml
161-0362	Precision Protein™ Unstained Standards, 1.5 ml
161-0372	Precision Protein Prestained Standards, 0.5 ml
161-0380	StrepTactin-HRP, 0.3 ml
161-0781	10% Tween 20, 1 L

Blotting membranes

Nitrocellulose membrane (0.45 µm)

162-0115	Roll, 30 cm x 3.5 m, 1
162-0113	Sheets, 20 x 20 cm, 5
162-0116	Sheets, 15 x 15 cm, 10
162-0114	Sheets,15 x 9.2 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
162-0234	Nitrocellulose/filter paper sandwich 8.5 x 13.5 cm, 20

162-0235	Nitrocellulose/filter paper sandwich 8.5 x 13.5 cm, 50
162-0214	Nitrocellulose/filter paper sandwich 7 x 8.5 cm, 20
162-0215	Nitrocellulose/filter paper sandwich 7 x 8.5 cm, 50

Nitrocellulose membrane (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 8.5 cm, 10
162-0150	Sheets, 20 x 20 cm, 5
162-0232	Nitrocellulose/filter paper sandwich 8.5 x 13.5 cm, 20
162-0233	Nitrocellulose/filter paper sandwich 8.5 x 13.5 cm, 50
162-0212	Nitrocellulose/filter paper sandwich 7 x 8.5 cm, 20
162-0213	Nitrocellulose/filter paper sandwich 7 x 8.5 cm, 50

Immun-Blot PVDF membrane (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
162-0218	Immun-Blot membrane/filter paper sandwich 7 x 8.5 cm, 20
162-0219	Immun-Blot membrane/filter paper sandwich 7 x 8.5 cm, 50
162-0238	Immun-Blot membrane/filter paper sandwich 8.5 x 13.5 cm, 20
162-0239	Immun-Blot membrane/filter paper sandwich 8.5 x 13.5 cm, 50

1.5 Storage and Stability of Components

Substrate	1 year at 4°C
HRP conjugated antibody (lyophilized)	1 year at 4°C
HRP conjugated antibody (reconstituted)	Aliquot and store at -20°C for 1 year
TBS (10x)	1 year at 4°C or room temperature
Nonfat dry milk	1 year at 4°C or room temperature
Tween 20	1 year at 4°C or room temperature

Section 2
Assay Instructions

2.1 Experimental strategy and general recommendations —

Water Purity – Use only deionized distilled (dd) water to prepare all solutions. In addition, care should be taken to prevent horseradish peroxidase contamination of assay solutions. Ideally, dd water should be autoclaved or sterile filtered prior to use in buffers and solutions.

Membrane Selection — The Immun-Star HRP kit is designed for use with nitrocellulose and PVDF membranes.

Primary Antibody — The recommended starting dilution for primary antibody is 1:1,000 – 1:6,000 from a 1 mg/ml stock; optimal dilution factors must be determined empirically.

Secondary Antibody — The recommended starting dilution for the secondary antibody is 1:15,000–1:30,000 from a 1 mg/ml stock. **The optimization of the**

appropriate dilution of your secondary antibody is very critical to reduce background noise.

Washes — Continuous **strong** agitation should be used during all washes. For best results, an orbital shaker should be employed to maintain a uniform exposure of the membrane to the solution.

Detergents — Tween 20 is essential in washing to eliminate overall background and nonspecific hydrophobic interactions. At 0.05% to 0.1%, Tween 20 will not disrupt binding of primary antibodies to antigens or antigens to the membrane, but will optimize detection sensitivity by eliminating nonspecific reactions. Increased concentrations of Tween 20 (up to 0.3%) can be used if background problems persist. Alternative detergents should not be used.

Molecular Weight Standards — Bio-Rad Precision Protein unstained standards are recommended for molecular weight determinations with the Immun-Star HRP assay. These standards contain an integral *Strep*-tag sequence to which StrepTactin-HRP conjugate binds. A chemiluminescent signal is produced upon reaction of the substrate with horseradish peroxidase (HRP).

Since the StrepTactin conjugate can be added together with the secondary antibody solution, no extra steps are needed to detect the standards.

Prestained Precision Protein standards and Kaleidoscope™ standards can also be used to assess the transfer efficiency of samples.

Tips —

When deciding on your blocker, remember that grocery store-purchased brands of milk are often vitamin fortified and contain biotin, which should not be used with avidin-biotin detection systems due to high background problems; other blockers may be substituted, such as 3% BSA or 3% gelatin.

Do **not** use azide as a preservative in any of your buffers as it is an inhibitor of the HRP enzyme.

Use good-quality plastic wrap when incubating with substrate.

The antibody concentrations required for this assay will be more dilute than for the colorimetric HRP assays.

They must be optimized if switching from a colorimetric system to chemiluminescence to prevent excessively strong signal.

The sensitivity of this system makes it important to be **very careful** during pipetting when loading samples, making samples, etc. Any small amount of protein cross-contamination will show up on your blot.

If you see a glowing green light on your blot when exposed to X-rays in the darkroom, this means the signal is very strong and the blot may require less than a 10 sec exposure. Alternatively, let the blot sit for 30 min before taking a picture. **Do not allow blot to dry out at any time.**

Working solutions:

Tris-Buffered Saline, (1x TBS) — (20 mM Tris, 150 mM NaCl, pH 7.5)
If using Bio-Rad liquid concentrated 10x TBS (170-6435), add 100 ml of 10x TBS to 900 ml of dd water.

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

OR

1X TBS

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

1X PBS

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 (170-6531) or 5 ml of 10% Tween 20 (161-0781) 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 non-fat dry milk; this is enough for one 8.5 x 13.5 cm Criterion™ blot.

Primary Antibody Solution — 1:1,000–1:6,000 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 1:15,000 to 1:30,000 (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 buffer) is sufficient for one 8.5 x 13.5 cm Criterion blot.

*To test activity of the system, mix 1 ml of Immun-Star HRP working solution (0.5 ml of peroxide buffer and 0.5 ml of enhancer) with 2–4 µl of undiluted HRP conjugate in a clear plastic tube. The mixture should emit a blue light in the dark.

***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. **Antigen Application —** Apply antigen to the membrane surface using one of the three basic methods described below. A small amount of known antigen or primary antibody dotted on one corner of the membrane prior to blocking will produce a positive result if the procedure is successful.

    a. **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, Mini Trans-Blot cell or Trans-Blot SD apparatus.

    b. **Microfiltration Blotting —** The Immun-Star assay can easily be adapted for use in the Bio-Dot or Bio-Dot SF apparatus. This instrument allows rapid, reproducible application of antigen to the membrane of up to 96 samples.

c. **Dot–Blotting —** Cut the membrane sheet to the appropriate size. Draw a grid on the membrane with a pencil. Wet the dry membrane by slowly sliding the membrane into TBS. Remove the thoroughly wetted membrane from the TBS and allow to dry on a piece of filter paper for 5 min. Apply antigen sample to each grid square using a syringe or variable pipet. Allow membrane to dry completely before proceeding on to the blocking step. For PVDF, wet the membrane in 100% methanol before beginning the procedure and rewet with methanol after letting the membrane dry.

2. **Blocking —** Block with 5% nonfat dry milk dissolved in TTBS (0.05% Tween 20) for 50 min at room temperature on a shaker. Decant blocker.

3. **Primary Antibody Incubation —** Incubate the membrane with
primary antibody in TTBS for 50 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.

5. **Secondary Antibody Incubation —** Incubate the membrane with secondary antibody, 1:15,000–1:30,000 dilution in TTBS , for 45 min at room temperature with gentle agitation. Decant secondary antibody.

6. **Wash —** Wash the membrane with TTBS for 6 x 10 min with **strong** agitation, 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 (12 ml is sufficient for a 8.5 cm x 13.5 cm Criterion gel). Incubate the membrane in the substrate mixture for 3–5 min. Make sure the surface of the blot is completely covered with substrate (no air bubbles; do not allow blot to dry out)

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.)

8. **Film exposure —** Place the membrane (transfer-side down) in a clear plastic sheet protector or good quality plastic wrap, covering membrane carefully. Squeeze out any excess substrate that may exist between the surface of the membrane and the protector sheet. Expose to X-ray film to visualize the bands, initial exposure time of 30 sec to 2 min. Do not allow membrane to dry out.

   Alternatively, an imager such as the Bio-Rad VersaDoc™ imaging system can be used instead of film. Longer exposure may be required.

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 proper 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	Store at proper temperature, avoid repeated freeze-thaw cycles
		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 off bound antigen from the membrane. Decrease amount of Tween 20 in the assay.

Problem	Probable Cause	Recommended Solution
		Check transfer technique, equipment and buffer dilutions
		Stain membrane with protein stain such as Ponceau to ensure transfer efficiency
		Transfer of proteins onto the membrane was incomplete. Use Precision Protein prestained standards to monitor transfer efficiency, stain gel to ensure transfer occurred.
Weak signal	Check all points above	
	Insufficient protein loaded	Load more protein on the gel

Problem	Probable Cause	Recommended Solution
	Azide inhibits HRP	Do NOT use azide to prevent bacterial contamination of solutions
High background	Exposure time was too long	Decrease exposure time
	Blocking was insufficient	Increase time of blocking step and/or the concentration of blocker used
	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
	Secondary antibody was used at an excessive concentration	Use the recommended dilution of conjugate or determine optimal dilution experimentally

Problem	Probable Cause	Recommended Solution
	Contamination occurred during transfer	Refer to blotting apparatus instruction manual for recommendations
	Solutions and buffers are contaminated	Autoclave or sterile filter dd water prior to making solutions
		Use blotting grade nonfat dry milk, included in the kit, for blocking. This product has been QC tested for acceptable background temperatures
		Avoid bacterial contamination of all solutions by storing at the appropriate temperatures
Signal too strong	Protein is overloaded	Load less protein in the gel

Problem	Probable Cause	Recommended Solution
	Antibody is too concentrated	Optimize antibody solution; see page 4 for recommendations
	Incorrect gel running and blotting conditions	Check gel and buffer recipes, optimize conditions
	Inappropriate incubation time with substrate	Decrease the incubation time

Section 4
Ordering Information

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Tween is a trademark of ICI Americas, Inc.

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