Immun-Star™ AP Chemiluminescent Protein Detection Systems

For Use With Nitrocellulose and PVDF Membranes

Instruction Manual

Catalog #

170-5010, 170-5011, 170-5012,
170-5013, 170-5014, 170-5015,
170-5018, 170-5056, and 170-5057

BIO-RAD

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*** Note important blocking solution instructions on page 13. High concentrations of blocker can lead to high background on blots.**

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Section 1
Preparation

1.1 Introduction

The Immun-Star chemiluminescent detection system is a sensitive, nonisotopic method for immunodetection of specific antigens immobilized on nitrocellulose or PVDF membrane. The system uses the powerful CDP-*Star* chemiluminescent substrate and enhancer, which is activated by an alkaline phosphatase enzyme conjugate. Drawing on Bio-Rad's expertise in protein blotting, the substrate and enhancer are incorporated into a sensitive and easy-to-use western blotting detection protocol.

The Immun-Star anti-mouse and Immun-Star anti-rabbit chemiluminescent detection kits provide enough reagents to assay 2,500 cm^2 of membrane or approximately 50 mini blots of ~50 cm^2 each. The blotting reagents pack provides all the complementary buffer reagents needed for western blotting, matched in quantity to the detection kits. We also offer the Immun-Star Intro chemiluminescent kits, with enough of all reagents needed for 2 large blots or 8 mini blots. The Immun-Star assay kits are for laboratory use only.

1.2 Method Overview

The first step in western blotting is the transfer of antigen onto a solid support membrane by one of several methods. The transfer can be done electrophoretically, following separation

of the antigen in a polyacrylamide or agarose gel, passively by directly spotting the antigen onto a membrane, or by vacuum filtration using a microfiltration apparatus. Following antigen binding, the remaining protein binding sites on the membrane surface are blocked with nonfat dry milk, or other protein blocking agents.

The membrane with bound antigen is then incubated with a primary antibody specific for the antigen to be detected. The blot is washed to remove unbound antibody and incubated with the respective second antibody, which has been conjugated to alkaline phosphatase (AP). The membrane is then treated with the chemiluminescent substrate alone for PVDF membranes, and with substrate and enhancer for nitrocellulose. The concentrated enhancer is added to the 1x substrate solution and does not add any extra incubation or wash steps. The blot is then used to expose X-ray or instant film, or imaged by an imager capable of detecting chemiluminescent signals, such as the Bio-Rad VersaDoc™ or ChemiDoc™ system.

1.3 Immun-Star Products

Catalog #	Description
170-5010	Immun-Star Goat Anti-Mouse -AP Detection Kit
170-5011	Immun-Star Goat Anti-Rabbit -AP Detection Kit
170-5012	Immun-Star AP Substrate Pack
170-5018	Immun-Star AP Substrate
170-5013	Immun-Star Goat Anti-Mouse -AP Intro Kit
170-5014	Immun-Star Goat Anti-Rabbit -AP Intro Kit

Catalog #	Description
170-5015	Blotting Reagents Pack
170-5056	GAR-AP Blotting Starter Kit
170-5057	GAM-AP Blotting Starter Kit

1.4 Complementary Products

Catalog #	Description

Individual Blotting Grade Reagents

170-6518	Goat Anti-Rabbit IgG (H+L)-AP, 1 ml
170-6520	Goat Anti-Mouse IgG (H+L)-AP, 1 ml
170-6521	Goat Anti-Human IgG (H+L)-AP, 1 ml
170-6435	Premixed Tris-Buffered Saline, 10x, 1 L
170-6531	Tween 20, 100 ml
170-6404	Blotting Grade Blocker, nonfat dry milk, 300 g

Blotting Membranes

Nitrocellulose Membrane, 0.45 µm

162-0113	Sheets, 20 x 20 cm, 5
162-0114	Sheets, 9.2 x 15 cm, 10
162-0115	Roll, 33 cm x 3 m, 1
162-0116	Sheets, 15 x 15 cm, 10
162-0117	Sheets, 9 x 12, 10
162-0145	Sheets, 7 x 8.4 cm, 10
162-0148	Sheets, 11.5 x 16 cm, 10

Nitrocellulose Membrane, 0.2 µm

162-0112	Roll, 33 cm x 3 m, 1
162-0146	Sheets, 7 x 8.4 cm, 10
162-0147	Sheets, 13.5 x 16.5 cm, 10

Immun-Blot PVDF Membranes

162-0175	Sheets, 10 x 15 cm, 10

Catalog #	Description
162-0176	Sheets, 20 x 20 cm, 10
162-0177	Roll, 26 cm x 3.3 m, 1
162-0174	Sheets, 7 x 8.4 cm, 10
Blotting Standards	
161-0306	Biotinylated Standards, low range, 250 µl
161-0308	Biotinylated Standards Kit (AP), low range
161-0311	Biotinylated Standards, high range, 250 µl
161-0313	Biotinylated Standards Kit (AP), high range
161-0319	Biotinylated Standards, broad range, 250 µl
161-0322	Biotinylated Standards Kit (AP), broad range
161-0363	Precision Plus Protein™ Unstained Standards
161-0382	Precision Protein™ StrepTactin-AP conjugate
170-6533	Avidin-AP, 1 ml
161-0324	Kaleidoscope™ Prestained Standards
161-0325	Kaleidoscope Polypeptide Standards
161-0375	Precision Plus Protein Kaleidoscope Standards
161-0305	Prestained SDS-PAGE Standards, low range
161-0309	Prestained SDS-PAGE Standards, high range
161-0318	Prestained SDS-PAGE Standards, broad range

1.5 Storage and Stability of Components

Description	Quantity Provided	Storage	Shelf Life
Catalog #170-5010/170-5011			
Immun-Star Detection Kits – 2,500 cm^2			
• Immun-Star chemiluminescent substrate	125 ml	4°C	1 yr
• Immun-Star enhancer**	6.25 ml	4°C	1 yr
• AP conjugated antibody*	0.5 ml	-20°C	1 yr

Description	Quantity Provided	Storage	Shelf Life
Catalog #170-5012			
Immun-Star Substrate Pack – 2,500 cm^2			
• Immun-Star chemiluminescent substrate	125 ml	4°C	1 yr
• Immun-Star enhancer**	6.25 ml	4°C	1 yr
Catalog #170-5018			
Immun-Star Substrate – 2,500 cm^2			
• Immun-Star substrate	125 ml	4°C	1 yr
Catalog #170-5013/170-5014			
Immun-Star Introduction Kits – 8 mini blots			
• Immun-Star chemiluminescent substrate	20 ml	4°C	1 yr
• Immun-Star enhancer**	1 ml	4°C	1 yr
• AP conjugated antibody*	0.1 ml	-20°C	1 yr
• TBS (10x)	220 ml	4°C or RT	1 yr
• Nonfat dry milk	2 g	4°C or RT	1 yr
• Tween 20	2.5 ml	4°C or RT	1 yr
Catalog #170-5015			
Blotting Reagents Pack – 2,500 cm^2			
• 10x TBS	2 x 1 L	RT	1 yr
• Tween 20	15 ml	RT	1 yr
• Nonfat dry milk blocker	10 g	RT	1 yr

* Store conjugate at -20°C until first use, then aliquot and store at -20°C. Avoid repeat freeze/thaw cycles. 4°C storage is also acceptable.

**Immun-Star enhancer is used with nitrocellulose membrane only.

1.6 Safety Instructions

1. Read the entire instruction manual before beginning the assay.
2. Wear gloves and protective clothing, such as a laboratory coat and goggles, when preparing and working with the solutions in the assay. The Immun-Star enhancer contains diethanolamine, which can cause skin and eye irritation. In case of contact, immediately flush the skin or eyes with copious amounts of water for at least 15 min, and remove contaminated clothing.

   Note: See Material Data Safety Sheet on diethanolamine for additional information.
3. Work in well-ventilated areas. Avoid inhalation of vapors when handling solutions containing diethanolamine.
4. Do not mouth-pipet any solutions.

Section 2
Assay Instructions

2.1 Experimental Strategy and General Recommendations

Temperature. All steps are performed at room temperature (22–25°C) unless indicated otherwise in the instructions. If a lower assay temperature is preferred, it is advisable to double the incubation and wash times for each 10°C decrease in temperature.

Water Purity. Use only deionized distilled water to prepare all solutions. In addition, care should be taken to prevent alkaline phosphatase contamination of assay solutions. Ideally, distilled deionized water (ddH_2O) should be autoclaved or sterile-filtered prior to use in buffers and solutions.

Membrane Selection. The Immun-Star assay is specially designed for use with nitrocellulose and PVDF membranes. This kit is not compatible with nylon membrane. The use of Immun-Star enhancer is required when performing blots on nitrocellulose membrane. Longer exposure times will be necessary if the enhancer is not used, with resulting interference from background development. If proteins are transferred to PVDF membrane, use of the enhancer is not necessary. All other steps in the assay procedure remain the same.

Primary Antibody. Generally, when serum or tissue culture supernatants are the source of primary antibody, a 1:100–1:1,000 dilution of the primary antibody in buffer is used for detection of antigens on the membrane surface. For chromatographically purified monospecific antibodies, a 1:500–1:10,000 dilution in buffer is used for antigen detection. A 1:1,000–1:100,000 dilution is used when ascites fluid is the source of antibody. Optimal dilution factors must be determined experimentally. The optimal antibody concentration is usually considered to be the greatest dilution of antibody reagent that still results in a strong positive signal without significant membrane background or nonspecific reactions.

Blotting Grade Conjugates. The conjugates supplied by Bio-Rad should be used in the concentrations indicated in Section 2.3. Using a conjugate at higher concentrations may result in an overall increase in background without any increase in detection sensitivity.

Washes and Incubations. Continuous gentle agitation should be used during all reactions. 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 reactions. At 0.05%–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 substituted. The wash between the blocking step and incubation with the first antibody is essential and should not be altered.

Molecular Weight Standards. Biotinylated SDS-PAGE standards and Precision Plus Protein™ unstained standards are recommended for molecular weight determinations with the Immun-Star assays. The Biotinylated SDS-PAGE standards are detected by binding avidin-AP or streptavidin-AP to the biotinylated proteins, which will produce a chemiluminescent signal upon reaction with the substrate. The Precision Plus Protein

unstained standards are detected by incubating with StrepTactin-AP. Because the avidin-AP (or streptavidin-AP) and StrepTactin-AP can be directly added to the second antibody solution, no extra steps are needed to detect the biotinylated or Precision Plus Protein unstained standards. The standards should be diluted 1:50 in electrophoresis sample buffer (**note:** this is a higher dilution than recommended in the biotinylated standards manual). Load 10 µl per lane for mini gels and 15 µl per lane for full size gels.

Prestained SDS-PAGE and Kaleidoscope prestained standards can also be used for assessing the transfer efficiency of samples.

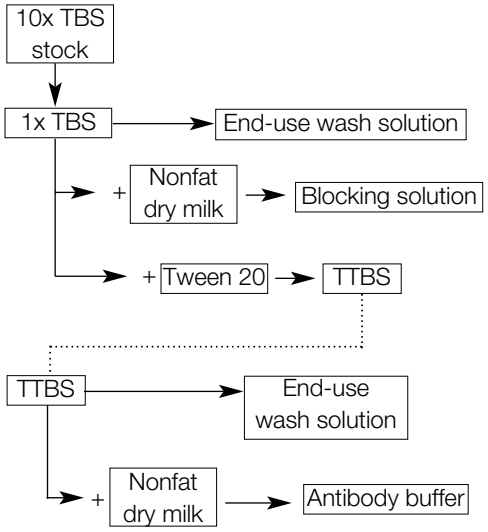
Total Protein Detection. In the identification of specific antigens, total protein staining is required to correlate the signal detected to a complex protein mixture. Any of the common total protein staining methods will work to give a profile of the blot, including colloidal gold, Amido Black, and Ponceau S. If you want to generate a chemiluminescent total protein blot, there are Biotin Blot™ systems that allow visualization by chemiluminescent signal. The chemiluminescent detection method uses NHS-biotin to biotinylate all the proteins on the membrane. The biotinylated proteins are detected with avidin-AP or streptavidin-AP and visualized with the chemiluminescent substrate (see Appendix 1 for details).

2.2 Reagent Flowchart

Several of the solutions you will make are end-use solutions and are also used to make up other solutions. For example, 1x TBS is used as a wash solution, for the blocking solution, and to make TTBS. In turn, TTBS is used both as a wash solution (end use), and as part of the antibody buffer.

This reagent flowchart shows the overall picture of the use of some of the reagents. Since there are many ways to go about making solutions, this flowchart can help you make best use of your time and reagents. The straightforward solutions were left out of the flowchart.

Refer to Section 2.3, Working Solutions, for the exact quantities and contents of all the solutions.



Reagent Flowchart. (Does not include all reagents in this kit.)

2.3 Working Solutions (based on 200 cm^2 of membrane; one large blot, or four mini gel blots)

This kit has been designed to blot 2,500 cm^2 of membrane, or approximately 50 mini blots. The Working Solutions section of this manual has been based on enough of each solution to blot 200 cm^2 of membrane at 0.5 ml of solution per cm^2 for each step, except the substrate/enhancer step, which requires only 0.05 ml per cm^2. For practical reference, 200 cm^2 of membrane is approximately the size of one large western blot, or four mini gel western blots. If a larger or smaller number of blots is used, vary the quantity of reagents by the appropriate amount.

Since any blotting procedure is best when it is performed with fresh solutions, it is preferable to make up the solutions needed for the day's blots. If solutions containing proteins are made and held over a period of days, they must be refrigerated. On the day that the solutions are going to be used, they should be warmed to room temperature before use to avoid adding any new variables to the blotting procedure.

Tips

It is important that enough reagent be used to cover the entire blot. In order to make most efficient use of the reagents, the container used for wash and incubation steps should fit the blot as closely as possible.

The enhancer used in this procedure is difficult to wash from containers, so care should be taken to use disposable or

dedicated-use containers to avoid contaminating other experiments.

Because of the high sensitivity of the chemiluminescent assay, care should be taken to avoid alkaline phosphatase contamination of the buffers and solutions used in the procedure. For best results, the ddH_2O used to prepare solutions should be autoclaved or filtered prior to use in this assay.

Tris-Buffered Saline (1x TBS)

(20 mM Tris, 500 mM NaCl, pH 7.5)

If using Bio-Rad liquid concentrate 10x TBS, add 110 ml of 10x TBS to 990 ml of ddH_2O. Label this bottle "1x TBS".

If using solid TBS powder, follow instructions to make 1,100 ml of a 1x TBS solution. Typically, a 1x solution is 32.3 g of TBS brought up to 1 L of solution with ddH_2O.

Wash Solution (TTBS)

(20 mM Tris, 500 mM NaCl, 0.1% Tween 20, pH 7.5)

Add 0.8 ml of Tween 20 to 800 ml of 1x TBS. Label this bottle "TTBS".

Blocking Solution

(0.2% nonfat dry milk in TBS)

Add 0.2 g of nonfat dry milk to 100 ml of 1x TBS. Stir until dissolved. Label this solution "Blocking Solution".

Suggestions for other blockers:
casein – 0.2% in TBS, gelatin – 3% in TBS or BSA or 3% in TBS

Antibody Buffer

(0.2% nonfat dry milk in TTBS)

Add 0.4 g of nonfat dry milk to 200 ml TTBS. Stir until dissolved. Label this solution “Antibody Buffer”.

Primary Antibody Solution

Dilute the primary antibody to the appropriate titer in 100 ml of antibody buffer. Label this solution “Primary Antibody Solution”.

Second Antibody Conjugate Solution (1:3,000)

Add 33 µl of the second antibody conjugate to 100 ml of antibody buffer. Label this solution “Second Antibody Solution”.

Substrate Solution for PVDF membrane blots

Immun-Star generates a very fast light signal on PVDF membrane; therefore, the use of the enhancer is not necessary. Use 10 ml of chemiluminescent substrate per 200 cm^2. The substrate is provided ready to use.

Substrate Solution for nitrocellulose membrane blots

Add 500 µl of the enhancer reagent to 10 ml of Immun-Star chemiluminescent substrate. Label this solution “Substrate Solution”. This solution can be stored at 4°C for up to 1 week.

2.4 Detailed Assay Procedure

Note: Before beginning, 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 reaction 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 Trans-Blot® cell, Mini Trans-Blot® cell, Criterion® blotter, or Trans-Blot SD cell. If desired, cut the wet membrane into 0.6–0.8 cm wide strips. Immerse the strips or the entire sheet in TBS before proceeding to the blocking step.

    b. **Microfiltration blotting** – the Immun-Star assay can easily be adapted for use in the Bio-Dot® and Bio-Dot SF apparatus. These instruments allow rapid, reproducible applications of up to 96 samples on one membrane sheet. The membrane should be removed from the apparatus after antigen application. Because nonfat dry milk cannot be filtered through the membrane in the Bio-Dot or Bio-Dot SF apparatus, the blocking and incubation steps should be carried out in a separate container.

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 at a 45° angle into TBS. (PVDF membranes must first be wetted in methanol; consult membrane instructions for complete information.) Remove the thoroughly wetted membrane from the TBS and dry it on filter paper for approximately 5 min. Apply antigen sample to each grid square using a syringe or a variable pipet, by displacing a 1 µl drop of sample to the tip of the syringe or pipet and gently touching it to the surface of the membrane. If the sample is very dilute, it is possible to apply successive 1 µl doses at the same spot by letting the previous sample application dry completely before adding an additional dose. In all cases, the membrane should be allowed to dry completely before proceeding to the blocking step. For PVDF following this drying step, rewet with 50% methanol before transferring to the TBS solution in step 2 below.

2. **Wash** – following transfer, wash the membrane in TBS for 5–10 min with gentle agitation at room temperature (RT). Decant the wash solution and repeat the wash step one more time. These washes are important to reduce spotted or blotchy background problems.

3. **Blocking step** – after the antigen is applied, using one of the above methods, immerse the membrane, at a

45° angle, into the blocking solution. Gently agitate the solution using an orbital shaker platform and incubate for 30 min to 1 hr at RT, or block overnight at 4°C.

4. **Wash** – decant the blocking solution and add TTBS to the membrane. Wash for 5–10 min with gentle agitation at RT.
5. **Primary antibody incubation** – decant the TTBS and add the primary antibody solution to the membrane. Incubate 1–2 hr with gentle agitation at RT. Overnight incubation may be preferred, since longer incubation periods may increase the sensitivity of detection. The optimum conditions of dilution and incubation time must be determined experimentally. For overnight incubations, 4°C is recommended.
6. **Wash** – decant the primary antibody solution and add TTBS to the membrane. Wash for 5–10 min with gentle agitation at RT. Decant the wash solution and repeat the wash step, with additional TTBS.
7. **Second antibody conjugate incubation** – decant the TTBS and add the second antibody solution. Incubate for 30 min to 2 hr using gentle agitation at RT.
8. **Wash** – decant the conjugate solution and add TTBS to the membrane. Wash for 5–10 min with gentle agitation at RT. Decant the wash solution and repeat the wash step two more times with additional TTBS.

9. **Blot development** – remove the membrane and drain excess liquid without letting the blot dry. Place on a piece of Saran wrap on a level surface. Pipet the substrate solution onto the membrane. Incubate 5 min. Remove the membrane, draining the excess liquid from the blot. Seal in a heat-sealable bag or in Saran wrap. Do not allow the membrane to dry, because this will result in a loss of signal.
10. **Film exposure/imaging** – expose X-ray or instant film to the blot at RT. Exposure time will depend on the type of membrane used and the protein concentration. Table 1 lists recommended exposure times; however, optimal conditions should be determined for each particular application. Develop the film according to the manufacturer's instructions. Alternatively, a Bio-Rad imager such as the Bio-Rad VersaDoc™ or ChemiDoc™ system can be used to capture the signal.

Table 1. Typical exposure times for nitrocellulose and PVDF membranes.

Membrane	With Immun-Star Enhancer	Without Enhancer
Nitrocellulose	1–10 min	12–24 hr
PVDF	Not recommended	1–10 min

Section 3
Troubleshooting

3.1 Troubleshooting Guide

Problem	Probable Cause	Recommended Solution
1. No reaction or weak signal.	a. Exposure time was too short.	i. Increase the exposure time.
	b. Immun-Star enhancer was not used with nitrocellulose membrane.	i. Use the Immun-Star enhancer assay protocol. Exposure times will vary; for example, a nitrocellulose blot without enhancer would require a much longer exposure time to generate a signal comparable to that generated with enhancer in 5 min.

Troubleshooting Guide *(continued)*

Problem	Probable Cause	Recommended Solution
	c. Blot was allowed to dry after incubation with the chemiluminescent substrate.	i. Use heat-sealable bags to prevent drying of the membrane.
	d. Chemiluminescent substrate solution is inactive.	i. Store the reagent at the proper temperature, 4°C. To test activity of substrate, mix 1 ml of substrate with 1–2 µl of undiluted conjugate and look for light emission in the darkroom.
	e. Primary antibody solution is inactive or nonsaturating.	i. Store the antibody solution at the proper temperature. Avoid bacterial contamination, heat inactivation, and repeated freeze-thaw cycles.

Troubleshooting Guide *(continued)*

Problem	Probable Cause	Recommended Solution
		ii. Antibody titer was too low. Increase the concentration of the antibody used in the assay.
		iii. Tween 20 may affect the reactivity of some antibodies. Eliminate Tween 20 from the assay (except the wash after the blocking step).
	f. Conjugate is inactive.	i. Store the conjugate at the proper temperature as recommended in Section 1.5. Avoid repeated freeze-thaw cycles.

Troubleshooting Guide *(continued)*

Problem	Probable Cause	Recommended Solution
		ii. The concentration of the conjugate was nonsaturating. Increase the concentration of the conjugate used in the assay.
		iii. Conjugate may be contaminated, causing inactivation of the antibody or enzyme. Tap water may cause inactivation; use only ddH_2O to prepare all solutions.
	g. Little or no antigen is bound to the membrane.	i. Tween 20 may wash bound antigen from the membrane. Eliminate Tween 20 from the assay (except the wash after the blocking step).

Troubleshooting Guide *(continued)*

Problem	Probable Cause	Recommended Solution
		ii. Transfer of protein onto the membrane was incomplete. Stain gel to assure transfer of protein. Use prestained standards to monitor transfer efficiency. Consult the appropriate instrument manual for proper procedures and recommendations.
	h. Primary antibody is not specific or does not recognize denatured antigens (common with monoclonals).	i. Loss of reactivity may have occurred during electrophoretic transfer. Pretest the reactivity of the antibody against the antigen by a dot blot.

Troubleshooting Guide *(continued)*

Problem	Probable Cause	Recommended Solution
2. High background.	a. Exposure time was too long.	i. Decrease exposure time.
		ii. The use of Immun-Star enhancer is required when using nitrocellulose membranes. This will allow decreased film exposure times.
	b. TBS washes after transfer were omitted or insufficient.	i. The washes are critical to reduce spotted or blotchy background development.
	c. Blocking was insufficient.	i. Increase the duration of the blocking step and/or the concentration of blocker used.
		ii. Try a different blocking reagent, such as casein, gelatin, or BSA.

Troubleshooting Guide *(continued)*

Problem	Probable Cause	Recommended Solution
	d. Wash stringency was insufficient.	i. Tween 20 is necessary in wash steps to reduce background. The concentration can be increased up to 0.3% if background persists.
		ii. Increase the number and length of washes.
	e. Second antibody conjugate was used at an excessive concentration.	i. Use the recommended dilution, or determine the optimal dilution experimentally.
	f. Contamination occurred during transfer.	i. Refer to the instrument instruction manual for recommendations.
	g. Solutions and buffers are contaminated with alkaline phosphatase.	i. Autoclave or sterile-filter ddH_2O prior to use in making solutions.

Troubleshooting Guide *(continued)*

Problem	Probable Cause	Recommended Solution
		ii. Use blotting grade nonfat dry milk blocker, which has been quality-control tested for acceptable background levels.
		iii. Avoid bacterial contamination of all solutions by storing at recommended temperatures.

References and Acknowledgements

1. Bronstein, I., Juo, R. R., Voyta, J. C. and Edwards, B., Bioluminescence and Chemiluminescence: Current status, John Wiley, Chichester, England (1991).

Note on Electrophoresis and Blotting Equipment

Bio-Rad provides a complete line of electrophoresis, electrophoretic transfer, and microfiltration apparatus that can be used with the Immun-Star assays. For more information in the U.S. contact Bio-Rad Laboratories technical support at 1-800-4BIORAD (1-800-424-6723), or contact your local Bio-Rad representative.

Appendix 1
Total Protein Detection Procedure

Required Reagents

Catalog #	Description
170-6533	Avidin-AP, 1 ml
or	
170-3554	Streptavidin-AP, 0.5 ml
170-6529	N-Hydroxysuccinimide Biotinate (NHS-Biotin), in dimethylformamide, 75 mM, 4 ml
170-6435	TBS Buffer Solution, 10x, 1 L
161-0715	Tween 20, 100 ml

Additional Reagents Required

Sodium borate, 10-hydrate ($Na_2B_4O_7 \bullet 10\ H_2O$), ACS reagent grade

Sodium chloride (NaCl), ACS reagent grade

Working Solutions

Borate-Tween (1x BT)

(0.05 M $Na_2B_4O_7 \bullet 10\ H_2O$, 0.5 M NaCl, 0.2% Tween 20, pH 9.3)

Dissolve 38.14 g $Na_2B_4O_7 \bullet 10\ H_2O$ and 58.44 g NaCl in 1.9 L ddH_2O. Add 4 ml Tween 20, then bring to a final volume of 2 L with ddH_2O and mix. Label this solution “1x BT”.

Tris-buffered saline (TBS)

(20 mM Tris, 500 mM NaCl, pH 7.5)

Add 120 ml of 10x TBS to 1,080 ml of ddH_2O. Label this bottle “1x TBS”.

Wash solution (TTBS)

(20 mM Tris, 500 mM NaCl, 0.2% Tween 20, pH 7.5)

Add 2 ml of Tween 20 to 1 L of TBS. Label this solution “TTBS”.

Avidin-AP or Streptavidin-AP solution

Add 100 µl of avidin-AP or streptavidin-AP to 100 ml TTBS.

Substrate solution for nitrocellulose membrane blots

Add 150 µl of the enhancer reagent to 3 ml of Immun-Star chemiluminescent substrate. Label this solution “Substrate Solution”.

For PVDF membrane blots, use the Immun-Star chemiluminescent substrate without the enhancer reagent.

Total Protein Detection Procedure

Note: Before beginning, read through the entire procedure. The following procedure is based on 100 ml of each solution, which is sufficient volume to assay one 10 x 15 cm membrane.

1. Wash the membrane in 100 ml 1x BT solution for 10 min. If the membrane has been in a buffer containing amines, repeat the wash two more times.
2. Decant the wash solution and replace it with fresh 1x BT solution. While agitating the incubation vessel, add 200 µl of NHS-biotin. Incubate the membrane for 15 min with constant agitation. **Do not prepare the BT solution containing NHS-biotin before use, as the biotin reagent is hydrolyzed in aqueous solution.**

   Note: To prevent hygroscopic accumulation of water in the NHS-biotin reagent, equilibrate the vial to room temperature before use and remove reagent with a sterile syringe.
3. Wash the membrane in 100 ml of 1x BT for 5 min. Repeat the wash step.
4. Wash the membrane in 100 ml of TTBS for 5 min. Repeat the wash step.
5. Prepare the avidin-AP or streptavidin-AP solution. Incubate the membrane in the solution for 1 hr with agitation.
6. Wash the membrane in 100 ml of TTBS for 5 min. Repeat the wash step.
7. Wash the membrane in 100 ml of TBS for 5 min. Repeat the wash step.

8. **Blot development** – remove the membrane and drain excess liquid without letting the blot dry. Place on a piece of Saran wrap on a flat, level surface. Pipet the substrate solution onto the membrane. Incubate 5 min. Remove the membrane, draining the excess liquid from the blot. Seal in a heat-sealable bag or in Saran wrap. Do not allow the membrane to dry because this will result in a loss of signal.
9. **Film exposure** – expose X-ray or instant film to the blot at RT. Exposure time will depend on the type of membrane used and on the protein concentration. Optimal conditions should be determined for each particular application. Develop the film according to the manufacturer's instructions.

   For further information, including a complete troubleshooting guide, consult the Biotin-Blot total protein detection kit manual.

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *(800) 4BIORAD* ***Australia*** *02 9914 2800*
Austria *(01)-877 89 01* ***Belgium*** *09-385 55 11* ***Brazil*** *55 21 2527 3454*
Canada *(905) 712-2771* ***China*** *(86 21) 6426 0808*
Czech Republic *+ 420 2 41 43 05 32* ***Denmark*** *44 52 10 00*
Finland *09 804 22 00* ***France*** *01 47 95 69 65* ***Germany*** *089 318 84-0*
Greece *30 210 777 4396* ***Hong Kong*** *(852) 2789 3300*
Hungary *36 1 455 8800* ***India*** *(91-124)-2398112/3/4, 5018111, 6450092/93*
Israel *03 951 4127* ***Italy*** *39 02 216091* ***Japan*** *03-5811-6270*
Korea *82-2-3473-4460* ***Latin America*** *305-894-5950*
Mexico *55-52-00-05-20* ***The Netherlands*** *0318-540666*
New Zealand *64 9 415 2280* ***Norway*** *23 38 41 30*
Poland *+ 48 22 331 99 99* ***Portugal*** *351-21-472-7700*
Russia *7 095 721 1404* ***Singapore*** *65-64153188*
South Africa *00 27 11 4428508* ***Spain*** *34 91 590 52 00*
Sweden *08 555 12700* ***Switzerland*** *061 717 95 55*
Taiwan *(886 2) 2578 7189/2578 7241* ***United Kingdom*** *020 8328 2000*

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