Immun-Lite™ II Chemiluminescent Protein Detection System

For Use with Nitrocellulose and Membranes

Instruction Manual

Catalog Numbers
170-6477
170-6478
170-6479

BIO-RAD

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Section 1
Preparation

1.1 Introduction

The Immun-Lite II chemiluminescent detection system is a sensitive, non-isotopic kit for immunodetection of specific antigens immobilized on nitrocellulose or PVDF membrane. The Immun-Lite II kits are available for rabbit, mouse, or human primary antibody systems.

The Immun-Lite assay is fast and simple. Antigen is transferred to the membrane. The transfer can be done electrophoretically, following separation of the antigen in a polyacrylamide or agarose gel, passively by directly spotting the antigen to 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 non-fat dry milk.

The membrane with bound antigen is then incubated with first antibody, specific for the antigen to be detected. The blot is washed to remove unbound antibody and incubated with the respective GAR, GAM, or GAH second antibody, which has been conjugated to alkaline phosphatase (AP). The membrane is treated with Immun-Lite enhancer and then incubated with the chemiluminescent substrate, CSPD.[1,2] The use of Immun-Lite enhancer is required when using nitrocellulose or PVDF membranes. Without the enhancer, exposure times are significantly longer to obtain a comparable signal. The blot is then exposed to X-ray or instant film.

The Immun-Lite kits provide all the necessary reagents for the chemiluminescent assay in an easy to use form. Each kit provides enough reagent to assay 2,000 cm^2 of membrane. The Immun-Lite kits are for research use only.

1.2 Product Information

Catalog Number	Product Description
	Immun-Lite II Assay Kit
170-6477	**Goat Anti-Rabbit IgG (H+L)-AP**
170-6478	**Goat Anti-Mouse IgG (H+L)-AP**
170-6479	**Goat Anti-Human IgG (H+L)-AP**
	Immun-Lite Assay Kit, with Immun-Lite membrane
170-6470	**Goat Anti-Rabbit IgG (H+L)-AP**
170-6471	**Goat Anti-Mouse IgG (H+L)-AP**
170-6472	**Goat Anti-Human IgG (H+L)-AP**
	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**, non-fat dry milk, 300 g
170-6473	**Chemiluminescent Substrate Kit**
	Blotting Membranes
	Nitrocellulose Membrane, 0.45 mm
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
162-0115	**Sheets**, 15 x 9.2 cm, 10
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 mm
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
	PVDF Membrane
162-0180	**Sheets**, 10 x 15 cm, 10
162-0181	**Sheets**, 15 x 15 cm, 10
162-0182	**Sheets**, 20 x 20 cm, 10
162-0184	**Roll**, 24 cm x 3.3 m, 1
162-0185	**Sheets**, 20 x 20 cm, 3

Catalog Number	Product Description
	Blotting Standards
161-0306	**Biotinylated Standards**, low range, 250 µl
161-0307	**Biotinylated Standards Kit (HRP)**, low range
161-0308	**Biotinylated Standards Kit (AP)**, low range
161-0311	**Biotinylated Standards**, high range, 250 µl
161-0312	**Biotinylated Standards Kit (HRP)**, high range
161-0313	**Biotinylated Standards Kit (AP)**, high range
161-0319	**Biotinylated Standards**, broad range, 250 µl
161-0321	**Biotinylated Standards Kit (HRP)**, broad range
161-0322	**Biotinylated Standards Kit (AP)**, broad range
170-6528	**Avidin-HRP**, 2 ml
170-6533	**Avidin-AP**, 1 ml
161-0305	**Prestained SDS-PAGE Standards**, low range
161-0309	**Prestained SDS-PAGE Standards**, high range
161-0318	**Prestained SDS-PAGE Standards**, broad range

1.3 Storage and Stability of Kit Components

Note: This kit contains enough reagents to blot 2,000 cm^2 of membrane. The working solutions for each step should be used in the ratio of: 0.25 ml/cm^2 of membrane for the Substrate and Enhancer solutions; 0.5 ml/cm^2 of membrane for all other reagents. For example, for 200 cm^2 of membrane, use 50 ml of Substrate Solution, and 100 ml of all other working solutions (Blocking Solution, Wash Solution, and Antibody Solutions).

Product Description	Quantity Provided	Storage	Shelf Life
Tris-Buffered Saline, 10x, pH 7.5	2 L	RT	> 1 yr
Blotting Grade Blocker	75 g	RT	> 1 yr
Tween-20	5 ml	RT	> 1 yr
Conjugate Solution*	0.5 ml	4 °C	1 yr
Chemiluminescent Substrate	1 ml	4 °C	1 yr
Substrate Dilution Buffer, 25x, pH 10	40 ml	4 °C	1 yr
Immun-Lite Enhancer, 25x	40 ml	4 °C	1 yr

* Each kit contains the appropriate antibody conjugate solution. These reagents will be shipped frozen and can be stored at -20 °C until their initial use. When thawed, store at 4 °C. Avoid repeated freeze-thaw cycles.

1.4 Safety Instructions

Read the entire instruction manual before beginning the assay.

1. Wear gloves and protective clothing, such as a laboratory coat and goggles, when preparing and working with the solutions in the assay. The Substrate Dilution Buffer and Immun-Lite enhancer contain 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 minutes, and remove contaminated clothing.

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

1.5 Solutions

This kit has been designed to run 2,000 cm^2 of membrane. The reagent preparation section of this manual has been set to make enough of each solution for 200 cm^2 of membrane. This correlates to approximately one large style Western blot, or four mini-gel Western blots. If a larger or smaller amount of membrane blots is being 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. Some guidelines are given for the expected shelf lives of working solutions. 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 the best results, the dd water used to prepare solutions should be autoclaved or filtered prior to use in this assay.

Stock Solutions

Tris-buffered saline, 10x (10x TBS)

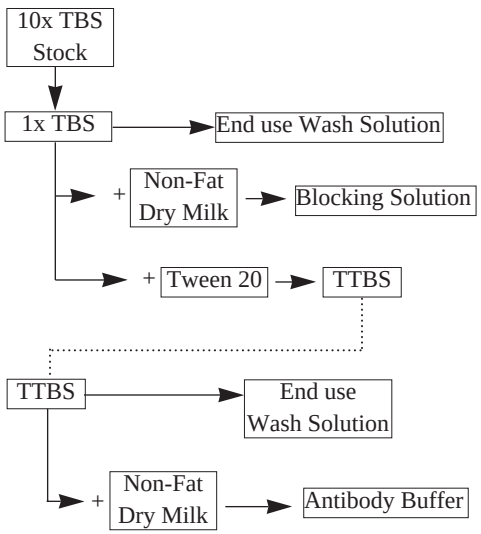
(200 mM Tris, 5 M NaCl, pH 7.5)

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

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 for a wash, 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 being made. Since there are many ways to go about making solutions, this flowchart can help you make best use of your time and reagents. The straight forward solutions were left out of the flowchart.

Refer to the detailed descriptions of the Working Solutions for the exact quantities and contents of all the solutions.



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

Tris-buffered saline, (TBS)

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

Add 140 ml of 10x TBS to 1,260 ml of dd water. Label this bottle "1x TBS".

Blocking solution

(5% non-fat dry milk in TBS)

Add 5.0 g of non-fat dry milk to 100 ml of 1x TBS. Stir until dissolved. Label this solution "Blocking Solution".

Wash solution (TTBS)

(20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5)

Add 500 µl of Tween-20 to 1000 ml of 1x TBS. Label this bottle "TTBS".

Antibody buffer

(1% non-fat dry milk in TTBS)

Add 2.0 g of non-fat dry milk to 200 ml TTBS. Stir until dissolved. Label this solution "Antibody Buffer".

First antibody solution

Dilute the first antibody to the appropriate titer in 100 ml of Antibody Buffer. Label this solution "First 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".

Enhancer solution, 1x (contains 0.02% sodium azide)

Mix 4 ml of 25x Immun-Lite enhancer Solution with 96 ml of dd water. Label this solution "Immun-Lite enhancer". Do not reuse this solution.

Substrate dilution buffer, 1x

(0.1 M diethanolamine, 1 mM $MgCl_2$, 0.02% sodium azide, pH 10).

Mix 4 ml of 25x Substrate Dilution Buffer with 96 ml of dd water. Label this solution "1x Substrate Dilution Buffer". This solution can be stored at 4 °C for up to 1 month.

Substrate solution

Add 180 µl of the chemiluminescent substrate reagent to 100 ml of 1x Substrate Dilution Buffer. Label this solution "Substrate Solution". This solution can be stored at 4 °C for up to 1 week.

Section 2
Immun-Lite Assay

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 required, 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, dd water should be autoclaved or sterile filtered prior to use in buffers and solutions.

Membrane Selection - the Immun-Lite II assay is specially designed for use with nitrocellulose or PVDF membranes. The use of Immun-Lite enhancer is required when performing blots on these types of membrane. Prolonged exposure times will be necessary if this step is omitted, with resultant interference from background development. If proteins are transferred to Immun-Lite nylon membrane, the use of Immun-Lite enhancer is not required. All other steps in the assay procedure will remain the same.

First 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 - 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 the greatest dilution of antibody reagent still resulting in a strong positive signal without membrane background or non-specific reactions.

Blotting Grade Conjugates - the conjugates supplied by Bio-Rad should be used in the concentrations indicated in Section 1.5. 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 non-specific hydrophobic reactions. At 0.05%, Tween-20 will not disrupt binding of primary antibodies to antigens or antigens to the membrane, but will optimize detection sensitivity by eliminating non-specific 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 are recommended for molecular weight determinations with the Immun-Lite assay. These 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. Because the Avidin-AP (or Streptavidin-AP) can be directly added to the second antibody solution, no extra steps are needed to detect the biotinylated standards. They 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 standards can also be used for assessing the transfer efficiency of samples, and for approximating the molecular weight of the blotted proteins.

Total Protein Detection - in the identification of specific antigens, total protein staining is required to correlate the signal detected to a complex protein mixture. The recommended staining 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 complete details.

2.2 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 indicate 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, 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-Lite assay can easily be adapted for use in the Bio-Dot® and Bio-Dot SF apparatus. This instrument allows rapid, reproducible applications of up to 96 samples on one membrane sheet. The membrane should be removed from the apparatus after antigen application. Because non-fat 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 wet 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 minutes. Apply antigen sample to each grid square using a syringe or a variable pipette, by displacing 1 µl of sample to the tip of the syringe or pipette as a drop 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.
2. **Wash -** following transfer, wash the membrane in TBS for 5 to 10 minutes 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 minutes to 1 hour at RT.
4. **Wash -** decant the blocking solution and add TTBS to the membrane. Wash for 5 to 10 minutes with gentle agitation at RT. Decant the wash solution and repeat the wash step with additional TTBS.
5. **First antibody incubation -** decant the TTBS and add the first antibody solution to the membrane. Incubate 1 to 2 hours 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.
6. **Wash -** decant the first antibody solution and add TTBS to the membrane. Wash for 5 to 10 minutes with gentle agitation at RT. Decant the wash solution and repeat the wash step two more times with additional TTBS.
7. **Second antibody conjugate incubation -** decant the TTBS and add the second antibody solution. Incubate for 30 minutes to 2 hours using gentle agitation at RT.
8. **Wash -** decant the conjugate solution and add TTBS to the membrane. Wash for 5 to 10 minutes with gentle agitation at RT. Decant the wash solution and repeat the wash step two more times with additional TTBS.
9. **Enhancer -** decant the TTBS and add 1x Immun-Lite enhancer solution to the membrane. Incubate for 15 minutes with gentle agitation. Incubation times may vary from 10 to 30 minutes. A stronger signal may be obtained by increasing the incubation time with the enhancer. Do not reuse the enhancer solution.
10. **Final Rinse -** remove the membrane and quickly rinse in TTBS.
11. **Blot development -** immerse the membrane in the chemiluminescent substrate solution. Incubate the blot for 5 minutes with gentle agitation. Remove the membrane from the solution, draining excess liquid from the blot. Seal the membrane in a heat-sealable bag. Do not allow the membrane to dry. This will result in loss of signal.
12. **Film exposure -** expose the blot to x-ray or instant film at room temperature. 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.

Table 1. Typical exposure times for nitrocellulose and PVDF membranes

Membrane	With Immun-Lite Enhancer	Without Immun-Lite Enhancer
Nitrocellulose	10 - 45 minutes	12 - 24 hours
PVDF	10 minutes	1 hour

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-Lite Enhancer was not used with nitrocellulose or PVDF membranes.	i. Include the use of Immun-Lite enhancer in the assay protocol. Exposure times will vary; for example, a nitrocellulose blot without enhancer would require an overnight exposure to generate a signal comparable to that Immun-Lite membrane would produce in 5 minutes.
	c. Blot was allowed to dry after incubation with the chemiluminescent substrate.	i. Use heat sealable bags to prevent drying of the membrane. Wrapping the blot in cellophane is not sufficient to keep the membrane wet.

Troubleshooting Guide *(continued)*

Problem	Probable Cause	Recommended Solution
	d. Chemiluminescent substrate solution is inactive.	i. Store the reagent at the proper storage temperature. ii. Tap water can inactivate the substrate solution. Use only distilled, deionized water to prepare the solution.
	e. First antibody solution is inactive or non-saturating.	i. Store the antibody solution at the proper storage temperature. Avoid bacterial contamination, heat inactivation, and repeated freeze-thaw cycles. 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 storage temperature as recommended in Section 1.3. Avoid repeated freeze-thaw cycles. ii. The concentration of the conjugate was non-saturating. Increase the concentration of the conjugate used in the assay.

Troubleshooting Guide *(continued)*

Problem	Probable Cause	Recommended Solution
		iii. Conjugate may be contaminated, causing inactivation of the antibody or enzyme. Tap water may cause inactivation; use only distilled, deionized water 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). 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. First antibody is not specific or does not recognize denatured antigens (common with monoclonals).	i. Loss of reactivity may have occurred during electrophoretic transfer. Pre-test the reactivity of the antibody against both antigen byf a dot-blot.
2. High background.	a. Exposure time was too long.	i. Decrease exposure time. native and denatured

Troubleshooting Guide *(continued)*

Problem	Probable Cause	Recommended Solution
		ii. The use of Immun-Lite enhancer is required when using nitrocellulose or PVDF 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 time of the blocking step and/or the concentration of blocker used.
	d. Blot was washed in the absence of Tween-20.	i. Tween-20 is necessary in wash steps to reduce background. The concentration can be increased if background persists.
	e. Second antibody conjugate was used at an excessive concentration.	i. Use the recommended dilution, or determine the optimal dilution experimentally.
	f. Chemiluminescent substrate was prepared at too high a concentration.	i. Use correct dilution (see Section 1.5).
	g. Contamination occur-red during transfer.	i. Refer to the instrument instruction manual for recommendations.
	h. Solutions and buffers are contaminated with alkaline phosphatase.	i. Autoclave or sterile filter dd water prior to use in making solutions.

Troubleshooting Guide *(continued)*

Problem	Probable Cause	Recommended Solution
		ii. Use blotting grade non-fat 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.

Section 4
References

1. Bronstein, I., Juo, R. R., Voyta, J. C. and Edwards, B., Bioluminescence and Chemiluminescence: Current status, John Wiley, Chichester, England (1991).
2. CSPD is a registered trademark of Tropix, Inc.

Section 5
Electrophoresis and Blotting Equipment

Bio-Rad provides a complete line of electrophoresis, electrophoretic transfer, and microfiltration apparatus that can be used with the Immun-Lite assay. For more information contact Bio-Rad Laboratories Technical Service toll-free at 1-800-4BIORAD (1-800-424-6723) or contact your local Bio-Rad representative.

Catalog Number	Product Description
	Vertical Electrophoresis Cells
165-1801	**PROTEAN® II xi Cell**
165-2940	**Mini-PROTEAN II Cell**
165-2944	**Mini-PROTEAN II Module**
	Transfer Cells
170-3910	**Trans-Blot Transfer Cell**
170-3946	**Trans-Blot Transfer Cell with Plate Electrodes**
170-3939	**Trans-Blot Transfer Cell with Plate Electrodes**, complete
170-3930	**Mini Trans-Blot Transfer Cell**
170-3935	**Mini Trans-Blot Module**
170-3940	**Trans-Blot SD Semi-Dry Transfer Cell**
	Microfiltration and Screening Apparatus
170-6542	**Bio-Dot SF Slot Format Apparatus**
170-6545	**Bio-Dot Apparatus**
170-4017	**Mini-PROTEAN II Multiscreen Apparatus**
	Incubation Apparatus
170-4037	**Large Incubation Tray**
170-4039	**Large Incubation Tray Lid**
170-4041	**Small Incubation Tray**
170-4045	**Small Incubation Tray Lid**
170-3902	**Mini-Incubation Trays**, 20
170-3903	**Mini-Incubation Trays**, 100

Appendix 1
Total Protein Detection Procedure

Required Reagents

Catalog Number	Product 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
170-6430	**Premixed Tris-Buffered Saline**, 10x
170-6435	**TBS Buffer Solution**, 10x, 1 L
161-0715	**Tween-20**, 100 ml

Additional Reagents Required

Sodium borate, 10-hydrate ($Na_2B_4O_7$•10 H_2O) - ACS reagent grade
Sodium chloride (NaCl) - ACS reagent grade

Solutions

Stock Solutions

Tris-buffered saline, 10x (10x TBS)

(200 mM Tris, 5 M NaCl, pH 7.5)

Working Solutions

Borate-Tween (1xBT)

(0.05 M $Na_2B_4O_7$•10H_2O, 0.5 M NaCl, 0.2% Tween-20, pH 9.3)
Dissolve 38.14 g $Na_2B_4O_7$•10H_2O and 58.44 g NaCl in 1.9 L dd water. Add 4 ml Tween-20, then bring to a final volume of 2 L with dd water 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 dd water. Label this bottle “1x TBS”.

Wash solution (TTBS)

(20 mM Tris, 500 mM NaCl, 0.2% Tween-20, 5% MPO, pH 7.5)

Add 2 ml of Tween-20 and 50 ml MPO to 1L 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 dilution buffer, 1x (contains 0.02% sodium azide)

(0.1 M diethanolamine, 1 mM $MgCl_2$, 0.02% sodium azide, pH 10).

Mix 4 ml of 25x Substrate Dilution Buffer with 96 ml of dd water. Label this solution "1x Substrate Dilution Buffer". This solution can be stored at 4 °C for up to 1 month.

Substrate solution

Add 180 µl of the chemiluminescent substrate reagent to 100 ml of 1x Substrate Dilution Buffer. Label this solution "Substrate Solution". This solution can be stored at 4 °C for up to 1 week.

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 minutes. 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 minutes 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 minutes. Repeat the wash step.
4. Wash the membrane in 100 ml of TTBS for 5 minutes. Repeat the wash step.

5. Prepare the Avidin-AP or Streptavidin-AP solution. Incubate the membrane in the solution for 1 hour with agitation.
6. Wash the membrane in 100 ml of TTBS for 5 minutes. Repeat the wash step.
7. Wash the membrane in 100 ml of TBS for 5 minutes. Repeat the wash step.
8. Decant the TTBS and add 1x Immun-Lite Enhancer solution to the membrane. Incubate for 15 minutes with gentle agitation. Incubation times may vary from 10 to 30 minutes. A stronger signal may be obtained by increasing the incubation time with the enhancer. Do not reuse the enhancer solution.
9. Remove the membrane and quickly rinse in TTBS.
10. Prepare the chemiluminescent substrate solution.
11. Immerse the membrane in the chemiluminescent substrate solution. Incubate the blot with agitation for 5 minutes. Remove the membrane from the solution, draining excess liquid from the blot. Seal the membrane in a heat sealable bag. Do not allow the membrane to dry. This will result in loss of signal. Expose the blot to x-ray or instant film at room temperature. 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

Life Science Group

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