Amplified Alkaline Phosphatase Goat Anti-Rabbit Immun-Blot® Assay Kit

Instruction Manual

Catalog Number 170-6412

For Technical Service

Call Your Local Bio-Rad Office or

in the U.S. Call **1-800-4BIORAD**

(1-800-424-6723)

Table of Contents

Section 1 Preparation....................1

1.1 Introduction 1

1.2 Product Information 2

1.3 Storage and Stability of Kit Components 2

1.4 Safety Instructions 3

1.5 Solutions....................3

Section 2 Immun-Blot Assay 5

2.1 Experimental Strategy and General Recommendations....................5

2.2 Detailed Assay Procedure....................7

2.3 Detailed Color Development Procedure....................9

Section 3 Troubleshooting 10

3.1 Test for Monitoring Reagent Activity 10

3.2 Troubleshooting Guide....................11

Section 4 References....................15

Section 5 Electrophoresis and Blotting Equipment....................16

Section 1
Preparation

1.1 Introduction

The Amplified Alkaline Phosphatase Immun-Blot assay kit is an enzyme immunoassay system using the latest in biotin-streptavidin technology[1-3] to achieve sensitive qualitative detection of specific antigens following dot-blotting[4] or electrophoretic blotting[5-13] to nitrocellulose membrane. The combination of streptavidin and biotin has proven to be a convenient Western blotting tool that allows excellent sensitivity and specificity while reducing nonspecific background.[14-15] The kit provides all necessary components in an easy-to-use form. Each kit provides enough reagent to assay 200 membrane strips. The Amplified Alkaline Phosphatase Immun-Blot kit is for research use only.

The amplified alkaline phosphatase assay is similar to other Immun-Blot systems. The assay is fast and simple. Antigen is transferred and bound to the nitrocellulose membrane. This transfer can be done electrophoretically, following separation of antigen in a polyacrylamide or agarose gel, passively by directly spotting the antigen onto the membrane, or by vacuum filtration using a microfiltration apparatus. Following binding of antigen, the remaining unbound membrane sites are blocked with non-fat dry milk. Bound antigen is then incubated with the first antibody specific for the antigen to be detected. The blot is washed to remove unbound antibody and incubated with a biotinylated antibody specific to the first antibody. Following this incubation the membrane is again washed and incubated with the streptavidin-biotinylated alkaline phosphatase complex which will bind to the biotin of the second antibody. Finally this solution is removed and

the membrane thoroughly washed. BCIP/NBT substrate is used to visualize the target antigen. The developed membrane will show purple bands or dots against a white background.

1.2 Product Information

Catalog Number	Product Description
170-6412	**Amplified Alkaline Phosphatase Goat Anti-Rabbit Immun-Blot Assay Kit,** without nitrocellulose
	Individual Blotting Grade Reagents
170-6401	**Blotting Grade Affinity Purified Biotinylated Goat Anti-Rabbit IgG (H+L),** human IgG adsorbed, 1 ml
170-6403	**Blotting Grade Biotinylated Alkaline Phosphatase,** 1 ml
170-6404	**Blotting Grade Blocker,** non-fat dry milk, 300 g
170-6408	**Blotting Grade Streptavidin,** 1 mg
170-6435	**Premixed Tris-Buffered Saline,** 10x, 1 L
170-6531	**Tween-20,** 100 ml
170-6432	**Alkaline Phosphatase Conjugate Substrate Kit,** 1 L

Note: See Section 1.3 for a complete list of reagents included in the kits.

1.3 Storage and Stability of Kit Components

Note: Each kit contains all the reagents necessary to complete 200 assays. One assay uses a 0.75 x 9.2 cm membrane strip with 5 ml of each working solution per incubation step.

Product Description	Quantity	Storage	Shelf Life
Biotinylated Goat Anti-Rabbit Antibody[1]	0.5 ml	4 °C	1 year
Biotinylated Alkaline Phosphatase[1]	0.5 ml	4 °C	1 year
Streptavidin[2] (rehydrated)	0.5 ml	-20 °C	6 months
Blotting Grade Blocker	75 g	RT	1 year
Tris-Buffered Saline, 10x	1 L	4 °C	1 year
Tween-20	5 ml	RT	1 year
AP Color Reagents A and B[3]	10 ml	-20 °C	1 year
AP Color Development Buffer, 25x	40 ml	4 °C	1 year

1. Conjugate solutions are shipped frozen. Once thawed, store at 4 °C. Avoid repeated freeze-thaw cycles.
2. Aliquot samples to prevent repeated freeze-thaw cycles; storage at 4 °C will reduce shelf life to 3 months.
3. AP color reagent A contains NBT in aqueous DMF (dimethylformamide); AP color reagent B contains BCIP in DMF.

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. DMF and BCIP can cause skin and eye irritation, and contact should be avoided. 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 Safety Data Sheets on DMF for additional information.
2. Work in well-ventilated areas. Avoid inhalation of vapors when handling solutions containing DMF and BCIP.
3. Do not mouth pipet any solutions.

1.5 Solutions

The following solutions must be prepared for each kit. The working solution volumes are based on 20 assays developing 0.75 x 9.2 cm nitrocellulose strips. For each strip, the assay uses 5 ml of each working solution per incubation step. Use at least 0.5 ml solution per square centimeter (cm^2) of membrane. For best results, the membrane must be completely covered with solution in all wash and incubation steps. Larger volumes can be used for convenience. However, all volumes should be increased proportionately to insure that all kit reagents are consumed at the same rate.

Stock Solutions

Tris-buffered saline, 10x (10x TBS) (200 mM Tris, 5 M NaCl, pH 7.5).

Color development buffer, 25x concentrate.

Working Solutions (based on 20 assays of 5 ml each)

Tris-buffered saline (TBS) (20 mM Tris, 500 mM NaCl, pH 7.5): Add 100 ml of 10x TBS to 900 ml of dd water. Label this bottle “1x TBS”.

Wash solution (TTBS) (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5): Add 450 µl Tween-20 to 900 ml of 1x TBS. Label this bottle “TTBS”.

Blocking solution (5% non-fat dry milk in TBS): Add 5.0 g of non-fat dry milk to 100 ml of TBS. Stir until dissolved. Label this solution “Blocking Solution”.

First antibody solution: Dilute the first antibody to the appropriate titer in 100 ml of TTBS. Label this solution “First Antibody Solution”.

Second antibody solution (1:3,000): Add 33 µl of the biotinylated goat anti-rabbit antibody to 100 ml of TTBS. Label this solution “Second Antibody Solution”.

Streptavidin-biotinylated alkaline phosphatase complex (1:3,000 dilution for each): Add 33 µl of streptavidin to 100 ml TTBS. Label this “Streptavidin Solution”. Add 33 µl of biotinylated-alkaline phosphatase to the Streptavidin Solution just prepared. Allow the complex to incubate for 1 to 3 hours at RT before using. If allowed to form over 3 hours, remake the solution.

AP color development buffer: The stock AP color development buffer solution is a 25x concentrate. It should be diluted with filtered, deionized water. Dilute to a 1x working solution by adding 1 part AP color development buffer concentrate to 24 parts filtered, deionized water. Example, to make 100 ml of 1x AP color development buffer, mix 96 ml of filtered, deionized water with 4 ml of 25x

AP color development buffer concentrate. Mix well. Store excess solution at 4 °C.

Since the AP color development buffer is better stored for longer periods as a concentrate, make as much 1x solution as is practical and needed for the planned experiments.

An alternative is to calculate the amount of 25x concentrate needed for the specific experiment being done. In this method, all the color reagents can be added to the same vial for the color development reaction.

Section 2
Immun-Blot Assay

2.1 Experimental Strategy and General Recommendations

1. **Background**—three types of background are common to immune blot detection:
   a. High membrane coloration—high backgrounds usually result when the blocking period is too short, when Tween-20 is absent from the appropriate buffers and washes, or when excessive amounts of antibody and/or enzyme are used.
   b. Non-specific antibody binding—evidenced by extra banding or high coloration in the separate lanes. This background is usually due to impure or cross-reactive antibodies, incubations with excessive antibody and/or enzyme concentrations, or when Tween-20 is absent from the appropriate buffers and washes.

   c. Non-specific conjugate binding—evidenced by band development in the absence of first antibody. This background results when second antibody and/or enzyme is used in excess or when Tween-20 is absent from the appropriate buffers and washes.
2. **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.
3. **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.
4. **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 1:500-1:10,000 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 the greatest dilution of antibody reagent still resulting in a strong positive signal without membrane background or non-specific reactions.

5. **Conjugates**—the conjugates supplied by Bio-Rad should be used in the concentrations recommended in Section 1.5. Using a conjugate at higher concentrations may result in an overall increase in background without any increase in detection sensitivity.
6. **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.
7. **Addition of 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. Alternative detergents, or concentrations of Tween-20 other than 0.05%, should not be substituted. The wash between the blocking step and incubation with first antibody is essential and should not be altered.
8. **Molecular Weight standards**—several types of blotting standards are available from Bio-Rad. The Prestained and Kaleidoscope Standards can be used to assess the transfer efficiency in your blotting system. For accurate molecular weight determinations, use Biotinylated SDS-PAGE Standards. These standards will be simultaneously detected by reaction with the streptavidin-biotinylated alkaline phosphatase complex used in the amplified Immun-Blot protocol.

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 develop color if the procedure is successful. This is an excellent check on the operation of the assay and will help you to gauge the rate of color development.
   a. **Electrophoretic blotting**—the antigens of interest are electrophoretically transferred to the membrane from a gel support (*i.e.* SDS-PAGE gel, IEF gel, or native gel) using the Trans-Blot®, Mini Trans-Blot®, or Trans-Blot SD cell. If desired, cut the wet nitrocellulose 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-Blot 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 nitrocellulose sheet to the appropriate size. Draw a grid on the membrane with a pencil. Typical grids consist of 1 x 1 cm squares. Wet the dry membrane by slowly sliding the membrane at a 45° angle into TBS. Remove the thoroughly wetted membrane from the buffer and dry it on filter paper for approximately 5 minutes. Apply sample antigen to each grid square using a syringe or 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 nitrocellulose 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 nitrocellulose membrane should be allowed to dry completely before proceeding to the blocking step.

2. **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 1 hour at room temperature (RT). An overnight incubation will not harm the blot.
3. **Wash**—decant the blocking solution and add TTBS to the membrane. Wash for 5–10 minutes with gentle agitation at RT. Decant and repeat the wash step with additional TTBS.
4. **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.

5. **Wash**—decant the first antibody solution and add TTBS to the membrane. Wash for 5–10 minutes with gentle agitation at RT. Decant and repeat the wash step with additional TTBS.
6. **Second antibody incubation**—decant the TTBS and add the biotinylated goat anti-rabbit antibody solution to the membrane. Incubate for 1 to 2 hours with gentle agitation at RT.
7. **Formation of the streptavidin-biotinylated alkaline phosphatase complex**—while the blot is in the second antibody solution, prepare the streptavidin-biotinylated alkaline phosphatase complex. (See Section 1.5.) Allow complex to form for 1 hour at RT. (Note: do not leave more than 3 hours.)
8. **Wash**—decant the second antibody solution and add TTBS to the membrane. Wash for 5–10 minutes with gentle agitation at RT. Decant and repeat the wash step with additional TTBS.
9. **Streptavidin-biotinylated alkaline phosphatase complex***—decant the TTBS and add the streptavidin-biotinylated alkaline phosphatase complex to the membrane. Incubate for 1 to 2 hours with gentle agitation at RT.
10. **Final washes**—Decant the complex solution and add TTBS to the membrane. Wash for 5–10 minutes with gentle agitation at RT. Decant and repeat the wash step three more times with additional TTBS.

* Save the used complex solution, at 4 °C, for testing in case results are unsatisfactory. See Section 3.1.

2.3 Detailed Color Development Procedure

1. **Developer preparation**—immediately before use, add 1.0 ml of AP color development reagent A and 1.0 ml of color development reagent B to 100 ml of color development buffer at RT. This solution can be stored at 4 °C overnight, but prompt use is recommended.
2. **Color development**—immerse the nitrocellulose membrane in the color development solution.** Protein concentrations greater than 100 ng will immediately become visible as purple bands or dots. Lower concentrations of protein will take longer, but should be visible with 30 minutes. Should a large amount of precipitate form before color development is complete, decant the color development solution and add additional, freshly prepared, color development solution. The precipitate, which is usually generated by high concentrations of alkaline phosphatase on the membrane surface, will settle on the membrane and can produce unusually high backgrounds.
3. **Wash**—stop the development by immersing the membrane in dd water for 10 minutes with gentle agitation. Change the water at least once during the 10 minute period to remove residual color development solution.
4. **Reading, drying, and storage**—take photographs while the membrane is wet to enhance the purple color. Acceptable photographs can be produced using Polaroid Type 108, Polacolor 2 Land Film, at f8 and 1 second exposure. Film should be developed for 1 minute. Dry the membrane on filter paper and store between polyester sheets (*e.g.* Gel Support Film, catalog number 170-4251). Protect from light to minimize fading.

** Save used color development solution for testing in case results are unsatisfactory. See Section 3.1.

Section 3 Troubleshooting

3.1 Tests for Monitoring Reagent Activity

1. **Activity test for the color development solution.**

   Combine 1.0 ml of the color development solution with 10 µl of full strength biotinylated alkaline phosphatase. The color should develop immediately. If color fails to develop within a few minutes, the color development solution is inactive. Make up fresh working solution and repeat the color development assay.
2. **Enzyme activity test for the enzyme complex.**

   Combine 1.0 ml of the color development solution (tested in step 1) and 1.0 ml of the prepared complex (streptavidin-biotinylated alkaline phosphatase solution). A light purple tinge should develop within 15 minutes. If color fails to develop with 25 minutes, the prepared complex solution is suspect. Repeat the procedure with a freshly prepared complex solution.
3. **Activity test for the first antibody solution.**

   Use an RID, Ouchterlony immunodiffusion, precipitation, or ELISA test to determine reactivity of the antibody with the antigen. If possible, repeat the Immun-Blot procedure with a more concentrated first antibody solution.

3.2 Troubleshooting Guide

Problem	Probable Cause	Recommended Solution
1. No reaction or weak color development	a. Color development solution is inactive (see Section 3.1)	i. Color development reagents must be stored at the proper temperature (see Section 1.3)
		ii. Avoid bacterial contamination of the color development buffers by storage at 4 °C
		iii. Tap water can inactivate the color development solution. Use only distilled, deionized water to prepare the solutions.
	b. First antibody solution is inactive or non-saturating (Section 3.1)	i. Antibody is improperly stored. Avoid bacterial contamination, inactivation, and repeated freeze-thaw cycles.
		ii. Antibody titer is too low. Increase the concentration of antibody used in the assay.

Problem	Probable Cause	Recommended Solution
		iii. Tween-20 may affect the reactivity of some antibodies. Eliminate Tween-20 from the assay (except the wash after blocking).
	c. Streptavidin-biotinylated alkaline phosphatase is inactive (see complex Section 3.1)	i. Components are improperly stored. Review Section 1.3. Do not subject to repeated freeze-thaw cycles.

Problem	Probable Cause	Recommended Solution
		ii. Complex may be contaminated, causing inactivation of the streptavidin or the biotinylated alkaline phosphatase. Always use a clean pipet tip for each stock solution when preparing the complex. Contaminated stock streptavidin or biotinylated alkaline phosphatase solutions will not produce a working complex.
		iii. Tap water may cause inactivation. Use only distilled, deionized water.

Problem	Probable Cause	Recommended Solution
	d. Little or no antigen is bound to the membrane	i. Transfer of protein onto membrane was incomplete. Stain the gel to assure transfer of protein. Use prestained standards to monitor transfer efficiency. Consult the Trans-Blot, Trans-Blot SD or Mini Trans-Blot manual for proper electrophoretic transfer procedures. Refer to the Bio-Dot or Bio-Dot SF manual for proper dot blotting procedures.
tiv-	e. First antibody is not specific or does not recognize denatured antigens (common monoclonals)	i. Loss of reactivity may have occurred during electrophoretic transfer. Pretest the reactivity of the antibody against both native and denatured antigen by a dot blot. Refer to the Trans-Blot manual for the transfer of native antigens.

Problem	Probable Cause	Recommended Solution
	f. Antigen is too dilute	i. Increase the amount of antigen in the assay.
2. High background (refer to experimental strategy section)	a. Blocking is insufficient	Increase the blocking step to 90 minutes
	b. Nitrocellulose is left in the color development solution too long	Remove the membrane when the reaction appears to be complete. If precipitate in the development solution appears, decant the solution and use fresh reagent.
	c. Blot is washed in the absence of Tween-20	Tween-20 is necessary in the washes to reduce background
	d. Streptavidin-alkaline phosphatase complex is used at an excessive concentration	Use the recommended 1:3,000 dilution
	e. Contamination occurred during transfer	Refer to the Trans-Blot, Trans-Blot SD, or Mini Trans-Blot manual

Problem	Probable Cause	Recommended Solution
	f. Use of poor quality, mixed ester nitrocellulose will cause increased background	Use pure nitrocellulose from Bio-Rad

Section 4 References

1. Chaiet, L. and Wolf, F. J., *Archives of Biochem. and Biophysics,* **106,** 1-5 (1964).
2. Guesdon, J. L., Ternynck, T. and Avrameas, S., *J of Histochem. and Cytochem.,* **27,** 8, 1131-1139 (1979).
3. Fuccillo, D. A., *BioTechniques,* **3,** 6, 494-501 (1985).
4. Hawkes, R., Niday, E. and Gordon, J., *Anal. Biochem.,* **119,** 142 (1982).
5. Towbin, H., Staehlin, T. and Gordon, J., *PNAS,* **76,** 4350 (1979).
6. Glass, W. F., Briggs, R. C. and Hnilica, L. S., *Anal. Biochem.,* **115,** 219 (1981).
7. Karcher, D., Lowenthal, A., Thormar, H. and Noppe, M., *J. Immunol. Meth.,* **43,** 175 (1981).
8. Legocki, R. P. and Verma, D. S. P., *Anal. Biochem.,* **111,** 385 (1981).
9. Reiser, J. and Wardale, J., *Eur. J. Biochem.,* **114,** 569 (1981).
10. Olmstead, J. B., *J. Biol. Chem.,* **256,** 11955 (1981).
11. Symington, J. M., Green, J. and Brackmann, K., *PNAS,* **78,** 177 (1981).
12. Burnette, W. N., *Anal. Biochem.,* **112,** 195 (1981).

13. Warren, J. S., Yabroff, K. R., Remick, D. G., Kunkel, S. L., *et. al.*, *J. Clin. Invest.,* **84,** 1873-1882 (1989).

14. Remick, D. G., Strieter, R. M., Eskandari, M. K., *et. al.*, *Am. J. Pathol.,* **136,** 1, 49-60 (1990).

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 Amplified AP Immun-Blot 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.

Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547
LIT232 Rev F