Immun-Blot®
Assay Kit

Instruction
Manual

BIO-RAD

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Section 1
Preparation

1.1 Introduction

The Immun-Blot assay kits are enzyme immunoassay kits optimized for the detection of specific antigens, at picogram levels, following electrophoretic blotting or dot blotting to a membrane. The kits provide all necessary components and chemicals, in an easy-to-use form, for detection of selected purified proteins using a double antibody or antibody-binding protein blotting assay.

The Immun-Blot alkaline phosphatase and Immun-Blot horseradish peroxidase assay systems can be used to detect rabbit, mouse, or human immune complexes.[1] They will give sensible detection of specific antigens or cloned translation products following dot-blotting,[2] electrophoretic blotting,[3-11] filter affinity transfers, [12] and *in situ* colony or plaque lifts.

The Immun-Blot assay is fast and simple. Antigen is transferred and bound to the membrane. This transfer can be done electrophoretically, following separation of the antigen in a polyacrylamide or agarose gel, or passively by either directly spotting the antigen to a membrane or by overlaying a phage plate or lysed bacterial colony with nitrocellulose membrane to bind expressed proteins. Following binding of antigen, the remaining protein binding sites on the membrane surface are blocked with gelatin or equivalent proteins which will not react with the primary and secondary antibodies. BSA (not included in the kit) is used as a blocker when the Bio-Dot® or Bio-Dot SF apparatus is used for blotting, since gelatin will obstruct filtration through blotting membranes.

The membrane with bound antigen is then incubated with first antibody, specific for the antigen to be detected. The membrane is

washed to remove unbound antibody and incubated with the respective GAR, GAM, or GAH second antibody, or Protein A or Protein G, which has been conjugated to alkaline phosphatase (AP) or horseradish peroxidase (HRP). In the case of AP, a color development reagent containing 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium (NBT) yields purple bands or "dots" on a white background. The HRP color development reagent, containing 4-chloro-1-naphthol and hydrogen peroxidase, also yields purple bands against a white background.

1.2 Product Information

Catalog Number	Product Description
Immun-Blot Assay Kit	
170-6460	**Goat Anti-Rabbit IgG (H+L) AP**
170-6461	**Goat Anti-Mouse IgG (H+L) AP**
170-6462	**Goat Anti-Human IgG (H+L) AP**
170-6463	**Goat Anti-Rabbit IgG (H+L) HRP**
170-6464	**Goat Anti-Mouse IgG (H+L) HRP**
170-6465	**Goat Anti-Human IgG (H+L) HRP**
170-6466	**Protein A-HRP**
170-6467	**Protein G-HRP**
Individual Blotting Grade Reagents	
170-6518	**Goat Anti-Rabbit IgG (H+L) AP Conjugate**
170-6520	**Goat Anti-Mouse IgG (H+L) AP Conjugate**
170-6521	**Goat Anti-Human IgG (H+L) AP Conjugate**
170-6515	**Goat Anti-Rabbit IgG (H+L) HRP Conjugate**
170-6516	**Goat Anti-Mouse IgG (H+L) HRP Conjugate**
172-1050	**Goat Anti-Human IgG (H+L) HRP Conjugate**
170-6522	**Protein A-HRP Conjugate**

Catalog Number	Product Description
Individual Blotting Grade Reagents (continued)	
170-6425	**Protein G-HRP Conjugate**
170-6435	**Premixed Tris Buffered Saline,** 10x, 1 L
170-6431	**Horseradish Peroxidase Conjugate Substrate Kit**
170-6432	**Alkaline Phosphate Conjugate Substrate Kit**
170-6537	**Gelatin,** Blotting Grade, 200 g
170-6531	**Tween-20,** Blotting Grade, 100 ml
Blotting Membranes	
Nitrocellulose Membrane (0.45 µm)	
162-0115	**Roll,** 33 cm x 3 m, 1
162-0145	**Sheets,** 7 x 8.4 cm, 10
162-0117	**Sheets,** 9 x 12 cm, 10
162-0114	**Sheets,** 15 x 9.2 cm, 10
162-0116	**Sheets,** 15 x 15 cm, 10
162-0113	**Sheets,** 20 x 20 cm, 5
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
Blotting Media	
Supported Nitrocellulose Membrane (0.45 µm)	
162-0190	**Sheets**, 7 x 8.4 cm, 10
162-0191	**Sheets**, 10 x 15 cm, 10
162-0192	**Sheets**, 15 x 15 cm, 10
162-0193	**Sheets**, 20 x 20 cm, 10
162-0194	**Roll**, 30 cm x 3 m, 1

Catalog Number	Product Description
	Supported Nitrocellulose Membrane (0.2 µm)
162-0195	**Sheets**, 7 x 8.4 cm, 10
162-0196	**Sheets**, 15 x 15 cm, 10
162-0197	**Roll**, 30 cm x 3 m, 1
	PVDF Membrane (0.2 µm)
162-0180	**Sheets**, 10 x 15 cm, 10
162-0181	**Sheets**, 15 x 15 cm, 10
162-0183	**Sheets**, 20 x 20 cm, 10
162-0184	**Roll**, 24 cm x 3.3 m, 1
162-0185	**Sheets**, 20 x 20 cm, 3
162-0186	**Sheets**, 7 x 8.4 cm, 10

1.3 Materials Provided with the Immun-Blot Kit Storage and Stability of Kit Components

Product Description	Quantity Provided	Storage	Shelf Life
Components supplied in all kits			
Tris buffered saline, 10x, pH 7.5	1 L	4 °C	>1 yr
Gelatin, Blotting grade	50 g	RT	>1 yr
Tween-20, Blotting grade	5 ml	RT	>1 yr
Conjugate solution*	0.5 ml	4 °C	1 yr
Components supplied in alkaline phosphatase kits			
AP color reagent A (contains NBT in aqueous dimethylformamide [DMF], containing magnesium chloride)	10 ml	-20 °C	1 yr
AP color reagent B (contains BCIP in DMF)	10 ml	-20 °C	1 yr
AP color development buffer, 25x	40 ml	4 °C	>1 yr

Product Description	Quantity Provided	Storage	Shelf Life
Components supplied in horseradish peroxidase kits			
HRP color regent A (contains 4-chloro-1-naphthol in diethylene glycol)	200 ml	-20 °C	1 yr
HRP color reagents B (contains hydrogen peroxide)	10 ml	4 °C	1 yr
HRP color development buffer, 10x	100 ml	4 °C	>1 yr
Additional components			
Citrate buffered saline (CBS), 3x, pH 5.5 (provided in the Protein G HRP kit only)	100 g	RT	>1 yr

* Each kit provides a different conjugate solution. These reagents are shipped frozen, and can be stored at -20 °C until their initial use. Once thawed, store the conjugate solution at 4 °C. Avoid repeated freeze-thaw cycles, which will cause breakdown of the product.

Note: Each kit contains all reagents necessary to complete 200 x 5 ml assays. An excess of conjugate has been supplied in each kit. BSA must be substituted for gelatin when blocking the membrane in the Bio-Dot or Bio-Dot SF microfiltration apparatus.

1.4 Safety Instructions

Read 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, diethylene glycol, 4-chloro-1-naphthol, 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.

2. Work in well-ventilated areas. Avoid inhalation of vapors when handling solutions containing DMF, diethylene glycol, 4-chloro-1-naphthol, 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. It is advisable to use at least 0.5 ml of 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	HRP color development buffer, 10x AP color development buffer, 25x
Color development reagents	Ready to use as provided in the kit.
Citrate buffered saline (CBS)	With the Protein G-HRP assay kit, add the contents of the citrate buffered saline to a 1 L bottle, add 900 ml dd water, agitate to dissolve, and quesce to 1.0 L with dd water. Label this solution “3x CBS.” Store at 4 °C.

Working solution (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 a 1 L bottle and quesce to 1.0 L with dd water. Label this bottle “TBS.”
Citrate buffered saline (CBS)	(20 mM citrate, 500 mM NaCl, pH 5.5) For the Protein G-HRP assay, add 100 ml of 3x CBS to 200 ml dd water. Label this solution “CBS.”
HRP Color development buffer	The HRP color development buffer solution is a 10x concentrate. It should be diluted with filtered, deionized water. Dilute to a 1x working solution by adding 1 part HRP color development buffer concentrate to 9 parts filtered, deionized water. For example, to make 100 ml of 1x HRP color development buffer, mix 90 ml of filtered, deionized water with 10 ml of 10x HRP color development buffer concentrate. Mix well. Store excess solution at 4 °C. Since the HRP 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.
AP Color development buffer	The 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. For 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 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.
Wash solution (TTBS)	(20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5) Add 350 µl of Tween-20 to 700 ml to 1x TBS. Label this bottle "TTBS." For the Protein G-HRP assay, make 400 ml of TTBS instead, using 200 µl of Tween-20. Also prepare 300 ml of TCBS by adding 150 µl of Tween-20 to 300 ml of CBS. Label this bottle "TCBS."
Blocking solution	(3% gelatin - TBS) Add 3.0 g of gelatin to 100 ml of TBS and heat to 50 °C, with stirring, until dissolved. A microwave oven will quickly solubilize the gelatin, but do not heat above 65 °C. Label this solution "Blocking solution - 3% gelatin in TBS."
Antibody Buffer	(1% gelatin - TTBS) Add 2.0 g of gelatin to 200 ml of TTBS and heat, with stirring to 50 °C until dissolved. Again, you can use a microwave oven. Label this solution "Antibody buffer - 1% gelatin in TTBS." Store at 4 °C. For the Protein G-HRP assay, prepare only 100 ml of 1% gelatin in TTBS and prepare 100 ml of 1% gelatin in TCBS.
First antibody solution	Dissolve the first antibody to the appropriate titer in 100 ml of antibody buffer. Label this solution "First antibody solution."

Second antibody conjugate solution or Protein A-HRP conjugate solution	Prepare the second antibody solution by dissolving 33 µl of the antibody conjugate in 100 ml of antibody buffer, if applicable.
Protein G-HRP conjugate solution	Dissolve 33 µl of the conjugate solution in 100 ml of 1% gelatin in TCBS, if applicable.

Note: If a bacteriostat is needed, 0.01% thimerosal should be used. Sodium azide is potent inhibitor of horseradish peroxidase.

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 membrane backgrounds usually result when the blocking period is too short, when Tween-20 is absent from the appropriate buffers and washes, or when excessive amount of conjugate are used.
   b. **Non-specific antibody binding -** Usually 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 concentrations, or an absence of Tween-20 from the appropriate buffers and washes.
   c. **Non-specific conjugate binding -** This background is evidenced by band development in the absence of first antibody. It results when conjugate 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. In low temperature incubations, it may be necessary to substitute BSA for gelatin.
3. **Wash Purity -** Poor quality water can contain inhibitors of enzymatic color development. Use only deionized, distilled water to prepare all 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 antibody buffer is used for detection of antigens on the membrane surface. For chromatographically purified monospecific antibodies, a 1:500-1:10,000 dilution in antibody 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 nonspecific reactions.
5. **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.
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 nitrocellulose, 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 blocking the nitrocellulose with TBS - 3% gelatin and probing with first antibody is essential and should not be altered.
8. **Color development reagents -** Immun-Blot assay kits are provided with one of two color development reagent systems. All color development reagents are ready to use after preparing a stock solution of AP or HRP color development buffer.
9. **Technical Service -** Contact Bio-Rad Laboratories Technical Services Group in Hercules, California, toll free 1-800-4BIORAD, or your local Bio-Rad representative, if you require assistance.

2.2 Detailed Assay Procedure

Note: Before beginning, read through the entire procedure.

1. **Antigen applications -** 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. **Dot-blotting -** Cut the nitrocellulose sheet to appropriate size (*i.e.* 0.9 x 9.2 cm strips, 3 x 5 cm rectangles, or Petri dish circles). It is advisable to draw a grid on the membrane with a pencil. Typical grids consist of 1 x 1 cm squares. Next, the dry nitrocellulose is wetted by slowly sliding the membrane at a 45° angle into TBS. Remove the thoroughly wetted membrane from the TBS and dry it on filter paper for approximately 5 minutes.

Apply sample antigen 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 nitrocellulose membrane. If the antigen 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.

b. **Electrophoretic blotting -** The anitgens of interest are electrophoretically transferred to the membrane from a gel support (*i.e.* SDS-PAGE gel, IEF gels, or native gels) 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 proceeding to the blocking step.

c. **Microfiltration blotting -** The Immun-Blot assay kits can easily be adapted for use in the Bio-Dot or Bio-Dot SF apparatus. These instruments allow rapid, reproducible applications of up to 96 samples on one membrane sheet. All applications and washes, except color development, are carried out in the apparatus.

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 30 minutes to 1 hour at room temperature (RT).
3. **Wash -** Decant the blocking solution and add TTBS to the membrane. Wash for 5-10 minutes with gently agitation at RT.
4. **First antibody -** Decent the TTBS and add first antibody solution to the membrane. Incubate 1 to 2 hours with gently agitation.

Overnight incubation may be preferred, since longer incubation periods may increase the sensitivity of detection.

5. **Washes -** Remove the unbound first antibody by washing the membrane in TTBS for 5 minutes at RT. Decant the wash solution and repeat the wash with another portion of TTBS for 5 minutes at RT. Protein G-HRP assays, these washes should be done in TCBS instead of TTBS. The optimal binding pH of Protein G is 5.5. Therefore, the Protein G-HRP binding step should be carried out in the citrate buffer.[13]
6. **Conjugate binding step -** Decant the TTBS and add the second antibody solution, Protein A-HRP conjugate solution, or Protein G-HRP conjugate solution, and incubate 30 minutes to 2 hours using gently agitation at RT. The optimum conditions of dilution and incubation time must be determined experimentally.
7. **Final washes -** Remove the conjugate solution*, and wash the membrane in TTBS for 5 minutes with gently agitation at RT. Decant the solution and wash with another portion of TTBS for 5 minutes at RT. Just prior to color development, wash the membrane in TBS for 5 minutes at RT with gentle agitation to remove residual Tween-20 from the membrane surface.

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

2.3 Detailed Color Development Procedure

Procedure for Alkaline Phosphatase Blots

1. **Developer preparation -** Immediately before use, add 1.0 ml of AP color reagent A and 1.0 ml of AP color reagent B to 100 ml 1x of AP color development buffer at RT. This solution can be stored at 4 °C overnight, but prompt use is recommended.

2. **Color development -** Immerse the 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 within 30 minutes. To maximize sensitivity, the incubation in color development solution can be extended to 4 hours. 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. **Washing -** 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. Protect from light to minimize fading.

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

Procedure for Horseradish Peroxidase Blots

1. **Developer preparation -** Add 600 µl HRP color reagent B to 100 ml of 1x HRP color development buffer at RT. Add this solution to 20 ml of HRP color reagent A immediately prior to use. This solution cannot be successfully stored. HRP color development solution will precipitate out of solution if kept at 0-4 °C, and will

be inactivated with exposure to direct light. Warm the HRP color development buffer to RT prior to addition of the color reagent solutions. HRP color reagents A and B should be kept at their proper storage temperature prior to mixing to maximize their activity and prolong their shelf life. Protect the color development solution from light.

2. **Color development -** Immerse the membrane in the color development solution.* Protein concentrations greater that 100 ng will immediately become visible as purple bands or dots. Lower concentrations of protein will take longer, but should be visible within 30 minutes. Avoid development periods longer than 45 minutes as bleaching or fading of color in positive reactions will occur. If a heavy precipitate forms in the color development solution, a fresh solution should be prepared and used immediately.
3. **Washing -** 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. 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 second antibody conjugate or Protein A or Protein G conjugate. The color reaction 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 conjugate solution.**

   Combine 1.0 ml of the color development solution (tested in step 1) and 1.0 ml of the 1:3,000 dilution conjugate solution. A light blue tinge should develop within 15 minutes. If color fails to develop within 25 minutes, the conjugate solution is suspect. Repeat the procedure with a freshly prepared dilution of conjugate.

3. **Activity test for the first antibody solution.**

   Use a 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	Color development solution is inactive (see Section 3.1).	Color development reagents must be stored at the proper temperature (see Section 1.3).
		HRP color development solution will precipitate out of solution if kept at 0-4 °C, and detection sensitivities may be decreased. Warm the HRP color development buffer to RT prior to addition of the color reagent solutions. HRP color reagents A and B should be kept at their proper storage temperature prior to mixing to maximize their activity and prolong their shelf life.
		Avoid bacterial contamination of the color development buffers by storage at 4 °C.
		Tap water can inactivate the color development solution. Use only distilled, deionized water to prepare the solutions.
	First antibody solution is inactive or non-saturating (see Section 3.1).	Antibody is improperly stored. Avoid bacterial contamination, heat inactivation, and repeated freeze-thaw cycles.
		Antibody titer is too low. Increase the concentration of antibody used in the assay.

Problem	Probable cause	Recommended solution
		Tween-20 may affect the reactivity of some antibodies. Eliminate Tween-20 from the assay (except the wash and after blocking).
	Conjugate is inactive (see Section 3.1).	Conjugate is improperly stored. Store at 4 °C. Avoid heat inactivation. Do not sub-ject to repeated freeze-thaw cycles.
		The concentration of conjugate is non-saturating. Increase the conjugate concentration used in the assay.
		Conjugate may be contaminated, causing inactivation of the antibody (of Protein A or Protein G) or the enzyme.
		Tap water may cause inactivation. Use only distilled, deionized water.
		Azide is a potent inhibitor of horseradish peroxidase. Use thimerosal as a bacteriostat.
	Little or no antigen is bound to the membrane.	Tween-20 may wash bound antigen from the membrane. Eliminate Tween-20 from the assay (except the wash after blocking).

Problem	Probable cause	Recommended solution
		Transfer of protein onto the 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.
	First antibody is not specific or does not recognize denatured antigens (common with monoclonals).	Loss of activity may have occurred during electrophoretic transfer. Pre-test 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.
	Antigen is too dilute.	Increase the amount of antigen in the assay.
2. **High background (refer to experimental strategy section)**	Blocking is insufficient.	Increase the blocking step to 60 minutes.
	Gelatin blocking solution is old.	Prepare and use fresh blocking solution.
	Nitrocellulose is left in the color development solution too long.	Remove the membrane when the reaction appears to be complete. If precipitate in the color development solution appears, decant the solution and use fresh reagent.

Problem	Probable cause	Recommended solution
	Blot is washed in the absence of Tween-20.	Tween-20 is necessary in the washes to reduce back ground.
	Conjugate is used at an excessive concentration.	Use the recommended 1:3,000 dilution.
	Contamination occurred during transfer.	Refer to the Trans-Blot, Trans-Blot SD, or Mini Trans-Blot manual.
	Use of poor quality, mixed ester nitrocellulose will cause increased background.	Use pure nitrocellulose from Bio-Rad.

Section 4
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