



Promega

## Technical Manual

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# ProtoBlot<sup>®</sup> II AP System with Stabilized Substrate

including *Western Express*<sup>®</sup> Fast Blotting Protocol

INSTRUCTIONS FOR USE OF PRODUCTS W3940, W3950 AND W3960.



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# ProtoBlot® II AP System with Stabilized Substrate

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Please visit the web site to verify that you are using the most current version of this Technical Manual. Please contact Promega Technical Services if you have questions on use of this system. E-mail [techserv@promega.com](mailto:techserv@promega.com).

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## 1. Description

The ProtoBlot® II AP Systems with Stabilized Substrate improve upon the simplicity of the original ProtoBlot® Western Blot AP Systems by incorporating the premixed, ready-to-use Western Blue® Stabilized Substrate for Alkaline Phosphatase into a convenient, reliable immunodetection system. The ProtoBlot® II System is designed for the rapid, sensitive detection of proteins or other macromolecular antigens immobilized on nitrocellulose or polyvinylidene fluoride (PVDF) membranes, either transferred from gels after electrophoresis (Western blots; 1,2) or bound directly from solution (“dot” blots).

Two protocols are provided: a standard immunodetection protocol and the *Western Express*® Fast Blotting Protocol. The *Western Express*® Protocol is a modification of the standard protocol, which significantly reduces the time required to perform immunodetection to as little as 32 minutes. This protocol is designed to optimize detection time for the sensitivity of detection required.

Applications of the *Western Express*<sup>®</sup> Protocol include:

- Fast Western and dot blots
- Quick assays of column chromatography fractions on dot blots
- Screening monoclonal antibodies

In general, 1ng of antigen on a Western blot and 200pg on a dot blot can be detected using a 32-minute *Western Express*<sup>®</sup> Protocol. Using the standard protocol, 50pg of antigen on a Western blot and 5pg on a dot blot can be detected in 2.5–3 hours.

The ProtoBlot<sup>®</sup> II AP Systems with Stabilized Substrate are based on the enzyme-linked immunodetection of antigen-specific antibodies (supplied by the researcher) using anti-IgG secondary antibodies conjugated to alkaline phosphatase (AP). Following incubations with the primary antibody and appropriate anti-IgG AP conjugate, Western Blue<sup>®</sup> Stabilized AP Substrate is applied directly to the blot for color development. Sites of antigen localization turn a dark purple color as the result of alkaline phosphatase activity.

Western Blue<sup>®</sup> Substrate contains NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) in a proprietary buffer and is stable at room temperature for up to one year. It is supplied premixed, fully diluted and ready to use. Western Blue<sup>®</sup> Substrate brings a new level of convenience and reliability to this procedure while providing the same sensitive detection levels seen with NBT/BCIP reagents, which require mixing before use.

Systems are available for the detection of human, mouse and rabbit antibodies. The blocking agents, bovine serum albumin (BSA) and Tween<sup>®</sup> 20, also are included with the systems.

## 2. Product Components and Storage Conditions

Product	Cat.#
ProtoBlot® II AP System with Stabilized Substrate, Human	W3940
ProtoBlot® II AP System with Stabilized Substrate, Mouse	W3950
ProtoBlot® II AP System with Stabilized Substrate, Rabbit	W3960

For Laboratory Use.

Each system contains reagents sufficient to process twenty 10 × 15cm membranes. The *Western Express*® Fast Blotting Protocol is included. All secondary antibody AP conjugates are affinity-purified from goat antisera. Includes:

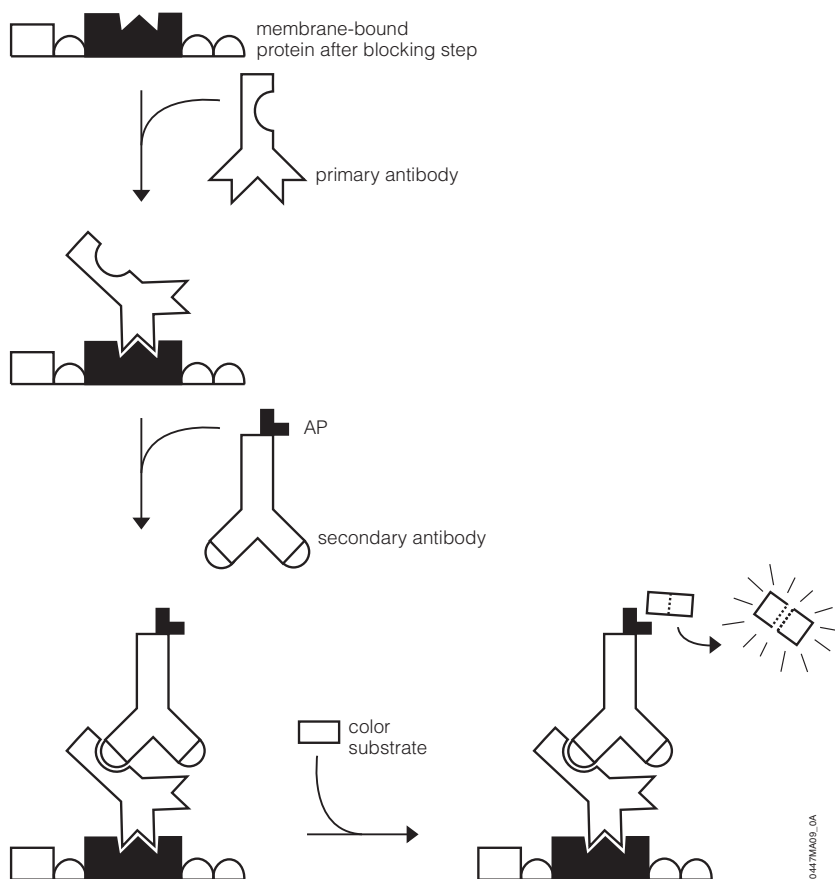
- 100µl Anti-Human or Anti-Mouse IgG (H + L) or Anti-Rabbit IgG (Fc), AP Conjugate (1mg/ml)
- 300ml Western Blue® Stabilized Substrate for Alkaline Phosphatase
- 5ml Tween® 20
- 10g Blot-Qualified BSA
- 1 Protocol

**Storage Conditions:** Store antibody conjugates at 4°C (undiluted).  
Store Western Blue® Substrate at room temperature (15–30°C).

## 3. General Considerations

In general, antigens immobilized on membranes are detected with antibodies in a three-step process (see Figure 1). First, the primary antibody, an IgG directed against the antigen in question, is added to bind potential antigenic sites. In the second step, a secondary antibody-enzyme conjugate, which recognizes general features of all IgGs (anti-IgG), is added to find locations where the primary antibody bound. In the third step, AP, the enzyme conjugated to the secondary antibody, catalyzes a colorimetric reaction when the appropriate substrate is added, resulting in the deposition of colored product on the membrane at the reaction site. This color provides a visual indication of potential primary antibody recognition.

Antigens can be immobilized (“blotted”) on nitrocellulose or PVDF membranes by one of several methods, depending on the requirements of the experiment. In all cases, the ability to detect a given antigen will depend on the amount of antigen per unit area of the membrane and on the characteristics of the primary antibody. Refer to Table 1 for detection sensitivities for dot blots [antigen spotted in 1µl (about 4mm)] and Western blots using this system and reasonably high-affinity, high-titer primary antibodies.



**Figure 1. Immunodetection of membrane-immobilized antigens.** The primary antibody, provided by the researcher, binds to a specific protein. The secondary antibody-alkaline phosphatase conjugate recognizes and binds to the primary antibody. Color development substrate is added and is acted upon by the alkaline phosphatase (AP), resulting in a localized colored precipitate on the membrane.

Several considerations should be taken into account when developing a blotting assay for a given antigen:antibody combination. For example, some antibodies (particularly monoclonals) recognize epitopes that may become buried or denatured when the antigen is bound to surfaces such as nitrocellulose or PVDF membranes. This effect is enhanced when blotting protein antigens out of SDS-containing gels. The sensitivity of the assay with even a “good” antibody can often be several-fold lower on Western blots (using denaturing gels) than on “dot” blots where native proteins are spotted directly on the membrane.

In practice, it is useful to blot a series of known amounts of antigen by the method required for the experiment and test several dilutions of primary antibody to maximize sensitivity and minimize background. If the primary

antibody is used at too high a concentration, higher backgrounds result. At too low a concentration, the positive signals will be weaker. In general, a range of dilutions from 1:200 to 1:10,000 will reveal the optimal levels for most sera, ascites fluids and purified antibodies, whereas hybridoma tissue culture fluids (which have much lower antibody concentrations) usually require dilutions from 1:10 to 1:100 for optimal performance.

#### 4. Transfer of Antigen to Membrane

Bind the antigen to either nitrocellulose or PVDF membrane. Three different methods are described below.

 Always wear gloves when handling membranes to avoid localized background problems.

##### Materials to Be Supplied by the User

(Solution compositions are provided in Section VII.A.)

- Tris-buffered saline (TBS)
- appropriate membranes
- filter paper

#### 4.A. Dot Blotting

##### Nitrocellulose Membranes

A solution containing the antigen (crude or purified) is spotted directly on a dry or moist nitrocellulose membrane, usually in a 1 $\mu$ l volume.

1. To prepare a moist nitrocellulose membrane, float the membrane in a container of TBS (without Tween<sup>®</sup> 20) until it is evenly wet, submerge it briefly, and place it on a piece of dry filter paper. Let the excess buffer drain for approximately 5 minutes before use.
2. Dry membranes can be placed on top of filter paper or the backing paper included with the nitrocellulose membranes. It is useful to mark the membrane with a pencil or ball point pen at convenient intervals as a guide in spotting.
3. Prepare appropriate dilutions of the sample in TBS and apply in 1 $\mu$ l volumes to the membrane using a micropipette. If more volume is required, add the sample in multiple applications over the same spot. Allow each spot to dry thoroughly before applying the next aliquot, so the spots remain compact. Allow all spots to dry before proceeding to the blocking step (Section V.A, Step 1, or V.B, Step 1).
4. An alternative method for making dot blots uses a filtration manifold, which allows multiple samples to be applied simultaneously in uniform spots. Several devices are commercially available for this purpose.

#### 4.A. Dot Blotting (continued)

##### PVDF Membranes

PVDF is a hydrophobic membrane that must be prewet before use and not allowed to dry out before sample application. It is helpful to label sample application positions with a pencil before wetting the membrane. Prewet the PVDF by floating the membrane in 100% methanol or ethanol and submerging it briefly. Transfer the membrane to a container of TBS (without Tween® 20), and submerge the membrane for 2 minutes.

1. To prevent the membrane from drying completely during spotting, place the wet membrane on top of two sheets of filter paper wetted in TBS. Place this wet sandwich on top of three sheets of dry filter paper (membrane up) to wick the surface moisture through the membrane.
2. Spot a solution containing the antigen (crude or purified) directly on the moist PVDF membrane, usually in a 1µl volume using a micropipette. If more volume is required, apply the sample in multiple applications over the same spot. Proceed to the blocking step (Section V.A, Step 1, or V.B, Step 1) while the membrane is still moist.
3. An alternative method for making dot blots is to use a filtration manifold, which allows multiple samples to be applied simultaneously in uniform spots. Several devices are commercially available for this purpose.

#### 4.B. Transfer from Gels: Western Blotting

Antigens are transferred from polyacrylamide or agarose gels to the membrane by passive diffusion or electrophoresis. Usually Western blots are made by electrophoretic transfer of proteins from SDS-polyacrylamide gels. Detailed procedures for electrophoretic blotting are usually included with commercial devices and can also be found in references 1, 3, 4 and 5.

A general discussion of Western blotting with PVDF membranes is found in reference 6. PVDF membranes must be prewet in methanol or ethanol before equilibrating in transfer buffer prior to transfer.

A method also is available (7) for transferring gels that have already been stained with Coomassie® brilliant blue after electrophoresis. Although the sensitivity of immunodetection is slightly lower using this method, it allows one to perform immunodetection experiments on samples that might otherwise be lost in a stained gel.


#### 4.C. Plaque/Colony Lift Immunoscreening

A detailed procedure for screening λgt11 expression libraries with antibodies is described in Chapter 4 of our *Protocols and Applications Guide*, Third Edition (8). Procedures for making colony lifts when immunoscreening plasmid libraries have been described by Esen *et al.* (9) and Helfman *et al.* (10).

**Note:** After transfer, the nitrocellulose membranes can be stored at 4°C, wrapped in plastic wrap. If PVDF membranes dry out, they must be rewet in methanol or ethanol followed by TBS before continuing with the blocking step. However, if PVDF membranes dry, there will be some loss of sensitivity.

## 5. Immunodetection of Antigens

For antibody incubations and the color development reaction, use just enough solution to submerge the membrane, protein side up. Usually, this volume is about 0.1–0.15ml/cm<sup>2</sup> of membrane surface (15ml for a 10 × 15cm membrane). Use at least twice this volume for blocking and washing steps.

 **Do not** allow the membranes to dry out during any of the subsequent steps. Perform all of the washing and incubation steps at room temperature with gentle shaking. We recommend using a shallow container that is slightly larger than the membrane.

### Materials to Be Supplied by the User

(Solution compositions are provided in Section VII.A.)

- Tris-buffered saline (TBS)
- TBST
- 1% BSA in TBST
- appropriate membranes

#### 5.A. Standard Protocol

If rewetting stored, dry nitrocellulose membranes containing transferred antigens, float the membrane on TBST until it is evenly wet, submerge it, and rinse briefly in the same buffer. For dry PVDF membranes, first rewet in ethanol or methanol followed by floating in TBS.

#### Blocking of Membranes

1. To saturate nonspecific protein binding sites, incubate the membrane in TBST + 1% Blot-Qualified BSA for 30 minutes for nitrocellulose membranes and 60 minutes for PVDF membranes.

#### Primary Antibody Binding

2. To bind primary antibody, replace the blocking solution (which can be reused several times) with TBST containing the appropriate dilution of primary antibody, and incubate for 60 minutes with gentle agitation.

**Note:** Often a 30-minute incubation is sufficient.

3. To remove unbound antibody, wash the membrane three times in TBST for 5–10 minutes each.

## 5.A. Standard Protocol (continued)

### Secondary Antibody Binding

4. Transfer the membrane to TBST containing the appropriate dilution of anti-IgG AP conjugate and incubate for 30 minutes with gentle agitation. (1:5,000 dilution is recommended.)
5. Wash the membrane in TBST three times for 5–10 minutes each to remove unbound secondary antibody.
6. Rinse briefly in two changes of TBS to remove Tween® 20 from the membrane surface. Residual Tween® 20 can affect depositing of the precipitated substrate and lead to smearing of bands. Deionized water can be substituted for TBS if it is neutral in pH.

### Color Reaction

7. Start the color reaction by incubating the membrane in Western Blue® Stabilized Substrate for Alkaline Phosphatase until the bands of interest have reached the desired intensity. A 10 × 15cm blot requires about 15ml of substrate solution. Protect the solution from strong light. Reactive areas will turn purple, usually within 1–15 minutes. Color development will continue for at least 24 hours, although membranes left overnight tend to have higher backgrounds.
8. When the color has developed to the desired intensity, stop the reaction by washing the membrane in deionized water for several minutes, changing the water at least once. The membrane can be photographed while still moist by placing it on top of a damp piece of filter paper on a glass plate. For storage, the membrane can be air-dried on filter paper. The bands and background will fade slightly upon drying but can be restored by moistening with water. (PVDF must be rewet first with methanol or ethanol.) Protect the membrane from light during prolonged storage.

## 5.B. Western Express® Fast Blotting Protocol

The *Western Express*® Protocol significantly reduces the time needed to perform immunodetection of blotted antigens. It can reduce the time required for immunodetection with the ProtoBlot® II System to 30 minutes to 2 hours, depending on the detection sensitivity required. This protocol is a modification of the standard protocol described above.

The *Western Express*® Protocol should be used for applications in which speed is more important than obtaining maximum detection sensitivity. Such applications include fast Western and dot blots, quick assays of column chromatography fractions using dot blots and screening monoclonal antibodies. Detection sensitivity varies depending on the protocol strategy employed. Table 1 shows protocol variations and the detection sensitivities obtained. Although there is usually a loss in sensitivity with this protocol, small amounts of blotted antigen can still be detected using AP-conjugated secondary antibodies.

## Strategy for *Western Express*<sup>®</sup> Protocol Optimization

The general strategy for optimizing the *Western Express*<sup>®</sup> Protocol is discussed below. The detection sensitivities obtained and the time needed for various protocol variations are shown in Table 1 at the end of this section. Optimization of the protocol will vary depending on the primary antibody used.

**Note:** The *Western Express*<sup>®</sup> Protocol was developed for use with high-quality nitrocellulose and Immobilon<sup>®</sup>-P PVDF membranes. Results may vary when using other membranes.

- Decrease blocking time. The blocking step can usually be reduced to a 1-minute incubation with little loss in sensitivity or increase in background. Use dilute primary and secondary antibodies (1:3,000 to 1:5,000 dilution of a 0.5–2mg/ml antibody) to minimize background.
- Reduce primary and secondary antibody incubation times. These incubation times can be reduced depending on the sensitivity required (see Table 1). The sensitivity decreases over a range of incubation times. The wash times also can be reduced; however washing less than 2 minutes may increase background.
- Combine the primary and secondary antibody incubation steps. If the primary antibody is of high purity and specificity (monoclonal or affinity-purified polyclonal) and well diluted (1:5,000 dilution of a 2mg/ml antibody), the primary and secondary antibody incubation steps can be combined, however there will be some loss in detection sensitivity. This procedure will not work with serum due to the high levels of nonspecific IgGs present.

If screening monoclonal antibodies to find one that will recognize denatured antigens, perform a dot blot using antigens denatured in a diluted SDS sample buffer (at least 1:20). (High levels of SDS in the sample buffer may block binding of the antibody.) Dilute the denatured antigens in transfer buffer and dot blot them onto a prepared PVDF membrane. We recommend making a dilution series of the antigen and spotting samples in the range of 10ng to 100pg. Also spot a dilution series of the native protein (in TBS) onto the membrane as a control. In some cases, native antigens will produce a positive signal, whereas SDS-denatured antigens give a negative signal. Continue with the *Western Express*<sup>®</sup> Protocol below.

If rewetting stored, dry nitrocellulose membranes containing antigens, float the membrane in TBST until it is evenly wet, submerge it, and rinse briefly in the same buffer. For dry PVDF membranes, first rewet in ethanol or methanol followed by floating in TBS.

## 5.B. *Western Express*<sup>®</sup> Fast Blotting Protocol (continued)

### Blocking of Membranes

1. To saturate nonspecific protein binding sites, incubate the membrane in TBST + 1% Blot-Qualified BSA for 1 minute.

**Note:** If the primary and secondary antibodies are more concentrated than recommended (1:3,000 to 1:5,000 for a 0.5–2mg/ml antibody), background may increase. If this occurs, increase the blocking time.

### Primary and Secondary Antibody Binding

Depending on the requirements of the experiment, the primary and secondary antibody incubation steps may be combined or performed separately. If these steps are combined, the amount of secondary antibody AP conjugate used should be determined experimentally.

A 1:1 stoichiometry of primary to secondary antibody is a good starting point. Antibody incubation times need to be optimized for each antibody and depend on the immunodetection sensitivity required.

We recommend diluting the antibodies in blocking buffer. This simplifies the incubation steps and allows blocking to continue during the antibody incubations.

- 2.a. If performing separate antibody incubations: Bind the primary antibody by diluting it directly into the blocking buffer. Incubate for 5–30 minutes with gentle agitation. Then, to remove unbound antibody, wash the membrane three times for 2 minutes each in TBST.  
b. If performing the primary and secondary antibody incubations together: Dilute the primary antibody into the blocking buffer as above, and also dilute the secondary antibody into the same solution at the experimentally determined concentration. Incubate 5–30 minutes.  
**Note:** After incubation, the primary/secondary antibody mixture can be stored at 4°C in 3mM sodium azide for several weeks and reused, although a slight reduction in detection sensitivity will be observed.
- 3.a. If performing separate antibody incubations: Transfer the membrane to blocking buffer containing the appropriate dilution of secondary antibody and incubate 5–30 minutes. Then remove unbound antibody by washing the membrane three times for 2 minutes each in TBST.  
b. If performing the primary and secondary antibody incubations together: Remove unbound antibody by washing the membrane three times for 2 minutes each in TBST.
4. Rinse the membrane briefly in TBS or deionized water, changing the buffer once, to remove residual Tween<sup>®</sup> 20.

## Color Reaction

5. Start the color reaction by incubating the membrane in Western Blue® Stabilized Substrate for Alkaline Phosphatase until the bands of interest have reached the desired intensity. A 10 × 15cm blot requires about 15ml of substrate solution. Protect the solution from strong light. Reactive areas will turn purple, usually within 1–15 minutes. Color development will continue for at least 24 hours, although membranes left overnight tend to have higher backgrounds.
6. When the color has developed to the desired intensity, stop the reaction by washing the membrane in deionized water for several minutes, changing the water at least once. The membrane can be photographed while still moist by placing it on top of a damp piece of filter paper on a glass plate. For storage, the membrane can be air-dried on filter paper. The bands and background will fade slightly upon drying but can be restored by moistening with water. (PVDF must be rewet first with methanol or ethanol.) Protect the membrane from light during prolonged storage.

**Table 1. Sensitivity of Detection with Western Express® Protocol Variations.**

Protocol Variations	A	B	C	D	E	F
<b>Blocking</b>	1 min	1 min	1 min	1 min	1 min	1 min
<b>1st antibody incubation</b>	60 min	30 min	15 min	5 min	-	-
<b>Wash*</b>	15 min	15 min	6 min	6 min	30 min	15 min
<b>2nd antibody incubation</b>	30 min	30 min	10 min	5 min	-	-
<b>Wash*</b>	15 min	15 min	6 min	6 min	6 min	6 min
<b>Dot blot sensitivity</b>	5pg	50pg	50pg	200pg	100pg	200pg
<b>Western blot sensitivity</b>	50pg	200pg	1ng	2ng	200pg	1ng
<b>Total time**</b>	130 min	101 min	48 min	33 min	47 min	32 min

\*Wash time equals the combined time of three separate washes.

\*\*Total time includes color development time. Color development was performed for 10 minutes with Western Blue® Substrate. The total time required for the standard protocol is 190 minutes, and the detection sensitivity is 50pg on a Western blot and 5pg on a dot blot.

See next page for protocol variations in Table 1.

**5.B. Western Express® Fast Blotting Protocol (continued)**

**Protocol Variations (Table 1)**

- A:** Standard protocol, but with 1-minute blocking step. (Standard blocking time is 60 minutes.)
- B:** Reduced 1st antibody incubation time.
- C:** Reduced 1st antibody incubation and 2nd antibody incubation times, reduced wash times.
- D:** Five-minute 1st and 2nd antibody incubations, reduced wash times.
- E:** 1st and 2nd antibody steps combined, 30-minute incubation followed by a short wash.
- F:** 1st and 2nd antibody steps combined, 15-minute incubation followed by a short wash.

## 6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: [www.promega.com](http://www.promega.com). E-mail: [techserv@promega.com](mailto:techserv@promega.com)

<b>Symptoms</b>	<b>Causes and Comments</b>
Signal weak or absent	<p>Primary antibody lost activity during storage or is of low titer. Store antisera/ascites at <math>-20^{\circ}\text{C}</math> in aliquots; avoid repeated freeze-thaw cycles. Use some other immunochemical assay, such as ELISA, immunoprecipitation, immunodiffusion, etc., to check reactivity toward the antigen. If positive, repeat the blot assay using higher concentrations of primary antibody.</p> <p>Primary antibody is of low affinity. Low-affinity antibodies are more affected by buffer conditions, incubation times, and relative concentrations than are high-affinity antibodies. Eliminate Tween<sup>®</sup> 20 from the buffers, increase the incubation time and concentration of the primary antibody. If Tween<sup>®</sup> 20 is eliminated from the wash buffer, it may be replaced by 0.1% BSA.</p> <p>Primary antibody binding is conformation-dependent. Some antibodies (particularly monoclonals) recognize epitopes involving secondary and/or tertiary structures that are altered when the antigen is applied to nitrocellulose and PVDF (see Section III). Test the antibody with dot blots made with native and denatured antigen. In some cases, native antigens will produce a positive signal, whereas SDS-denatured antigens will produce no signal. In other cases, an antibody that recognizes an antigen in solution (i.e., will immunoprecipitate the antigen) will not recognize the same antigen when it is bound to a surface, even under nondenaturing conditions. These antibodies generally produce no signal in ELISA as well as in blotting assays.</p> <p>Inefficient transfer of antigen out of gel. Stain the gel with Coomassie<sup>®</sup> blue or silver stain after transfer to monitor the disappearance of antigen from the gel. If too much antigen remains in the gel, longer transfer times or higher voltages may be required. SDS (0.1%) can sometimes be added to the transfer buffer to improve the transfer efficiency of proteins over 100kDa.</p>

## 6. Troubleshooting (continued)

Symptoms	Causes and Comments
Signal weak or absent (continued)	<p data-bbox="550 285 1021 502">Incubation time with antibodies is too short. Increase the length of incubation with the antibodies. If using the <i>Western Express</i><sup>®</sup> Protocol, there may not be enough antigen on the blot to detect. If detection is weak, reprobe the same blot using longer incubations, beginning at the primary antibody step.</p> <p data-bbox="550 521 1021 1226">Inefficient binding of antigen to membrane. This can be caused by insufficient contact or air bubbles between the gel and the membrane, or other conditions of transfer such as pH (more important to consider with native gels) or excess amounts of SDS in the gel or buffer. Proteins may be stained directly on nitrocellulose membranes with India ink (11) or with 0.1% Amido Black in 20% methanol, 10% acetic acid or colloidal gold. Proteins on PVDF can be stained with India ink or Coomassie<sup>®</sup> brilliant blue R-250 (0.2% Coomassie<sup>®</sup> brilliant blue R-250 in 45% methanol, 5% acetic acid for 30 seconds to 1 minute; destain in the same solution without stain). Alternatively, an extremely sensitive staining method involves derivatization of bound proteins with 1-fluoro-2,4-dinitrobenzene followed by incubation with rabbit anti-dinitrophenol antiserum, alkaline phosphatase conjugated goat anti-rabbit IgG and color development with NBT/BCIP (adaptation of method described in references 12 and 13).</p> <p data-bbox="550 1246 1021 1530">If the concentration of Tween<sup>®</sup> 20 in the buffers was greater than 0.05%, proteins may have eluted from PVDF membranes. The sensitivities of dot blot assays and Western blots using the same antigen:antibody system often differ. This is not necessarily due to the amount of antigen bound but may reflect differences in the relative affinity of the antibody for different antigen conformations (see next page).</p>

## 6. Troubleshooting (continued)

Symptoms	Causes and Comments
Signal weak or absent (continued)	<p>Anti-IgG AP conjugate activity is too low. Conjugate stored improperly. Store at 4°C. Avoid heat treatment and bacterial contamination. Test activity by adding 1ml of a 1:7,500 dilution in TBST to 1ml of color development solution. Intense purple color should appear within 5 minutes. (This is also a test for activity of the color development substrates.) Although unlikely, it is possible that some water may contain inhibitors. Use reagents of the highest quality available.</p> <p>Improper blocking agent was used. Some blocking proteins may have effects on antigen recognition by certain antibodies. If this is suspected, try a blocking protein other than BSA (such as casein, gelatin or serum) to saturate excess binding sites. If an agent other than ProtoBlot® II System Blot-Qualified BSA is used, include controls omitting the primary antibody, the anti-IgG conjugate and both antibodies from the procedure to check for possible effects on background.</p>
General purple background throughout the membrane (background too high)	<p>Color development reaction was too long. Stop the reaction when the color has reached the desired intensity.</p> <p>Poor-quality nitrocellulose. Use high-quality 100% pure nitrocellulose membranes.</p> <p>Poor-quality anti-IgG AP conjugates. Use ProtoBlot® II System secondary antibodies.</p> <p>Improper blocking:</p> <ul style="list-style-type: none"> <li>• Use ProtoBlot® II System Blot-Qualified BSA. The blocking step incubation time can be increased to 1–2 hours, if necessary. Some alternative blocking agents such as nonfat dry milk may contain alkaline phosphatase activity or IgGs that bind conjugates. Perform controls by omitting the primary antibody and the anti-IgG AP conjugate from the procedure.</li> <li>• Elimination of Tween® 20 from the buffers also can lead to increased background. Inclusion of 1% BSA in the antibody incubation steps will often improve the signal-to-background ratio.</li> </ul>

## 6. Troubleshooting (continued)

Symptoms	Causes and Comments
General purple background throughout the membrane (background too high; continued)	<p>Improper blocking (continued):</p> <ul style="list-style-type: none"> <li>• We recommend first using a blocking buffer containing 1% BSA and 0.05% Tween® 20 in TBS (1% BSA in TBST). If blocking is believed to be incomplete, try a blocking solution containing 5% BSA. (Millipore Corporation recommends blocking its Immobilon® PVDF membranes with 5% BSA.) We have found this works well. However, this method uses a large amount of BSA.</li> </ul>
	<p>Anti-IgG AP conjugate concentration too high. Use the secondary antibody at a 1:5,000 dilution. Generally, the background will increase significantly at concentrations higher than 1:2,500.</p>
	<p>Primary antibody binds nonspecifically even to blocked membranes. Perform the procedure using a blocked membrane carrying no bound antigen. A few IgGs and other immunoglobulins (particularly IgM) that will be recognized by the anti-mouse and anti-human AP conjugates are especially “sticky” and may be difficult to use for blotting.</p>
Unexpected spots or bands that appear to be positive (localized background)	<p>Sample has alkaline phosphatase activity. Omit the primary antibody and anti-IgG AP conjugate incubations from the procedure. If color development is significant, try to inactivate the alkaline phosphatase in the sample by either heating the blot at 80°C for 20 minutes or by incubating it in 0.1M acetic acid for 20 minutes followed by rinsing in TBST prior to the blocking step. If this is successful, test the effect of the treatment on primary antibody binding.</p>

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## 6. Troubleshooting (continued)

<u>Symptoms</u>	<u>Causes and Comments</u>
Unexpected spots or bands that appear to be positive (localized background; continued)	<p>Primary antibody recognizes epitopes shared by other species in the sample or contains a mixture of antibodies with multiple specificities. If the sample contains a mixture of antigens, the primary antibody may either contain IgGs that recognize molecules other than those being assayed, or the same IgG may cross-react with other molecules due to shared epitopes. It is sometimes difficult to distinguish between these possibilities, but several approaches are possible (for a more detailed discussion, see reference 14). The most straightforward is to design pre-adsorption experiments in which the primary antibody is incubated with various samples prior to being used in a blotting assay. For example, pre-incubate a crude sample not containing the antigen of interest with the antibody at various ratios, and then perform a blotting assay using the original crude antigen as the sample. If the antibody was heterogeneous and contained IgG directed against other antigens, the pre-adsorption (at high ratios) should remove those specificities without affecting the IgG of interest. Therefore, samples on the blot should be positive, and the background should be decreased relative to unadsorbed antibody. However, if the antibody recognizes epitopes shared by other antigens, the pre-adsorption (at some ratio) should remove the IgG of interest since it is the same IgG that reacts with the antigen being assayed. In this case, the signal using the pre-adsorbed antibody will be decreased. In a reciprocal experiment, highly purified antigen could be used to pre-adsorb the antibody, and the results of the blotting assay should complement those using the crude mixture for adsorption.</p>

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## 7. Appendix

### 7.A. Composition of Buffers and Solutions

Tris-buffered saline (TBS)		TBST	
20mM	Tris-HCl (pH 7.5)	20mM	Tris-HCl (pH 7.5)
150mM	NaCl	150mM	NaCl
		0.05%	Tween® 20
1% BSA in TBST			
1% (w/v)	Blot-Qualified BSA in TBST		

### 7.B. References

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## 7.C. Related Products

<b>Product</b>	<b>Size</b>	<b>Cat.#</b>
Anti-Human IgG (H + L) AP Conjugate	100µl	S3821
Anti-Mouse IgG (H + L) AP Conjugate	100µl	S3721
Anti-Rabbit IgG (Fc) AP Conjugate	100µl	S3731
Anti-Rat IgG (H + L) AP Conjugate	100µl	S3831
Donkey Anti-Goat IgG, AP	60µl	V1151

<b>Product</b>	<b>Size</b>	<b>Cat.#</b>
Blot-Qualified BSA	10g	W3841
Tween® 20	2.5ml	W3831

## Color Development Substrates

<b>Product</b>	<b>Size</b>	<b>Cat.#</b>
Western Blue® Stabilized Substrate for Alkaline Phosphatase	100ml	S3841
BCIP/NBT Color Development Substrate	1.25ml/2.5ml	S3771

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