

# Western blotting using the ProtoBlot<sup>®</sup> II AP System with Stabilized Substrate

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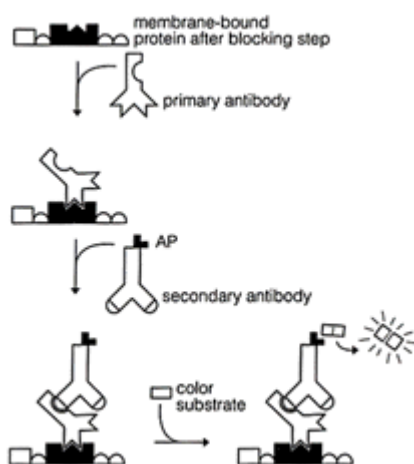
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*The ProtoBlot<sup>®</sup> II AP Systems with Stabilized Substrate are designed for the rapid, sensitive immunodetection of proteins or other macromolecular antigens immobilized on nitrocellulose or polyvinylidene fluoride (PVDF) membranes. The ProtoBlot II AP Systems improve upon the simplicity of the original ProtoBlot Western AP Systems by incorporating the premixed, ready-to-use Western Blue<sup>™</sup> Stabilized Substrate for Alkaline Phosphatase. When ProtoBlot II is used in Western blotting, less than 50pg of antigen can be detected after 10 minutes of color development, while overnight development increases the sensitivity further. Information is provided on the sensitivity levels of the ProtoBlot II System under a variety of conditions.*

## The ProtoBlot II AP System

Researchers have relied upon immunological techniques for the detection of a specific protein or other antigens in a complex mixture. Immunodetection of proteins and other macromolecules usually involves a number of steps which lead to the production of a signal that confirms the presence of an antigen of interest in a sample. Two popular immunodetection methods are "dot" blots, in which antigen-containing solutions are spotted directly onto a membrane, and Western blots (1,2), in which proteins are transferred to a membrane from acrylamide gels after electrophoresis. A method is also available for Western blotting gels which have been previously stained during electrophoresis with the ChromaPhor<sup>™</sup> Staining System or with Coomassie Brilliant Blue after electrophoresis (3). For in-depth discussions of Western blotting, see references 1-7.

The ProtoBlot II AP Systems with Stabilized Substrate are based on the enzyme-linked immunodetection of antibodies (supplied by the researcher) using anti-IgG secondary antibodies conjugated with alkaline phosphatase (AP). [Figure 1](#) shows schematically the steps involved in the enzyme-linked detection of an antigen on a blot using the system. After transfer of the antigen-containing sample to the membrane, unoccupied protein binding sites on the membrane are blocked with the BSA and Tween<sup>®</sup> 20 supplied in the system. Next, the primary antibody, an IgG directed against the antigen in question, is applied to bind potential antigenic sites. Then, unbound antibody is washed from the membrane. A secondary antibody-alkaline phosphatase conjugate is added to localize sites of primary antibody binding. Following another wash to remove unbound secondary antibody conjugate, Western Blue Stabilized AP Substrate is applied directly to the blot for color development.



**Figure 1. Steps involved in the enzyme-linked immunodetection of proteins or other macromolecular antigens using the ProtoBlot II System.**

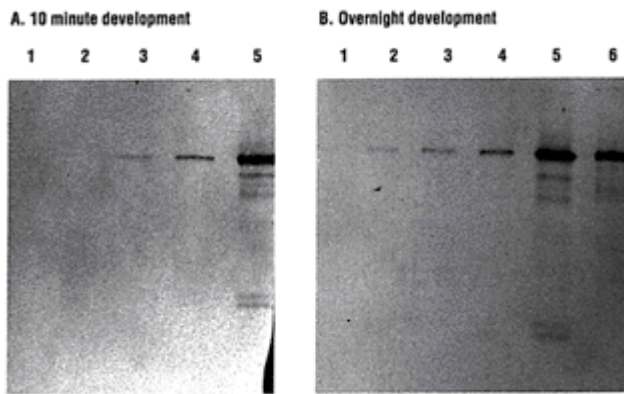
Alkaline phosphatase catalyzes a colorimetric reaction with the Western Blue Substrate resulting in the deposition of a dark purple precipitate on the membrane at the reaction site (8,9). This color provides a visual indication of primary antibody location. Reactive areas will turn purple, usually within 1-15 minutes. Color development will continue for at least 24 hours, although membranes left overnight tend to have higher backgrounds. In most cases, blotted proteins can be visualized in 2-3 hours using the ProtoBlot II System.

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

## Immunodetection of beta-galactosidase on a Western blot

Two Western blots were produced containing 10-1,000pg of purified beta-galactosidase and a beta-galactosidase sample which was produced in the TNT<sup>TM</sup> coupled transcription/translation system. The primary antibody used was monoclonal anti-beta-galactosidase from Promega. The remainder of the detection was performed using the ProtoBlot II AP System (Mouse) according to protocols included in the technical manual supplied with the system. The primary antibody and secondary antibody conjugate were both diluted 1:5,000 for use. Color development with Western Blue Substrate was allowed to proceed for either 10 minutes or overnight at room temperature.

The two Western blots are shown in [Figure 2](#) and demonstrate the sensitivity of detection of the ProtoBlot II System when used for Western blotting. After 10 minutes of color development, 50pg of beta-galactosidase was easily detected ([Figure 2A](#)). However, after overnight development, the lower limit of detection was 25pg of beta-galactosidase ([Figure 2B](#)). Bands containing less than 25pg of protein were observed; however, these bands do not reproduce well in publications. The minor bands seen in some lanes in [Figure 2](#) are proteolytic fragments which result during sample preparation.



**Figure 2. Sensitivity of Western blot detection of beta-galactosidase using the ProtoBlot II System.** After SDS-PAGE electrophoresis in an 8% minigel, the gel was electroblotted to nitrocellulose. The Western blot was produced according to the procedures in the technical manual supplied with the system. The AP color reaction was allowed to proceed for 10 minutes (Panel A) or overnight (Panel B).

For Panel A: Lane 1, 10pg of purified beta-galactosidase; lane 2, 25pg; lane 3, 50pg; lane 4, 100pg; Lane 5, 1000pg. Panel B: Lanes 1-5 are as in Panel A. Lane 6 contains 1 $\mu$ l of a 1:50 dilution of a coupled transcription/translation reaction producing beta-galactosidase.

## Levels of Detection Sensitivity Using ProtoBlot II

The level of sensitivity obtained using the ProtoBlot II AP System, as with any immunodetection system, varies with the method chosen. The sensitivity of an assay can be several-fold greater on dot blots than on Western blots containing the same protein. This difference in sensitivity can be due to the SDS present in the denaturing gels used to fractionate proteins before Western blotting. SDS destroys the native protein conformation, potentially altering the epitope recognized by the antibody. Furthermore, electroblotting from SDS-containing gels enhances the tendency of the epitope to become buried when the antigen is bound to surfaces such as nitrocellulose or PVDF membranes.

We have been able to detect down to 1pg of beta-galactosidase when serial dilutions of native beta-galactosidase were dotted directly onto a membrane and subjected to the immunodetection protocol used for the Western blots above. However, the best detection sensitivity we have been able to achieve on a Western blot is approximately 10pg. These observations are in general agreement with the variation in sensitivity seen with dot blots and Western blots.

A further apparent increase in the sensitivity of immunodetection can be shown by performing a dot blot using the primary antibody as the antigen. When the primary antibody was dotted onto the membrane and immunodetected using the ProtoBlot II AP System, a nearly 5-fold increase in sensitivity was seen compared to the standard immunodetection of an antigen on a dot blot. We have been able to detect 0.2pg of anti-beta-galactosidase antibody on a dot blot. The reduction in sensitivity seen when the full dot blotting protocol is performed is probably the result of some elution of the antigen-antibody complexes during the additional antibody incubation and washing steps required in the full procedure.

A summary of these sensitivity results is provided in [Table 1](#).

### Table 1. Levels of Detection Sensitivity Observed with the ProtoBlot II AP System for Various

## Methods

Procedure	Detection Level
Dot Blot of 1° Antibody	0.2pg
Dot Blot of Antigen	1pg
Western Blot of Antigen	10pg

In summary, the ProtoBlot II AP System can be used for the sensitive immunodetection of antigens on dot blots and Western blots with the added convenience offered by the stable Western Blue Substrate. As these results demonstrate, it is important to remember that sensitivity of any given system will vary with the conditions and methods chosen. The best conditions for performing an immunoblot using a particular antigen:antibody combination must be determined experimentally. The transfer, blocking conditions, antibody binding, washing steps, and color development steps can be adjusted depending upon the sensitivity and time requirements of the experiment, as well as the information sought.

## References:

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