SUPPLEMENTAL PROTOCOL

Enzyme Immunoassays, Hybridoma Screening, and Western Blots

The aim of this protocol is to provide guidelines for using the VECTASTAIN® ABC systems in enzyme immunoassays (including hybridoma screening) and protein blots.

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Enzyme Immunoassays and Hybridoma Screening

The ultrasensitive nature of the VECTASTAIN ABC systems reduces the amount of antigen and supernatant required for screening and allows an earlier detection of positive clones. Assay times can also be reduced.

The procedures outlined here describe methods using mouse monoclonal antibodies, but slight modifications will permit their use with any species of primary antibody.

Plating the Antigen

Generally proteins can be plated to plastic microtiter plates by adding an aliquot (e.g., 50 µl) of the antigen in an alkaline pH buffer (e.g., 50 mM bicarbonate, pH 9.6) to wells and incubating them for 1–2 h at room temperature (or 30 min at 37°C). Some proteins may require plating at 4°C overnight if the assay response is found to be poor with shorter incubation times. Cells (untreated or trypsinized) usually can be plated by incubating 5 x 10^4 cells per well for 16–24 h. After plating, cells may be fixed with formalin or glutaraldehyde for a few minutes, then washed with buffer.

After plating the antigen, non-specific interactions in the wells can be blocked by filling the wells with 1% normal serum or 0.1–0.5% immunohistochemical grade bovine serum albumin (Cat. No. SP-5050) in buffer.

Normally, antigen coated plates can be prepared in advance of the assay, sealed to prevent drying, and stored at 4°C until utilized. Positive control wells with mouse IgG or mouse serum should be included. Color development in the control well indicates that all of the reagents have been properly prepared.

Preparation of VECTASTAIN ABC Working Solutions

A number of different buffers can be used in the VECTASTAIN ABC system. Three common solutions are 10 mM sodium phosphate, pH 7.5, 0.9% saline (PBS); 100 mM Tris, pH 7.5, 0.15 M saline (TBS); or 10 mM HEPES, pH 7.5, 0.9% saline. The working solutions are prepared as follows:

- **Blocking Serum (Normal Serum):** add 1–4 drops (50–200 µl) of stock (yellow label) to 10 ml of buffer in mixing bottle (yellow label). This reagent can also be used as a diluent for the biotinylated secondary antibody.

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Preparation of VECTASTAIN ABC Working Solutions *(cont.)*

- **Biotinylated Antibody:** add 1 drop (50 µl) of stock (blue label) to 10 ml of buffer with 1% normal serum in mixing bottle (blue label). For VECTASTAIN ABC secondary antibodies, add 2 drops (100 µl) of biotinylated antibody stock (blue label) to 5 ml of buffer with 1% normal serum in mixing bottle.
- **VECTASTAIN ABC-HRP or VECTASTAIN ABC-AP:** add 2 drops (100 µl) of Reagent A to 10 ml of PBS, pH 7.5, containing 0.1% Tween® 20 in the ABC Reagent mixing bottle. Then add 2 drops (100 µl) of Reagent B to the same mixing bottle, mix immediately, and allow VECTASTAIN ABC reagent to stand for about 30 min before use.
- **VECTASTAIN® Elite® ABC-HRP:** add 2 drops (100 µl) of Reagent A to 5 ml of PBS, pH 7.5, containing 0.1% Tween 20 in the ABC Reagent mixing bottle. Then add 2 drops (100 µl) of Reagent B to the same mixing bottle, mix immediately, and allow VECTASTAIN ABC Elite to stand for about 30 min before use.

Enzyme Substrate Preparation

Substrates should be freshly prepared prior to use. Substrates are not included with the VECTASTAIN ABC Kits and are available separately. VECTASTAIN® Elite® ABC Universal PLUS Kit (Cat. No. PK-8200) is available with substrates included.

Horseradish Peroxidase Substrates:

TMB Substrate Kit, Peroxidase (HRP), (3,3', 5,5'-tetramethylbenzidine) (Cat. No. SK-4400) is available in a concentrated liquid form. Working solutions can be made following substrate kit instructions. A fresh working solution of ABTS [2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid)] can also be used for HRP enzyme detection systems. Alternatively, o-phenylenediamine dihydrochloride (OPD) substrate can be prepared as follows: 0.5 mg/ml OPD in 50 mM citrate/phosphate buffer, pH 5.3, 0.015% hydrogen peroxide.

Alkaline Phosphatase Substrate:

Make a working solution of 2 mg/ml p-nitrophenylphosphate in 100 mM sodium bicarbonate, pH 9.5, 10 mM magnesium chloride according to kit instructions.

Glucose Oxidase Substrate:

Prepare stock solutions in distilled water as follows: 75 mg/ml D-Glucose, 1 µg/ml HRP, and 2 mg/ml ABTS. To make substrate working solution, add 1 ml of D-Glucose, 0.5 ml HRP, 3 ml deionized water, and 0.5 ml ABTS to 5 ml 50 mM Citrate, pH 5.3 buffer.

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Screening Procedure

Standard Method

1. Plate wells with antigen as described above.
2. Block by filling wells to the top with 1–2% normal horse serum in PBS for 30 min at 37°C. Rinse wells with PBS.
3. Add an aliquot (50–100 µl) of hybridoma culture supernatant and incubate for 30–60 min. Rinse with PBS.
4. Add one drop of biotinylated anti-mouse IgG working solution (prepared as described above). Incubate for 15–30 min. Rinse 5x with PBS.
5. Add one drop of VECTASTAIN ABC reagent working solution. Incubate 15–30 min. Rinse 5x with PBS.
6. Add 100 µl of appropriate substrate solution. Incubate in the dark at room temperature (or at 37°C for alkaline phosphatase enzyme systems). Positive wells will develop color in 5–30 min. Quantitative measurements can be made at 405–415 nm for ABTS and pNPP; 650 nm (blue product) or 450 nm (yellow product) for TMB; and 490–495 nm for OPD.

All steps of this procedure may be carried out at room temperature. This protocol may require optimization for different antigens or culture supernatants. If less sensitivity is required or desired, shorter incubation times, higher dilution of reagents, or lower plating densities of antigen can be used. If greater sensitivity is desired, increased incubation temperature/time or higher concentration of reagents can be employed. The use of Tween 20 or other detergents in reagents other than suggested may reduce sensitivity or produce adverse background color.

Rapid Screening Method

Rates of binding and enzymatic activities generally are accelerated by higher temperature. A more rapid procedure can be accomplished by carrying out all incubations (with the exception of the substrate incubation) at 37°C to 45°C. Reagents should be diluted as described, and care should be taken that wells do not dry out. In some cases, incubation times may be reduced to 5–10 min and washes (except in Step 5) often can be omitted. It is suggested, however, that the standard screening procedure be used first before trying the rapid screening method.

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Rapid Screening Method *(cont.)*

Notes:

1. Do not allow the wells to dry out. If the protocol is interrupted, leave the reagent in the well or add PBS.
2. The alkaline phosphatase substrate 4-methylumbelliferyl-phosphate, which produces a fluorescent reaction product, can also be used in this assay at the same concentration and in the same buffer as the one described in this procedure.
3. For best results, serum from the species of the secondary antibody should be used for blocking. Usually 2% serum in PBS for 30 min will block all nonspecific sites. In some instances where the highest sensitivity is desired, blocking may require several hours. Alternatively, 0.1% solution of immunohistochemical grade Bovine Serum Albumin (Cat. No. SP-5050) can be used.
4. Tween 20 and other detergents should only be used in certain steps in the microtiter plate protocol. Detergents used throughout the protocol can produce higher backgrounds than if no detergents are used. The effect of detergents should be assessed at every stage to optimize the system.

Western Blot Detection Procedure

VECTASTAIN ABC systems can be used to detect antigens bound to nitrocellulose or PVDF membranes. The following procedure is suggested as a guideline for using the VECTASTAIN ABC-HRP, VECTASTAIN ABC-AP, or VECTASTAIN ABC-AmP reagent in such applications (see note 5). All incubations are at room temperature.

Preparation of VECTASTAIN ABC Working Solutions

The volumes of the reagents in the procedure below are optimized for the development of 100 cm^2 membranes. Volumes may be proportionally adjusted for blots of different sizes.

- **Phosphate buffered saline with Tween 20 (PBST):** PBST can be prepared a number of ways. Here, it refers to the following formulation: 10 mM Na2HPO4, pH 7.5, 150 mM NaCl, and 0.5% Tween 20.
- **Blocking Solutions:** prepare 100 ml of appropriate blocking solution. We recommend casein solution prepared by adding 10 ml of 10x Casein Solution (Cat. No. SP-5020) to 90 ml distilled water. 1% BSA (Cat. No. SP-5050) in PBST is also commonly used.
- **Primary Antibody:** dilute primary antibody in blocking solution (generally between 0.2–1 µg/ml or according to manufacturer's recommendations). Optimal dilution depends on the specific primary antibody and the amount of protein target on the membrane.
- **Biotinylated Secondary Antibody:** add 1 drop (50 µl) of stock (blue label) to 10 ml of blocking solution. For VECTASTAIN ABC secondary antibodies, add 2 drops (100 µl) of biotinylated antibody stock (blue label) to 5 ml of blocking solution.

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Western Blot Detection Procedure *(cont.)*

- **VECTASTAIN ABC-AP, VECTASTAIN ABC-HRP, or VECTASTAIN ABC-AmP:** add 2 drops (100 µl) of Reagent A to 10 ml of blocking solution in the ABC reagent mixing bottle. Then add 2 drops (100 µl) of Reagent B to the same mixing bottle, mix immediately, and allow VECTASTAIN ABC Reagent to stand for about 30 min before use.
- **VECTASTAIN Elite ABC-HRP:** add 2 drops (100 µl) of Reagent A to 5 ml of blocking solution in the ABC reagent mixing bottle. Then add 2 drops (100 µl) of Reagent B to the same mixing bottle, mix immediately, and allow VECTASTAIN ABC Elite to stand for about 30 min before use.

Enzyme Substrate Preparation

Substrates should be freshly prepared prior to use according to kit instructions. Substrates are not included with the VECTASTAIN ABC Kits and are available separately.

All Vector Laboratories substrates are supplied in convenient, easy-to-use dropper bottles. Vector Laboratories offers conventional as well as proprietary substrates producing the colors listed.

Chromogenic Peroxidase Substrates:

DAB (Brown or Gray-Black) • SK-4100

ImmPACT® DAB (Brown) • SK-4105

ImmPACT® DAB EqV * (Brown) • SK-4103

Vector® VIP (Purple) • SK-4600

ImmPACT® VIP* (Purple) • SK-4605

Vector® SG (Blue-Gray) • SK-4700

ImmPACT® SG* (Blue-Gray) • SK-4705

Vector® NovaRED® (Red) • SK-4800

ImmPACT NovaRED®* (Red) • SK-4805

AEC (Red) • SK-4200

ImmPACT® AEC* (Red) • SK-4205

ImmPACT® AMEC Red* (Red) • SK-4285

TMB (Blue) • SK-4400

** Can be used on nylon and PVDF membranes. Can cause wrinkling of some nitrocellulose membranes.*

Chromogenic Alkaline Phosphatase Substrates:

Vector® Red (Magenta) • SK-5100

ImmPACT® Vector® Red (Magenta) • SK-5105

Vector® Black (Brown-Black) • SK-5200

Vector® Blue (Blue) • SK-5300

BCIP/NBT (Indigo) • SK-5400

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Enzyme Substrate Preparation *(cont.)*

Chemiluminescent/Fluorescent Substrates:

The DuoLuX® substrates produce a reaction product which is highly chemiluminescent as well as fluorescent.

DuoLuX® Chemiluminescent/Fluorescent for Alkaline Phosphatase • SK-6605

DuoLuX® Chemiluminescent/Fluorescent for Peroxidase • SK-6604

Detection Protocol:

1. Remove the blot from the transfer apparatus and block the membrane in 10 ml blocking solution for 5 min at room temperature with gentle agitation.
2. Incubate the membrane in 10 ml of primary antibody solution for 30 min at room temperature with gentle agitation. Time can vary depending on concentration of primary antibody used.
3. Wash the membrane 3 x 5 min each in 10 ml PBST with gentle agitation.
   Note: The VECTASTAIN ABC Reagent should be prepared at this time as described above.
4. Transfer the membrane to the biotinylated secondary antibody solution. Incubate for 30 min with gentle agitation.
5. Wash the membrane 3 x 5 min each in 10 ml PBST with gentle agitation.
6. Transfer the membrane to the appropriate VECTASTAIN ABC Reagent. Incubate the membrane in this solution for 30 min with gentle agitation.
7. Wash the membrane 3 x 5 min each in 10 ml PBST with gentle agitation.
   Note: When using the VECTASTAIN ABC-AP reagent and AP substrate, we recommend using 100 mM Tris, pH 9.5 buffer for the final washing step.
8. Transfer the membrane to the substrate solution (made according to kit instructions).
   Note: We recommend using a separate staining vessel for this step. Incubation times vary with each substrate. For DuoLuX Chemiluminescent/Fluorescent Substrate development, please follow substrate kit instructions.
9. After signal development, wash the membrane with 2 changes of distilled water over 10 min and allow the membrane to air dry. Air drying removes a slight color from the nitrocellulose but does not reduce the specific staining. Membranes should be stored in the dark.

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Use of Biotinylated Lectins:

Lectins can be employed in a Western Blot technique using the VECTASTAIN ABC systems to localize glycoproteins. The steps are essentially identical to those described in the previous protocol with the following changes.

Avoid blocking the membrane with protein solutions that potentially could contain glycoproteins recognized by the lectin of interest. Some protein solutions to avoid include normal serum, non-fat dry milk, ovalbumin, and impure grades of bovine serum albumin. If a protein solution is used for blocking, it should be tested for background staining with the lectin of interest.

Carbo-Free™ Blocking Solution (Cat. No. SP-5040) or a 1% BSA (Cat. No. SP-5050) solution can be used instead of casein solution as the blocking agent, and TBST (TBS plus 0.1% Tween 20) can be used as the diluent and wash solution. Alternatively, TBST can be used as the blocking solution, diluent, and wash solution.

Substitute steps 2, 3, and 4 with an incubation of the blot with the biotinylated lectin (5–10 µg/ml in TBST) for 30–60 min. Then continue the procedure at Step 5 as described.

Notes:

5. Although most VECTASTAIN ABC Kits can be used for western blot applications, specific kits, based on an amplified alkaline phosphatase system, are available. The kits listed below utilize the VECTASTAIN ABC-AmP detection system with either the DuoLuX Chemiluminescent/Fluorescent substrate or the chromogenic substrate (BCIP/NBT). Kits are available for use with mouse or rabbit primary antibodies. Reagents sufficient to stain approximately twenty 100 cm^2 blots are provided in each kit.

   VECTASTAIN® ABC-AmP
   Standard (Reagents A and B only) • AK-6000

6. Some enzymes isolated from tissues may have covalently attached biotin as a cofactor. An Avidin/Biotin Blocking Kit (Cat. No. SP-2001) step prior to incubation with the primary antibody can eliminate this unwanted staining.
7. In the rare cases that, in the presence of Tween 20, a primary antibody binds poorly to an antigen or that antigen elutes from the membrane, PBST in steps 1, 2, and 4 can be replaced by protein blocking solutions like PBS or TBS containing 2–10% normal serum from the species of the biotinylated antibody. In all other steps PBST can be replaced by PBS or TBS. These substitutions, however, may give rise to slightly higher background.
8. For experimental systems using sheep, goat, or bovine primary antibodies, endogenous immunoglobulins in the non-fat dry milk preclude its use as a blocking reagent. In these cases we recommend 10x Casein Solution (Cat. No. SP-5020), Animal-Free Blocker® (Cat. No. SP-5030), or TBS with 5% Normal Rabbit Serum Blocking Solution (Cat. No. S-5000).

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