



ENZYME IMMUNOASSAYS, HYBRIDOMA SCREENING, AND WESTERN BLOTS

The aim of this brochure is to provide guidelines from which investigators can develop systems for enzyme immunoassays, including hybridoma screening, and transfer blots. Because there are many possible formats which can be used for these procedures, those outlined here describe methods using mouse monoclonal antibodies, but slight modifications will permit their use with any species of primary antibody.

THE BIOTIN/AVIDIN SYSTEM

Avidin is a 68,000 molecular weight glycoprotein with an extraordinarily high affinity ($10^{13}M^{-1}$) for the small molecular weight vitamin, biotin. Because this affinity is over one million times higher than that of antibody for most antigens, the binding of avidin to biotin (unlike antibody-antigen interactions) is essentially irreversible. In addition to this high affinity, the Biotin/Avidin System can be effectively exploited because avidin has four binding sites for biotin and most proteins (including antibodies and enzymes) can be conjugated with several molecules of biotin.

These aspects allow the use of three basic detection systems. In all three cases the first step in detecting a primary antibody is the addition of a biotinylated, affinity-purified secondary antibody. The biotinylated secondary antibody can then be detected using (1) a preformed macromolecular complex between avidin and biotinylated enzyme which still retains biotin-binding sites – the VECTASTAIN® ABC system, (2) avidin covalently conjugated to enzymes or (3) avidin acting as a bridge to biotinylated enzymes. Procedures using the VECTASTAIN® system will be detailed below. The choice of ABC system will depend on a number of factors including assay time, sensitivity required, and endogenous enzyme activities.

ENZYME IMMUNOASSAYS AND HYBRIDOMA SCREENING

The first step in screening mouse monoclonal antibodies is to attach the antigen of interest to plastic microtiter plates. Generally proteins can be plated by adding an aliquot (e.g. 50 μ l) of the antigen in an alkaline pH buffer (e.g. 50 mM bicarbonate, pH 9.6) to wells and incubating them for 1-2 hours at room temperature (or 30 min. at 37 °C). Cells (untreated or trypsinized) usually can be plated by incubating 5×10^4 cells per well for 16 to 24 hours. After plating, cells may be fixed with formalin or glutaraldehyde for a few minutes, then washed with buffer. After plating the antigen, potential “non-specific” binding sites on the wells can be blocked by adding 2% normal serum or 0.1% immunohistochemical grade bovine serum albumin (Cat. No. SP-5050) in diluting buffer up to the top of the wells. Normally, antigen coated plates can be prepared in advance of the assay, covered to prevent drying, and stored at 4 °C until utilized. It may be desirable to designate one of the wells as a positive control onto which is plated mouse IgG or mouse serum. Color development in the control well indicates that all of the reagents have been properly prepared. It should be emphasized that these protocols are guidelines. Other conditions may be required for the antigen of interest.

MICROTITER PLATE ASSAY PROCEDURE

VECTASTAIN® ABC Mouse IgG Kits are available with three different enzymes as markers – horseradish peroxidase (ABC or *ABC* ABC), alkaline phosphatase (ABC-AP), and glucose oxidase (ABC-GO). The procedure described in the remainder of this section can be employed with any of these VECTASTAIN® ABC Kits using the correct substrate for each enzyme. In the procedure, aliquots from hybridoma cultures or ascites fluid are added to blocked, antigen-coated wells of a microtiter plate. After incubation, the fluid is removed from the wells, the wells are rinsed, and an aliquot of biotinylated anti-mouse IgG is added and incubated. Then the biotinylated anti-immunoglobulin is removed, the wells are rinsed, and an aliquot of the VECTASTAIN® ABC Reagent is added to each well. After incubation, the wells are washed and substrate is added. Clonal supernatants or ascites fluid containing the specific monoclonal antibody are detected by positive color development in the corresponding wells. The ultrasensitive nature of the VECTASTAIN® ABC system reduces the amount of antigen and supernatant required for screening and allows an earlier detection of positive clones. Assay times can be reduced to less than 2 hours.

Although the procedures described herein are for screening mouse hybridoma cultures, the same system can be employed for screening rat or human hybridoma cultures by merely changing the biotinylated secondary antibody. A detailed protocol is outlined below.

Reagents supplied:

- Blocking Serum (Normal Serum) in yellow-labeled small bottle – 3 ml
- Biotinylated, Affinity-purified Anti-Immunoglobulin in blue-labeled small bottle – 1 ml (*See special instructions for Universal ABC kits.*)
- Reagent A (Avidin DH) – 2 ml
- Reagent B (Biotinylated Enzyme) – 2 ml

Reagents not supplied:

- Buffers
- Substrates

PREPARATION OF VECTASTAIN® ABC WORKING SOLUTIONS

For convenience, VECTASTAIN® ABC Kits include mixing bottles to prepare working solutions of reagents. As supplied, the drop dispenser tip is in an inverted position and is not inserted into the bottle. After the buffer and appropriate reagents are added to the bottle, insert the drop dispenser tip into the white or gray opaque cap in the correct orientation. Place the entire unit onto the bottle and twist on the cap. As the cap is tightened, the drop dispenser will snap into place. To remove the drop dispenser tip for refilling, merely press laterally with thumb until the tip snaps off. Care should be taken to replace the dispenser tip on the correct bottle to avoid cross-contaminating reagents. All bottles have color-coded labels to minimize inadvertent use of the wrong mixing bottle. To prevent evaporation, secure the white or gray opaque caps on the bottles when not in use. When dispensing drops, hold the bottle in an inverted vertical position and squeeze gently.

- A number of different buffers can be used in the VECTASTAIN® ABC system. Two of the most common are 10 mM sodium phosphate, pH 7.5, 0.9% saline (PBS) or 100 mM Tris, pH 7.5, 0.15 M saline (TBS).
- Blocking Serum (Normal Serum): add from one to four drops* of stock (yellow label) to 10 ml of buffer in mixing bottle (yellow label). This reagent can also be used as a diluent for biotinylated secondary antibodies. *One drop is approximately 50 μ l.
- Biotinylated Antibody: add one drop of stock (blue label) to 10 ml of buffer with 1% normal serum in mixing bottle (blue label). When using Universal ABC kits, follow kit instructions.
- VECTASTAIN® ABC Reagent: add exactly two drops of REAGENT A to 10 ml of PBS, pH 7.5, containing 0.1% Tween 20, in the ABC Reagent mixing bottle. (NOTE: Use 5 ml of buffer for the *ABC* ABC.) Then add exactly two drops of Reagent B to the same mixing bottle, mix immediately, and allow VECTASTAIN® ABC Reagent to stand for about 30 minutes before use.

NOTE: If more dilute reagents are used, first prepare the diluted biotinylated antibody and VECTASTAIN® ABC Reagent as described in the instructions. Subsequent dilutions should be made in a buffer containing 0.1% immunohistochemical grade bovine serum albumin (Cat. No. SP-5050). Further dilution of these reagents may require longer incubation times and/or higher incubation temperatures to achieve maximum sensitivities. Solutions containing sodium azide or other inhibitors of peroxidase activity should not be used in diluting the peroxidase substrate and VECTASTAIN® ABC Reagent, or for the washes after step 3.

PREPARATION OF SUBSTRATES

Substrates should be freshly prepared prior to use.

HORSERADISH PEROXIDASE SUBSTRATES:

ABTS [2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid)], TMB [3,3',5,5'-tetramethylbenzidine], and OPD [o-phenylenediamine dihydrochloride] are commonly used chromogenic substrates for peroxidase. Complete substrate kits for ABTS (Cat. No. SK-4500) and TMB (Cat. No. SK-4400) are available in concentrated liquid form. 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. The **DuoluX™** Chemiluminescent/Fluorescent Substrate (Cat. No. SK-6604) can be used as described in the **DuoluX™** product insert.

ALKALINE PHOSPHATASE SUBSTRATE:

2 mg/ml p-nitrophenylphosphate in 100 mM sodium bicarbonate, pH 9.5, 10 mM magnesium chloride. A concentrated stock solution of p-nitrophenylphosphate (pNPP) is available (Cat. No. SK-5900). The **DuoluX™** Chemiluminescent/Fluorescent Substrate (Cat. No. SK-6605) can be used as described in the **DuoluX™** product insert.

GLUCOSE OXIDASE SUBSTRATE:

Dissolve 7.5 mg/ml glucose in 50 mM citrate/phosphate buffer, pH 5.3 and allow to stand for 60 minutes at room temperature. Then add 0.1 mg/ml ABTS or 0.5 mg/ml OPD and 0.1 μ g/ml horseradish peroxidase.

SCREENING PROCEDURE

For screening mouse hybridoma cultures using a VECTASTAIN® ABC Kit, the following procedures are suggested.

• Standard Method

1. Coat wells with antigen as previously described.
2. Block nonspecific binding sites by filling wells to the top with 1-2% normal horse serum (blocking serum) in PBS for 30 minutes at 37 °C. Rinse wells with PBS.
3. Add an aliquot (50-100 μ l) of hybridoma culture supernatant and incubate for 30-60 minutes. Rinse with PBS.
4. Add one drop of biotinylated anti-mouse IgG diluted in PBS containing 1% normal horse serum. Incubate for 15-30 minutes, then rinse 5X with PBS.
5. Add one drop of VECTASTAIN® ABC Reagent made in PBS, 0.1% Tween 20. Incubate 15-30 minutes, then rinse 5X with PBS.
6. Add 100 μ l of appropriate substrate solution. Incubate at room temperature in the dark except in the case of the VECTASTAIN® ABC-AP where color development is best at 37 °C. Positive wells will develop color in 5-30 minutes. 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. (*See Note 8.*)

All steps of this procedure may be carried out at room temperature. This protocol may not be optimal for all 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 diluted as described should be used and care should be taken that wells are not inadvertently allowed to dry out. In some cases, incubation times may be reduced to 5-10 minutes 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.

NOTES:

1. At no time should the wells be allowed to dry out. If the protocol is interrupted, leave the reagent in the well or add PBS. Stock VECTASTAIN® ABC Kit reagents should be stored under refrigeration.
2. Most of the substrates described above produce a colored reaction product. Alkaline phosphatase can also be detected using the substrate 4-methylumbelliferyl-phosphate, which produces a fluorescent reaction product. This substrate can be used at the same concentration and in the same buffer as the one described in the procedure.
3. High background colors present the most troublesome problems in hybridoma screening. The following points might be helpful to keep in mind. The appropriate serum to block nonspecific sites on the wells should always be used. For best results the serum should be from the same species in which the secondary antibody is made (e.g. if the biotinylated anti-mouse IgG is produced in horse, horse serum should be used as the blocking serum). Usually 2% serum in PBS for 30 minutes will block all nonspecific sites. In some instances, where the highest sensitivity is desired, blocking may require several hours. A 0.1% solution of immunohistochemical grade bovine serum albumin (Cat. No. SP-5050) can be used instead of normal serum in many cases. If another grade is chosen, it should be tested for background prior to use.
4. The washes just before substrate addition are usually the most crucial since any free enzyme left in the wells will produce a color. The most common method is to rinse using a squirt bottle filled with washing buffer. Wells can be emptied by shaking them out sharply over the sink. We recommend tapered side, flat-bottomed plates.
5. Tween 20 and other detergents should be used only at certain places in the microtiter plate protocol. We have found that detergents used throughout the protocols generally produce higher backgrounds than if no detergents are used. The effect of detergents should be assessed at every stage to optimize the system. This differs from our nitrocellulose protocol.
6. The concentrations and incubation times of reactants described for the various enzyme systems are provided as guidelines. In some cases, the various components may need to be diluted further to reduce background color. For best results, reagents and buffers should be prepared fresh or diluted fresh from refrigerated stock solutions. We recommend using glass distilled water as deionized water can contain inhibitors of enzymes such as peroxidase.
7. The purity and source of the antigens, the specific enzyme used, and the time of color development are all factors in determining the enzyme immunoassay system of choice. The ideal system should be sensitive, rapid and produce little to no background. Assay conditions should always be adjusted to optimize the signal to background ratio.
8. When the **DuoLuX™** Chemiluminescent/Fluorescent substrates are used for detection, opaque plates should be utilized to avoid light contamination from adjacent wells. In step 6, 100 µl of the appropriate **DuoLuX™** substrate is added in the dark or in subdued light conditions and incubated at room temperature for 5 minutes. Detect the signal using a luminometer, imaging system, or X-ray film.

VECTASTAIN® ABC Reagents and Kits are designed to be used for research purposes only and are not intended for use in diagnostic procedures.

WESTERN BLOT DETECTION PROCEDURE

The highly sensitive VECTASTAIN® ABC systems can be used to detect antigens bound to nitrocellulose or PVDF membranes after they have been transferred from an electrophoresis gel, dot-blotted, transferred from culture dishes, or bound through filtration techniques. The following procedure is suggested as a guideline for the use of VECTASTAIN® ABC, VECTASTAIN® *Elite* ABC or VECTASTAIN® ABC-AP kits in such a system but it must be emphasized that optimization of conditions may be required for the antigen of interest in a given case. (See also Note 1.) All incubations are at room temperature.

- 1) Prepare approximately 200 ml of 1x casein block per 100 cm² of membrane by diluting 10x Casein Solution (Cat. No. SP-5020) with deionized H₂O.
Block the membrane by incubating in at least 10 ml/100 cm² 1x casein solution for 30 minutes with gentle agitation.
- 2) Transfer the membrane to a solution of the primary antibody in 1x casein. The dilution of antibody will have to be determined in each case by the user but it should be noted that the VECTASTAIN® ABC systems are very sensitive and lower dilutions than normal can often be employed. Incubate for 30 minutes with gentle agitation.
- 3) Wash with at least 3-4 changes of 1x casein over 15 minutes with gentle agitation.

Note: The VECTASTAIN® ABC Reagent (step 6) should be prepared at this time according to the instructions in the kit. Add 2 drops of reagent A to 10 ml (or 5 ml for VECTASTAIN® *Elite* ABC) of 1x casein and then add 2 drops of reagent B. Mix immediately and allow 30 minutes for complex formation before use.

- 4) Transfer the membrane to a dilute solution of biotinylated secondary antibody in 1x casein. This solution can be prepared as outlined in the specific VECTASTAIN® ABC kit instructions (one drop into 10 ml of buffer or according to kit instructions) but using 1x casein as the diluent. Incubate for 30 minutes with gentle agitation.
- 5) Wash as in step 3.
- 6) Transfer the membrane to the VECTASTAIN® ABC, VECTASTAIN® *Elite* ABC, or VECTASTAIN® ABC-AP Reagent. Incubate the membrane in this solution for 30 minutes with gentle agitation.

- 7) Wash as in step 3.

Note: When using the VECTASTAIN® ABC-AP reagent and BCIP/NBT substrate, we recommend 100 mM Tris, pH 9.5 buffer, for the final washing step.

- 8) Transfer the membrane to the substrate solution.

Note: We recommend using a separate staining vessel for this step. Incubation times vary with each substrate. Follow instructions provided in the substrate kit.

- 9) Wash with 2 changes of distilled water over 10 minutes 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.

CHROMOGENIC SUBSTRATES FOR PEROXIDASE:

The most sensitive substrates are tetramethylbenzidine (TMB), diaminobenzidine plus nickel chloride (DAB-Ni), Vector® VIP, Vector® NovaRED™, and Vector® SG. A less sensitive substrate is 4-chloro-1-naphthol (4-CN). Complete substrate kits containing TMB (Cat. No. SK-4400), DAB (ImmPACT™ DAB, Cat. No. SK-4105), DAB-Ni (Cat. No. SK-4100), Vector® VIP (Cat. No. SK-4600), Vector® NovaRED™ (Cat. No. SK-4800), Vector® SG (Cat. No. SK-4700), or 4-CN (Cat. No. SK-4300) are available.

CHROMOGENIC SUBSTRATES FOR ALKALINE PHOSPHATASE:

Although a variety of alkaline phosphatase substrate systems can be employed, we recommend the Vector® BCIP/NBT Kit (Cat. No. SK-5400), which produces a blue reaction product, or the Vector® Black Substrate Kit (Cat. No. SK-5200).

CHEMILUMINESCENT/FLUORESCENT SUBSTRATES

The **DuoLuX™** Chemiluminescent/Fluorescent Substrates can be used with alkaline phosphatase (Cat. No. SK-6605) or peroxidase (Cat. No. SK-6604) detection systems. The **DuoLuX™** substrates produce a reaction product which is highly chemiluminescent as well as fluorescent.

USE OF BIOTINYLATED LECTINS:

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

(Use TTBS (TBS plus 0.1% Tween 20) instead of casein solution to block the membrane and in the diluents and washes. Alternatively, use Carbo-Free™ Blocking Solution (Cat. No. SP-5040) as the blocking agent and TTBS as a diluent and wash solution.

Avoid blocking the membrane with protein solutions that potentially could contain glycoproteins recognized by the lectin of interest. Some of these 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.

Steps 2,3, and 4 are replaced by incubating the blot with a 5-10 µg/ml solution of the biotinylated lectin in TTBS for 30-60 minutes. Then continue the procedure at Step 5 as described.

NOTES:

- 1) 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 a chromogenic substrate (BCIP/NBT). Kits are available for use with mouse or rabbit primary antibodies. Reagents sufficient to stain approximately twenty 100 cm² blots are provided in each kit.

Chemiluminescent/Fluorescent Detection (DuoLuX™) –	
Rabbit IgG	AK-6601
Mouse IgG	AK-6602

Chromogenic Detection (BCIP/NBT) –	
Rabbit IgG	AK-6401
Mouse IgG	AK-6402

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

- 2) Although it is unusual and is generally restricted to some monoclonal antibodies, it is possible that a particular primary antibody will not bind well to an antigen in the presence of Tween 20. In such cases the TTBS in steps 1, 2 and 4 can be replaced by phosphate- or Tris-buffered saline containing 2%-10% normal serum from the species in which the biotinylated antibody was raised. In all other steps the TTBS can be replaced by phosphate- or Tris-buffered saline. This procedure may give rise to a higher background on development of the color.
- 3) In rare cases some bands may stain nonspecifically. This can usually be eliminated by increasing the concentration of NaCl to 0.3-0.5 M in the VECTASTAIN® ABC Reagent. Alternatively, the membrane can be incubated in TBS containing 10% (w/v) non-fat dry milk for 2 hours at 37 °C prior to step 1 above. Overloaded gels may also produce spurious band staining. A range of concentrations of proteins should be applied to establish optimal results.
- 4) Some enzymes isolated from tissues may have covalently attached biotin as a cofactor. If high salt does not prevent the VECTASTAIN® ABC Reagent from binding to particular bands, use an Avidin/Biotin blocking step prior to the primary antibody step. Avidin and Biotin are available as a Blocking Kit (Cat. No. SP-2001).
- 5) For experimental systems using sheep, goat, or bovine antibodies, endogenous immunoglobulins in the non-fat dry milk preclude its use as a blocking reagent. In these cases we recommend TBS with 5% normal rabbit serum, 1x casein solution, or Animal-Free Blocker™ (Cat. No. SP-5030).
- 6) Tween 20 may elute some antigens from the membrane. If this occurs, use protein solutions for blocking and in the appropriate diluents.
- 7) Solutions containing sodium azide or other inhibitors of peroxidase activity should not be used in diluting the peroxidase substrate and VECTASTAIN® ABC Reagent or for the washes. Please also see note 6 of the screening procedure.
- 8) Stock VECTASTAIN® ABC Kit reagents should be stored under refrigeration and kept in the box in which they are supplied.
- 9) Do not add any substances to the VECTASTAIN® ABC reagents which may contain biotin or other inhibitors of Biotin/Avidin interactions. Such substances include serum, non-fat dry milk, culture media and some impure grades of bovine serum albumin.