

- If the reagents are to be diluted beyond the concentrations supplied, dilutions should be made in a buffer containing 0.1% immunohistochemical grade bovine serum albumin (Cat. No. SP-5050). Only immunohistochemical grade BSA should be used, as other preparations can contain undesired impurities. Dilution of these reagents may require longer incubation times and/or higher incubation temperatures to achieve maximum sensitivities.
- The section should be well prepared. Fixation (generally, in buffered formalin not exceeding 4 percent formaldehyde) should be sufficient to maintain the integrity of the section throughout the staining procedure but not so harsh as to destroy the antigen under study. During the staining procedure, do not allow the section to dry out. Use a humidified chamber for incubations. In some cases, use of Antigen Unmasking Solution (Cat. No. H-3300) and exposure to high temperatures can overcome loss of antigens due to fixation.
- To avoid adsorption of the antibody to the plastic or glass container in which the final dilution is made, the primary antibody may be diluted in buffers containing 0.1% immunohistochemical grade bovine serum albumin or dilute Blocking Serum.
- Use only freshly prepared buffers. Bacterial contamination which can occur in buffers stored at room temperature may affect the quality of the staining. It is recommended that the substrate solution be prepared with glass distilled water. Deionized water (even with low conductivities) may contain inhibitors of peroxidase and can reduce sensitivity.
- R.T.U. VECTASTAIN® *Elite* ABC Kit reagents should be stored under refrigeration. For best results, the R.T.U. VECTASTAIN® *Elite* ABC Kit reagents should be used before the date shown on the box. We recommend that they be kept in the box in which they were supplied. If reagents are removed from the box please note on them the date shown on the box so that specific lots of reagents can be traced.
- The universal biotinylated antibody in this kit is prepared in horse specifically for this VECTASTAIN® *Elite* Universal ABC Kit. This antibody should not be confused with other biotinylated antibodies sold as part of other kits.
- Sections of neuronal tissue or sections which are thicker than normal may require longer incubation times for optimal staining.
- Specimens should not be embedded in paraffin heated higher than 60 °C. Too much heat can destroy antigens.

- To prevent sections from detaching from the glass, slides can be treated with VECTABOND™ Reagent (Cat. No. SP-1800), a non-protein tissue section adhesive. Do not use egg albumin coated slides. Traces of egg white avidin may affect staining quality.
- After mounting, paraffin sections should be dried in a hot air oven at 50-56 °C. Some slide warmers contain "hot spots" that can overheat tissues.
- Hand lotions can cause sections to detach from slides or may prevent adequate penetration of reagents. Avoid touching rinse baths with oily hands.
- Paraffin tissue blocks should be stored in sealed containers in a cool location.
- Complete deparaffinization is important. Clearing agents and alcohol solutions should be changed regularly. All steps of the deparaffinization should be sufficiently long to completely remove the paraffin from the sections.
- To reduce the risk of introducing contaminants, avoid removing the drop dispensers from the bottles unless dispensing large volumes. Avoid pipetting reagents directly from their bottles.

Other VECTASTAIN® *Elite* ABC Kits (providing 110 ml of working solutions):

VECTASTAIN® <i>Elite</i> ABC Kit (Goat IgG)	1 Kit	PK-6105
VECTASTAIN® <i>Elite</i> ABC Kit (Human IgG)	1 Kit	PK-6103
VECTASTAIN® <i>Elite</i> ABC Kit (Mouse IgG)	1 Kit	PK-6102
VECTASTAIN® <i>Elite</i> ABC Kit (Rabbit IgG)	1 Kit	PK-6101
VECTASTAIN® <i>Elite</i> ABC Kit (Rat IgG)	1 Kit	PK-6104
VECTASTAIN® <i>Elite</i> ABC Kit (Sheep IgG)	1 Kit	PK-6106
VECTASTAIN® <i>Elite</i> ABC Kit (Universal)‡	1 Kit	PK-6200
VECTASTAIN® <i>Elite</i> ABC Kit (Standard)	1 Kit	PK-6100
This Standard Kit consists of only the ABC <i>Elite</i> Reagents.		
R.T.U. VECTASTAIN® <i>Elite</i> ABC Reagent	50 ml	PK-7100

Peroxidase Substrate Kits

Each kit provides sufficient stock reagents to prepare about 300 ml of substrate solution.

DAB Substrate	1 Kit	SK-4100
Vector® VIP Substrate	1 Kit	SK-4600
Vector® SG Substrate	1 Kit	SK-4700
Vector® NovaRED® Substrate	1 Kit	SK-4800
TMB Substrate	1 Kit	SK-4400
AEC Substrate	1 Kit	SK-4200

Other related reagents also available are:

Avidin/Biotin Blocking Kit (for blocking nonspecific binding of avidin)	1 Kit	SP-2001
Heat-treated, ultrafiltered normal serum from:		
Goat	20 ml	S-1000
Horse	20 ml	S-2000
Chicken	20 ml	S-3000
Swine	20 ml	S-4000
Rabbit	20 ml	S-5000

The following biotinylated antibodies can be used in conjunction with any VECTASTAIN® *Elite* ABC Kit:

Biotinylated "Universal" Anti-Mouse/Rabbit 2.1 mg IgG (H + L)† made in horse	2.1 mg	BA-1400
Biotinylated "Universal" Pan-Specific Anti-Mouse/Rabbit/Goat IgG (H + L) made in horse	2.2 ml	BA-1300
Biotinylated Anti-Cat IgG (H + L)* made in goat	1.5 mg	BA-9000
Biotinylated Anti-Chicken IgG (H + L) made in goat	1.5 mg	BA-9010
Biotinylated Anti-Goat IgG (H + L)**† made in rabbit	1.5 mg	BA-5000
Biotinylated Anti-Goat IgG (H + L)** made in horse	1.5 mg	BA-9500
Biotinylated Anti-Guinea Pig IgG (H + L) made in goat	1.5 mg	BA-7000
Biotinylated Anti-Hamster IgG (H + L) made in goat	1.5 mg	BA-9100
Biotinylated Anti-Horse IgG (H + L) made in goat	1.5 mg	BA-8000
Biotinylated Anti-Human IgA (α-chain specific) made in goat	0.5 mg	BA-3030
Biotinylated Anti-Human IgE (ε-chain specific) made in goat	0.5 mg	BA-3040
Biotinylated Anti-Human IgG (H + L)† made in goat	1.5 mg	BA-3000
Biotinylated Anti-Human IgG (γ-chain specific) made in goat	0.5 mg	BA-3080
Biotinylated Anti-Human IgM (μ-chain specific) made in goat	0.5 mg	BA-3020
Biotinylated Anti-Human Kappa Chain (κ-chain specific) made in goat	0.5 mg	BA-3060
Biotinylated Anti-Human Lambda Chain (λ-chain specific) made in goat	0.5 mg	BA-3070
Biotinylated Anti-Mouse IgG (H + L)† made in horse	1.5 mg	BA-2000
Biotinylated Anti-Mouse IgG (H + L) made in goat	1.5 mg	BA-9200
Biotinylated Anti-Mouse IgG (H + L) (Rat Adsorbed) made in horse	0.5 mg	BA-2001
Biotinylated Anti-Mouse IgG (γ-chain specific) made in horse	0.5 mg	BA-2080
Biotinylated Anti-Mouse IgM (μ-chain specific) made in goat	0.5 mg	BA-2020
Biotinylated Anti-Rabbit IgG (H + L)† made in goat	1.5 mg	BA-1000
Biotinylated Anti-Rat IgG (H + L)† made in rabbit	1.5 mg	BA-4000
Biotinylated Anti-Rat IgG (H + L) made in goat	1.5 mg	BA-9400
Biotinylated Anti-Rat IgG (H + L) (Mouse Adsorbed) made in rabbit	0.5 mg	BA-4001
Biotinylated Anti-Sheep IgG (H + L)† made in rabbit	1.5 mg	BA-6000
Biotinylated Anti-Sheep IgG (γ-chain specific) made in rabbit	0.5 mg	BA-6080
Biotinylated Anti-Swine IgG (H + L) made in goat	1.5 mg	BA-9020

* Use with Dog IgG primary antibodies.
 ** Use with Bovine IgG primary antibodies.
 † Antibodies included in VECTASTAIN® *Elite* ABC Kits.
 ‡ Mouse/Rabbit IgG

A complete catalog listing is available upon request.

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R.T.U. VECTASTAIN®
 UNIVERSAL
Elite
 ABC KIT
 Cat. No. PK-7200

INSTRUCTIONS FOR
 IMMUNOHISTOCHEMICAL STAINING

Introduction

The VECTASTAIN® ABC system is widely accepted as one of the most sensitive, economical and reliable immunoperoxidase systems available. For several years our efforts were devoted to improving the original ABC system. This research led to the development of the VECTASTAIN® *Elite* ABC Kit, which is considerably more sensitive than the original VECTASTAIN® ABC peroxidase system without increased background staining. This enhanced sensitivity is particularly important in the localization of antigens present in low amounts or in cases where the cost of primary antibodies is significant. The increased sensitivity also provides an option to substantially reduce staining times. The Ready-To-Use (R.T.U.) VECTASTAIN® *Elite* ABC Kit is based on the same patented principles as the other VECTASTAIN® ABC Kits.

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Avidin is a 68,000 molecular weight glycoprotein with an extraordinarily high affinity ($10^{15}M^{-1}$) 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 provide the potential for macromolecular complexes to be formed between avidin and biotinylated enzymes.

An immunoperoxidase procedure based on these properties was devised for localizing a variety of histologically significant antigens and other markers. (Hsu SM, Raine L, Fanger H: Am. J. Clin. Pathol. 75, 734-738, 1981; Hsu SM, Raine L, Fanger H: J. Histochem. Cytochem. 29, 577-580, 1981.) This technique employs unlabeled primary antibody, followed by biotinylated secondary antibody and then a preformed Avidin and Biotinylated horseradish peroxidase macromolecular Complex. This has been termed the ABC technique.

The R.T.U. VECTASTAIN® *Elite* ABC Kits contain Avidin DH and biotinylated horseradish peroxidase H reagents, which have been specially prepared to form ideal complexes for immunoperoxidase staining. Although the structure of the Avidin DH: biotinylated horseradish peroxidase H complex is still undefined, evidence suggests that it consists of many biotinylated horseradish peroxidase molecules crosslinked by avidin into a three dimensional array. The complex apparently has few exposed biotin residues but retains at least one biotin binding site. Formation of the complex is achieved by mixing Avidin DH and biotinylated horseradish peroxidase H in dilute solution and in defined amounts. The R.T.U. VECTASTAIN® *Elite* ABC Kits contain a stabilized preformed *Elite* ABC in ready-to-use form requiring no complex formation or dilution prior to use.

The most important factor to note concerning the VECTASTAIN® *Elite* ABC Kits is the increased sensitivity. For this reason primary antibodies can be diluted about fivefold higher than when used with the original VECTASTAIN® ABC peroxidase kit to produce equivalent staining intensity.

VECTASTAIN® *Elite* ABC Reagents and Kits are designed to be used for laboratory use only.

VECTASTAIN® WORKING SOLUTIONS

For convenience, the R.T.U. VECTASTAIN® *Elite* ABC Kits include ready-to-use working solutions of reagents. The working solutions are supplied in bottles fitted with drop dispenser tips. If the tip is to be removed, press laterally with thumb until the tip snaps off. When dispensing drops, hold the bottle in an inverted position and squeeze gently. Secure the caps on the bottles when they are not in use.

When dispensing reagents, apply a sufficient number of drops on the slide to cover the entire section. Slides should then be placed in a humidified chamber during the incubation period. Staining dishes, Coplin jars or automated tissue staining instruments may also be used in the staining procedure.

A number of different washing buffers can be used in the VECTASTAIN® *Elite* ABC system. One of the most common is 10 mM sodium phosphate, pH 7.5, 0.9% saline (PBS). The R.T.U. VECTASTAIN® Universal *Elite* ABC Kit consists of 50 ml of each of the following ready-to-use reagents:

- Prediluted normal horse serum (NHS)
- Prediluted biotinylated horse antibody which recognizes rabbit and mouse IgG (H+L).
- Ready-to-use, stabilized *Elite* ABC Reagent.

The R.T.U. VECTASTAIN® Universal *Elite* ABC Kit will stain approximately 500 sections.

ENZYME SUBSTRATES

A variety of chromogens are used to localize peroxidase in tissue sections. The most commonly used have been diaminobenzidine tetrahydrochloride (DAB) and 3-amino-9-ethyl carbazole (AEC). DAB produces a brown precipitate or a gray/black precipitate in the presence of some divalent cations. DAB is insoluble in alcohol and clearing agents, allowing sections to be permanently mounted. AEC produces a red reaction product but must be mounted in aqueous mounting media. There are three additional peroxidase substrates developed by Vector Labs that can be dehydrated and permanently mounted. Reagents in the Vector® VIP substrate kit produce an intense purple precipitate, those in the Vector® SG substrate kit produce a blue-gray reaction product, and those in Vector® NovaRED™ substrate kit produce a red precipitate. Combinations of these substrates can be used in multiple-labeling protocols. TMB peroxidase substrate produces a blue precipitate that also can be permanently mounted. All six chromogenic systems are available as kits in convenient dropper bottle formats. See product listing for catalog numbers of these substrate kits.

STAINING PROCEDURE FOR PARAFFIN SECTIONS

1. Deparaffinize and hydrate tissue sections through xylenes or other clearing agents and graded alcohol series.
2. Rinse for 5 minutes in tap water.
3. If quenching of endogenous peroxidase activity is required, incubate the sections for 30 minutes in 0.3% H₂O₂ in either methanol or water. Incubation times may be shortened by using higher concentrations of H₂O₂. If endogenous peroxidase activity does not present a problem, this step may be omitted.
4. Wash in buffer for 5 minutes.
5. Incubate sections for 20 minutes with diluted normal horse serum.
6. Blot excess serum from sections.
7. Incubate sections for 30 minutes with primary antibody diluted in buffer. (See note 6)
8. Wash slides for 5 minutes in buffer.
9. Incubate sections for 30 minutes with diluted biotinylated "universal" secondary antibody.
10. Wash slides for 5 minutes in buffer.
11. Incubate sections for 30 minutes with VECTASTAIN® R.T.U. *Elite* ABC Reagent.
12. Wash slides for 5 minutes in buffer.
13. Incubate sections in peroxidase substrate solution until desired stain intensity develops (see Note 2).
14. Rinse sections in tap water.
15. Counterstain, clear and mount.

STAINING PROCEDURE FOR FROZEN SECTIONS

This procedure is generally appropriate for frozen sections, cell smears or cytocentrifuge preparations.

1. Sections are air dried.
2. Immediately before staining, fix sections with acetone or the appropriate fixative for the antigen under study.
3. Transfer slides into buffer.

4. If quenching of endogenous peroxidase is required, use gentle H₂O₂ blocking to reduce the risk of antigen destruction or tissue loss: 0.3% H₂O₂ in 0.3% NHS in PBS for 5 minutes; or 0.3% H₂O₂ in methanol for 30 minutes, or use other published methods (eg. Andrew, S. M., Jasani, B., Histochem J. 1987, 19, 426-30). If necessary, H₂O₂ treatment may also be performed after the biotinylated secondary antibody step.
5. Follow steps 4-15 of the procedure recommended for paraffin sections.

After completion of the staining procedure, R.T.U. reagents should be stored together at 4 °C in their original kit box.

If unwanted staining occurs in the absence of biotinylated secondary antibody, endogenous protein-associated biotin may be present in the tissue. To eliminate this unwanted staining, use an Avidin/Biotin blocking step (Cat. No. SP-2001) between steps 6 and 7.

RAPID STAINING

Rapid staining of tissue sections can be achieved using the VECTASTAIN® Universal Quick Kit (Cat. No. PK-8800) or its ready-to-use form (Cat. No. PK-7800) or by using the VECTASTAIN® Universal *Elite* ABC Kit (Cat. No. PK-6200) with the rapid staining protocol.

NOTES:

1. Solutions containing sodium azide or other inhibitors of peroxidase activity should not be used in diluting the peroxidase substrate. Do not add normal serum, non-fat dried milk, culture media or other potential sources of biotin in the ABC reagent. This may result in reduced sensitivity.
2. Development times may differ depending upon the level of antigen, the intensity of the stain that is required or the substrate used. DAB generally should be developed for 2-10 minutes; Vector® VIP for 2-15 minutes; Vector® SG for 2-10 minutes; Vector® NovaRED™ for 2-15 minutes; AEC for 10-30 minutes; TMB for 5-20 minutes. Some counterstains may not be compatible with certain peroxidase substrates because of solubility of the reaction products or lack of color contrast. A counterstain compatibility chart is available upon request. Refer to the instructions in the respective substrate kits for further details.
3. In the presence of nickel ions, the precipitate formed by DAB is gray/black rather than brown. Adding Ni²⁺ may enhance the sensitivity of the staining procedure and, because of the difference in color from DAB alone, has been used in double-labeling techniques. The DAB Substrate Kit (Cat. No. SK-4100) contains nickel chloride and allows two colors to be introduced into the section.