



R.T.U. VECTASTAIN®

Elite®

ABC REAGENT

Cat. No. PK-7100

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 R.T.U. VECTASTAIN® *Elite* ABC Reagent is the same patented ABC Reagent as that contained in other VECTASTAIN® *Elite* ABC Kits, but in a ready-to-use form.

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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 reagent has been prepared from Avidin DH and biotinylated horseradish peroxidase H, the same reagents contained in the VECTASTAIN® *Elite* ABC kit. 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 is supplied as 50 ml in a ready-to-use form, in a bottle fitted with a drop dispenser tip. To remove the drop dispenser tip, press laterally with thumb until the tip snaps off. When dispensing drops, hold the bottle in an inverted position and squeeze gently. Secure the cap on the bottle when it is 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.

The R.T.U. VECTASTAIN® *Elite* ABC Reagent has been designed to be used with any of our extensive range of biotin-labeled reagents. The protocols below detail the use of normal serum and biotinylated secondary antibody from VECTASTAIN® ABC Kits, which are supplied in dropper bottles with instructions for diluting reagents. If purchased separately from the VECTASTAIN® ABC Kit, the biotinylated antibody is supplied lyophilized and should be reconstituted in 1 ml of distilled water to prepare the concentrated stock solution.

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). Refer to the specification sheet accompanying the product or to the instructions in the VECTASTAIN® ABC Kits for preparation of the blocking serum and biotinylated secondary antibody.

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

ENZYME SUBSTRATES

A variety of chromogens can be used to localize peroxidase substrates in tissue sections. Vector Laboratories offers the traditional substrates DAB and AEC as well as several proprietary substrates, producing colors as listed.

These substrates can be used as single labels or to introduce multiple colors in a tissue section.

DAB (Diaminobenzidine), SK-4100, brown
DAB + Ni²⁺, SK-4100, gray/black
Vector® VIP, SK-4600, purple
Vector® SG, SK-4700, blue-gray
Vector® NovaRED™, SK-4800, dark red
TMB, SK-4400, blue
AEC (3-amino-9-ethyl carbazole)*, SK-4200, red

Vector Laboratories also offers a line of peroxidase substrates with increased sensitivity.

ImmPACT™ DAB, SK-4105, brown
ImmPACT™ VIP, SK-4605, purple
ImmPACT™ SG, SK-4705, blue-gray
ImmPACT™ NovaRED™, SK-4805, dark red
ImmPACT™ AEC*, SK-4205, red

* AEC is soluble in alcohol and clearing agents and must be mounted in aqueous mounting media. All other substrates are not soluble in alcohol or clearing agents. They may be dehydrated, cleared, and permanently mounted.

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 (2.5%) normal blocking serum which was prepared from the species in which the secondary antibody is made.
6. Blot excess serum from sections.
7. Incubate sections for 30 minutes with primary antibody diluted in buffer containing 2.5% normal serum.
8. Wash slides for 5 minutes in buffer.
9. Incubate sections for 30 minutes with diluted biotinylated secondary antibody solution.
10. Wash slides for 5 minutes in buffer.
11. Incubate sections for 30 minutes with R.T.U. VECTASTAIN® *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% normal serum 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.

- Follow steps 4-15 of the procedure recommended for paraffin sections.

After completion of the staining procedure, the R.T.U. *Elite* ABC Reagent should be stored at 4 °C in the original 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 any of the VECTASTAIN® *Elite* ABC Kits with the rapid staining protocol.

NOTES:

- 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 to the ABC reagent. This may result in reduced sensitivity.
- Substrate development times may differ depending upon the level of antigen, the intensity of the stain that is required, or the substrate used. ImmPACT™ DAB and DAB generally should be developed for 2-10 minutes; ImmPACT™ VIP and Vector® VIP for 2-15 minutes; ImmPACT™ SG and Vector® SG for 2-10 minutes; ImmPACT™ NovaRED™ and Vector® NovaRED™ for 2-15 minutes; ImmPACT™ AEC and 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.
- 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.
- 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 or H-3301) 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.
- The R.T.U. VECTASTAIN® *Elite* ABC Reagent should be stored under refrigeration. For best results, the VECTASTAIN® *Elite* ABC Reagent should be used before the date shown on the box. We recommend that it be kept in the box in which it was supplied. If the reagent is removed from the box please note on it the date shown on the box so that specific lots can be traced.
- The affinity-purified biotinylated secondary antibody and the normal serum provided in VECTASTAIN® *Elite* ABC Kits can be purchased individually. The Avidin DH and biotinylated horseradish peroxidase H used to prepare the R.T.U. VECTASTAIN® *Elite* ABC Reagent are special. Do not confuse those in VECTASTAIN® ABC Kits with Cat. Nos. A-2000 and B-2004. We recommend using only ABC reagents provided in the VECTASTAIN® *Elite* ABC 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.
- If smaller volumes of working solutions are desired, it is recommended that a drop from the stock solution be dispensed into a small, conical plastic tube. A suitable aliquot can then be withdrawn. To reduce the risk of introducing contaminants, avoid removing the drop dispenser from the stock solution bottle, unless dispensing large volumes. Avoid pipetting *Elite* ABC reagent directly from the bottle.

The R.T.U. VECTASTAIN® *Elite* ABC Reagent and R.T.U. “Universal” Kit (PK-7200) contain 50 ml of working solutions. Other VECTASTAIN® *Elite* ABC Kits providing 110 ml of working solutions are:

VECTASTAIN® <i>Elite</i> ABC Kit (Standard)	1 Kit	PK-6100
This Standard Kit consists of only the ABC <i>Elite</i> Reagents.		
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

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

Biotinylated “Universal” Anti-Mouse/Rabbit 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 IgG (H + L)‡	1.5 mg	BA-3000

Biotinylated Anti-Mouse IgG (H + L)‡ made in horse†	1.5 mg	BA-2000
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-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
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.
- ‡ Chain-specific antibodies are also available.

Other related reagents also available are:

Antigen Unmasking Solution (dilutes to 25 liters)		
Citrate-based	250 ml	H-3300
High pH	250 ml	H-3301
Avidin/Biotin Blocking Kit	1 Kit	SP-2001
ImmEdge™ Hydrophobic Barrier Pen	2-pen set	H-4000
ImmPrint™ Histology Pen	5-pen set	H-6100
Vectabond™ Reagent (dilutes to 350 ml)	7 ml	SP-1800
VectaMount™ Mounting Medium	60 ml	H-5000
VectaMount™ AQ Mounting Medium	60 ml	H-5501
Vector® Hematoxylin	500 ml	H-3401
Vector® Hematoxylin QS	100 ml	H-3404
Vector® Methyl Green	500 ml	H-3402
Vector® Nuclear Fast Red	500 ml	H-3403
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

Peroxidase Substrate Kits

These kits provide 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

More sensitive versions of most of these substrates are also available as ImmPACT™ substrates:

ImmPACT™ DAB	120 ml	SK-4105
ImmPACT™ VIP Substrate	120 ml	SK-4605
ImmPACT™ SG Substrate	120 ml	SK-4705
ImmPACT™ NovaRED™ Substrate	120 ml	SK-4805
ImmPACT™ AEC Substrate	120 ml	SK-4205

Detailed product listings, specifications and protocols are available on our website: www.vectorlabs.com