Diaminobenzidine-Catechol
DAB-C Substrate Concentrate

Catalog No.	Size
71-00-13	10 mL

DESCRIPTION
DAB (3,3'-diaminobenzidine) deposits a brown specific stain in the presence of horseradish peroxidase (HRP)-labeled reporter reagents. DAB-C Substrate Concentrate provides an enhanced orange reaction when used as a component of the HistoMark ORANGE Substrate System. DAB-C Substrate Concentrate must be used with Enhance ORANGE Buffer Solution rather than standard Tris solutions for proper results. This substrate is useful for immunohistochemical or immunoblotting applications.

CONTENT
10 mL stable liquid concentrate at 25 mg/mL. Sufficient material supplied to prepare 500 mL Substrate Solution.
CAUTION: DAB is a suspected carcinogen. Avoid contact with skin or clothing. Follow recommended disposal procedures.

STORAGE/STABILITY
- DAB-C Substrate Concentrate is stable for a minimum of one year stored at 2 – 8°C.
- Solution should appear light to medium brown. Discard solution if color changes from brown to purple or if heavy precipitate develops.
- Warm to room temperature (24 – 28°C) before use.

REAGENTS NOT INCLUDED
1. Primary antibody.
2. HRP-labeled reagents.
3. Hydrogen Peroxide (H_2O_2) Solution (Catalog No. 71-00-09).
4. Enhance ORANGE Buffer Solution (Catalog No. 71-00-07).
5. Isopropyl alcohol.
6. Mounting media (aqueous or xylene-based).
7. Contrast BLUE Solution (Catalog No. 71-00-06).
8. 0.1 M Tris-HCl wash solution, pH 7.6:
   a. Dissolve 121 g Tris in 500 mL reagent quality water.
   b. Adjust pH to 7.6 with 2 mol/L HCI (approximately 300 mL).
   c. QS to 1 L with reagent quality water to obtain a 1 M stock.
   d. Dilute 1 part stock from Step 5c with 9 parts reagent quality water and mix well.

PREPARATION
a. Add 0.5 mL Enhance ORANGE Buffer Solution to 5 mL reagent quality water.
b. Add 0.1 mL DAB-C Solution.
c. Add 0.1 mL Peroxide Solution.
d. Mix thoroughly. Use solution immediately.

PROCEDURE
1. Rehydrate paraffin embedded sections through graded alcohol (3 minutes each in 100%, 80%, 40% and 20% EtOH) to water. Other samples listed below do not require rehydration. Frozen sections must be thoroughly dried before use.
2. To block endogenous peroxidase activity, immerse samples in diluted Blocking Solution as follows:

a. Frozen sections	45 seconds
b. Paraffin sections	4 minutes
c. Cytospin preparations	45 seconds
d. Blood films	45 seconds
e. Touch or squash preparations	1 minute
f. Floating or whole sections	5 minutes

3. Rinse five minutes in reagent quality water.
4. Soak in 0.1 M Tris-HCl or PBS 10 minutes.
5. Treat sample with primary antibody diluted in Tris-HCl or PBS 15 - 20 minutes.
   NOTE: Extended incubation may improve sensitivity.
6. Wash sample with Tris-HCl or PBS 10 minutes.
7. Incubate sample with biotin-labeled link antibody, directed against the primary antibody host species, 15 - 20 minutes. If using HRP-labeled secondary antibody, proceed to Step 9.
8. Wash as in Step 6.
9. Shake off excess buffer and incubate sample with HRP Streptavidin or HRP-labeled secondary antibody diluted in Tris-HCl or PBS, 15 - 20 minutes.
10. Wash as in Step 6. (Prepare Substrate Solution during this step.)
11. Shake off excess buffer and cover section with Substrate Solution.
12. Incubate 10 minutes at room temperature out of direct light.
13. Rinse slide 2 - 3 minutes in reagent quality water.
14. Counterstain with Contrast BLUE if desired. Paraffin embedded and frozen sections for 3 minutes; touch preparations, cytospin preparations and blood films for 30 - 45 seconds.
15. Rinse thoroughly in 2 - 3 changes of isopropyl alcohol or until excess stain is removed from slide. DO NOT USE WATER OR ETHANOL SOLUTIONS.
16. Air dry and mount in aqueous or xylene-based mounting medium.

RESULTS
Sites of enzyme activity range from bright orange to red-orange.
If counterstained, nuclei appear a contrasting blue.
Sections not reacted with primary antibody as a negative control should not develop an orange tint.
Further dilution of primary antibody or HRP-labeled reagent may be required to prevent excessive background.

NOTES

1. Always incorporate appropriate positive and negative controls.
2. The following method of disposal is recommended for solutions containing DAB:

a Add 100 mL of household bleach to 2 Liters of water. Pour this solution into a 1 gallon plastic bottle.

b. Pour waste DAB solution into the solution from Step 2a and mix by shaking. No more than 500 mL of DAB solution should be added. After last addition, allow container to stand at least 24 hours before discarding.

3. Instant development of brown color indicates that the primary antibody or peroxidase-labeled reagent must be further diluted.
4. Prolonged incubation in substrate may increase background and inhibit nuclear counterstaining.
5. As an alternative method to block endogenous peroxidase, incubate slides for 30 minutes in 0.3% (w/v) H_2O_2 in absolute methanol followed by a 10 - 15 minute rinse in 0.1 mol/L Tris-HCI, pH 7.6 or similar buffer.
6. KPL Universal Kits provide biotin-labeled secondary antibody, HRP-labeled Streptavidin, and Serum Block:

Universal Kit for use with	Catalog No.
Mouse Primary Antibody	71-00-18
Rabbit Primary Antibody	71-00-19
Rat Primary Antibody	71-00-20
Goat Primary Antibody	71-00-26

7. KPL DAB Reagent Set (Catalog No. 54-10-00) provides DAB, H_2O_2 and Tris solutions at use dilution in convenient dropper bottles.

PRINCIPLE

The application of antibodies and other proteins covalently coupled to horseradish peroxidase (HRP) in immunohistology is well documented 4. It is the most frequently used label for immunohistologic techniques. In the presence of peroxide (H_20_2), HRP catalyzes the oxidation of phenols, naphthols, diamines, aminophenols, indophenols, etc. forming chromogenic products visible by light microscopy. Most commonly employed are 3amino-9-ethylcarbazole5, p-phenylenediamine/catechol6, 4-chlorol-naphthol~ and diaminobenzidine (DAB)(8). Although a suspected carcinogen, DAB is the most widely accepted donor substrate for peroxidase immunohistochemistry, since it provides a reaction product insoluble in alcohols and xylene.
The oxidation of DAB results in formation of a free radical intermediate which polymerizes to form a brown product. DAB may be employed for demonstration of endogenous peroxidase and catalase activity; Cytochrome oxidase; Cupric ferrocyanide; and hemoproteins such as hemoglobin, myoglobin, and cytochrome c. Reaction with Osmium tetraoxide results in an electrom opaque osmium black useful for ultrastructure research.

The light brown DAB reaction can be difficult to visualize. Treatment of the DAB product with osmium, silver, cobalt or nickel will intensify final color. The procedure described in this insert employs nickel DAB enhancement, providing an orange reaction product for improved contrast.

REFERENCES

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PRODUCT SAFETY AND HANDLING

See MSDS (Material Safety Data Sheet) for this product.

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Gaithersburg, MD • USA
301-948-7755 • 800-638-3167 • FAX 301-948-0169