ORANGE Peroxidase Substrate Kit

Catalog Number KA0563

1000 slides

Version: 02

Intended for research use only

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Introduction and Background

A. Overview

The ORANGE Substrate System is an enhanced metal-bridging DAB procedure for visualization of horseradish peroxidase (HRP)-labeled reporter reagents. The substrate system provides a reddish-orange specific stain with green counterstain for immunohistochemical staining. This procedure employs nickel/catachol-based DAB enhancement, providing improved contrast compared to standard DAB procedures.

B. Test Principle

The application of antibodies and other proteins covalently coupled to horseradish peroxidase (HRP) in immunohistology is well documented (1-4). It is the most frequently used label for immunohistologic techniques. In the presence of peroxide (H_2O_2), HRP catalyzes the oxidation of phenols, naphthols, diamines, aminophenols, indophenols etc. forming chromogenic products visible by light microscopy. Most commonly employed are 3-amino-9-ethylcarbazole (5), p-phenylenediamine/catechol (6), 4-chloro-1-naphthol (7) 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 DAB oxidation product is light brown and may be difficult to visualize if low antigen density occurs. The HistoMark ORANGE system is based on oxidative coupling of aromatic amines and phenolic compounds by HRP in the presence of peroxides (9). Endogenous peroxidase activity is eliminated by controlled oxidation prior to application of antibodies (10).

C. Additional Required Materials

1. Primary antibody.
2. Biotin-labeled secondary antibody, HRP-labeled Streptavidin and Serum Block
3. Isopropyl alcohol.
4. Mounting media (aqueous or xylene-based).
5. 0.1 M Tris-HCl or PBS (See Preparation of reagent).

Material and Method

A. List of component

Component	Amount
Enhance ORANGE Buffer Solution	50 ml
DAB-C Solution	10 ml
Peroxide Solution	10 ml
Contrast GREEN Solution	50 ml
Blocking Solution Concentrate (10X)	10 ml

Sufficient reagents are supplied to prepare 500 ml Substrate Solution (approximately 1000 slides).

B. Preparation of reagent

- ✓ Substrate Solution (prepare immediately before use in Step 10):
  a. Add 0.5 ml Enhance ORANGE Buffer Solution to 5 ml reagent quality water.
  b. Add 0.1 ml of DAB-C Solution.
  c. Add 0.1 ml Peroxide Solution.
  d. Mix thoroughly. Use solution immediately.
- ✓ Contrast GREEN Solution: supplied at use dilution.
- ✓ Blocking Solution: Dilute 1:10 with reagent quality water i.e. 1 ml of Blocking Solution Concentrate to 9 ml reagent quality water. Diluted solution may be stored tightly capped at 2-8℃ for up to one week.
- ✓ 0.1 M Tris-HCl:
  a. Dissolve 121 g Tris in 500 mL reagent quality water.
  b. Adjust pH to 7.6 with 2 M HCl (approximately 300 mL).
  c. QS to 1 Liter with reagent quality water to obtain 1 M stock solution.
  d. Dilute 1 part stock from Step 5c with 9 parts reagent quality water and mix well.
- ✓ Phosphate Buffered Saline (PBS):
  a. Add PBS (0.01M), 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4.
  b. Adjust pH to 7.4 with 2 M HCl.
  c. QS to 1 L with reagent quality.

Stability and storage:

- ✓ Reagents are stable for a minimum of one year stored at 2-8℃. Discard solutions that become turbid.
- ✓ Warm all reagents to room temperature (24-28℃) before use.

Protocol

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. Block endogenous peroxidase activity, immersing 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 5 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 0.1 M Tris-HCl or PBS 10 minutes.
7. Incubate sample with biotin-labeled antibody, directed against the primary antibody host species, 15 - 20 minutes. If using HRP-labeled secondary antibody, go 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 in Contrast GREEN Solution: 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 with aqueous or Xylene-based mounting medium.

Note:

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 an orange color indicates that the primary antibody or peroxidase labeled reagent

must be further diluted.

4. Prolonge d 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 M Tris-HCl, pH 7.6 or PBS.

Result

- ✓ Sites of enzyme activity range from pale to vivid red-orange. Nuclei appear a contrasting pale green.
- ✓ 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.

References

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