Introduction and Background

A. Overview

RED Substrate System is designed for visualization of alkaline phosphatase-labeled (AP) reagents. RED is a New Fuchsin stain and Contrast BLUE is a hematoxylin counterstain. The substrate system provides a red specific stain with blue counterstain for immunohistochemical staining or immunoblotting.

B. Test Principle

The application of antibodies and other reagents such as avidin, streptavidin, etc., covalently coupled to calf intestine alkaline phosphatase in immunohistology is well documented (l, 2). The procedure described in this insert employs a simultaneous capture azo-dye technique, providing the research laboratory a method for precise localization of alkaline phosphatase labeled reagents (3, 4). Primary aryl amines, when reacted with alkyl nitrites in acid media, form azo compounds (5). These react with substituted naphthols to produce highly chromogenic insoluble dyes. In this procedure the phosphate ester of 6-bromo-2-hydroxy-3-naphthoic acid (Buffered Substrate Solution) is employed as substrate. Enzymatic hydrolysis, in the presence of hexazotized triaminotrimethyltriphenylmethane (PhThaloRED Solution) results in the formation of a brilliant red reaction product. Endogenous enzyme is eliminated by incorporation of levamisol (6). It should be noted that a levamisole-resistant alkaline phosphatase has been demonstrated in some malignant cells from serous effusions (7). Additional blocking measures may be required (8, 9).

C. Additional Required Materials

1. Primary antibody.
2. AP-labeled reagents: Biotin-labeled secondary antibody, AP-labeled Streptavidin, and Serum Block
3. Isopropyl alcohol.
4. Xylene-based mounting media.
5. 0.1 M Tris-HCl or PBS (See Preparation of reagent).
6. 1 M Citric Acid Free Acid (See Preparation of reagent).

Material and Method

A. List of component

Component	Amount
Activator Solution	10 mL
RED Solution	10 mL
Buffered Substrate Solution	50 mL
Contrast BLUE Solution	50 mL

B. Preparation of reagent

✓ Substrate Solution (prepare immediately before use in Step 10)

NOTE: Prior to preparation, if a light precipitate is visible in Buffered Substrate Solution, warm for 10 – 15 minutes in 37℃ waterbath. Mix thoroughly by inversion until completely in solution.

a. Add 0.5 mL Buffered Substrate Solution to 5 mL reagent quality water.
b. Mix 0.1 mL PhThaloBLUE Solution with 0.1 mL Activator Solution in a separate tube. Mix gently and allow standing 3 minutes.
c. After 3 minutes combine solutions from steps a. and b. Mix thoroughly and use immediately.

✓ Contrast BLUE Solution: supplied at use dilution.

✓ 0.1 M Tris-HCl:

a. Dissolve 121 g Tris in 500 mL reagent quality water.
b. Adjust pH to 7.6 with 2M HCl (approximately 300 mL).
c. QS to 1 Liter with reagent quality water to obtain 1M stock solution.
d. Dilute 1 part stock from Step 5c with 9 parts reagent quality water and mix well.

✓ 1 M Citric Acid Free Acid

a. Dissolve 192 g of citric acid free acid in 500 mL reagent quality water.
b. QS to 1L with reagent quality water.

Stability and storage:

✓ Reagents are stable for a minimum of one year stored at 2 - 8℃.

✓ Store Contrast BLUE Solution tightly capped at room temperature.

✓ Discard PhThaloRED Solution if solution turns red.

✓ Discard Activator Solution or Buffered Substrate Solution if yellow color develops.

✓ Warm all reagents to room temperature (24 - 28℃) before use.

✓ If a light precipitate is visible in Buffered Substrate Solution, warm for 10 – 15 minutes in 37℃ waterbath. Mix thoroughly by inversion until completely in solution.

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. RED reagents contain levamisole to block endogenous phosphatase activity. If additional blocking is required, apply Bouin's Solution or 1M citric acid free acid 1 - 10 minutes.
3. Rinse five minutes in reagent quality water.
4. Soak in 0.1 M Tris-HCl for 3 - 10 minutes.

NOTE: Inorganic phosphate inhibits alkaline phosphatase activity. Avoid use of PBS or any solution containing phosphates.

5. Treat sample with primary antibody diluted in Tris-HCl for 15 - 20 minutes.

NOTE: Extended incubation may improve sensitivity.

6. Wash sample with Tris-HCl 10 minutes.
7. Incubate sample with biotin-labeled antibody, directed against the primary antibody host species, 15 - 20 minutes. If using AP-labeled secondary antibody, proceed to Step 9.
8. Wash as in Step 6.
9. Shake off excess buffer and incubate sample with AP Streptavidin or AP-labeled secondary antibody diluted in Tris-HCl 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 BLUE Solution 30 seconds to 10 minutes.
15. Rinse thoroughly in reagent quality water.
16. Dip 10 times in 100% ethanol.
17. Dip 10 times in xylene or xylene equivalent.
18. Mount in xylene-based mounting medium.

Note:

1. Always incorporate appropriate positive and negative controls.
2. Use substrate reagents immediately after mixing.
3. Instant development of blue color indicates that the primary antibody or phosphatase-labeled reagent must be further diluted.
4. Prolonged incubation in substrate may increase background and inhibit nuclear counterstaining.

Result

- ✓ Sites of enzyme activity range from pale pink to red. Nuclei appear a contrasting pale blue.
- ✓ Sections not reacted with primary antibody as a negative control should not develop a red tint.

References

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