HistoMark® BLUE

For Localization of
Alkaline Phosphatase-Labeled Reagents

Catalog No.	Size
55-70-00	500 mL

DESCRIPTION

HistoMark® BLUE Substrate System is designed for visualization of alkaline phosphatase-labeled (AP) reagents. HistoMark® BLUE is a Fast Blue stain and Contrast RED is a nuclear counterstain formulated from nuclear Fast Red. The substrate system provides a blue specific stain with red counterstain for immunohistochemical staining or immunoblotting.

KIT COMPONENTS

Component	Catalog No.	Volume
PhThaloBLUE Solution	71-00-03	10 mL
Activator Solution	71-00-01	10 mL
Buffered Substrate Solution	71-00-04	50 mL
Contrast RED Solution	71-00-05	50 mL

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

STORAGE/STABILITY

- Reagents are stable for a minimum of one year stored at 2 - 8°C.
- Store Contrast RED Solution tightly capped at room temperature.
- Discard PhThaloBLUE Solution if black precipitate develops.
- Discard Activator Solution or Buffered Substrate Solution if yellow color develops.
- Warm all reagents to room temperature (24 - 28°C) before use.
- If a light precipitate is visible in Buffered Substrate Solution, warm for 10 – 15 minutes in 37°C waterbath. Mix thoroughly by inversion until completely in solution.

REAGENTS NOT INCLUDED

1. Primary antibody.
2. AP-labeled reagents: KPL HistoMark Biotin Streptavidin Systems provide biotin-labeled secondary antibody, AP-labeled Streptavidin, and Serum Block:

HistoMark Biotin Streptavidin Kit for use with	Cat. No.
Mouse Primary Antibody	71-00-39
Rabbit Primary Antibody	71-00-40
Rat Primary Antibody	71-00-41
Goat Primary Antibody	71-00-44

3. Isopropyl alcohol.
4. Aqueous or xylene-based mounting media.
5. 0.1 M Tris-HCl (see **BUFFER PREPARATION**)
6. 1 M Citric Acid Free Acid (see **BUFFER PREPARATION**)

PREPARATION

- 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°C 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 to stand 3 minutes.
  c. After 3 minutes combine solutions from steps a. and b.. Mix thoroughly and use immediately.
- Contrast RED Solution is supplied at use dilution.

PROCEDURE

1. Rehydrate paraffin embedded sections through graded alcohol (3 minutes each in 100%, 80%, 40% and 20% EtOH) to water. Other samples do not require rehydration. Frozen sections must be thoroughly dried before use.
2. HistoMark BLUE reagents contain levamisole to block endogenous phosphatase activity. If additional blocking is required, apply Bouin's Solution or 1M citric acid 1 - 10 minutes.
3. Rinse slide 5 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 for 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 RED Solution 5 - 10 minutes.
15. Rinse thoroughly in reagent quality water until excess stain is removed from slide.
16. Air dry and mount in aqueous mounting medium. DO NOT USE XYLENE BASED MOUNTING MEDIA.

RESULTS

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

NOTES

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.

BUFFER PREPARATION

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 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 Reagents Section, Step 5, 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.

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 diazotized 4'-amino-2-,5-diethoxybenzanilide (PhThaloBLUE Solution) results in the formation of a brilliant blue 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).

REFERENCES

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