HistoMark® RED

For Localization of
Alkaline Phosphatase-Labeled Reagents

Catalog No.	Size
5510-0036 (55-69-00)	500 mL

DESCRIPTION

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

KIT COMPONENTS

KPL PhThaloRED Solution	5510-0038 (71-00-02)
KPL Activator Solution	5570-0002 (71-00-01)
KPL Buffered Substrate Solution	5570-0003 (71-00-04)
KPL Contrast BLUE Solution	5540-0002 (71-00-06)

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 KPL Contrast BLUE Solution tightly capped at room temperature.
- Discard KPL PhThaloRED Solution if solution turns red.
- Discard KPL Activator Solution or KPL 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 KPL 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
3. Isopropyl alcohol.
4. 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 KPL 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 KPL Buffered Substrate Solution to 5 mL reagent quality water.

  b. Mix 0.1 mL KPL PhThaloRED Solution with 0.1 mL KPL 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.

- KPL Contrast BLUE Solution: 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. KPL HistoMark 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 slide 5 minutes in reagent quality water.
4. Soak in 0.1 M Tris-HCl 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 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.

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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 KPL 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.

RESULTS

- 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.

NOTES

1. Always incorporate appropriate positive and negative controls.
2. Use substrate reagents immediately after mixing.
3. Instant development of red 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 HCl (approximately 300 mL).
c. QS to 1 L with reagent quality water to obtain a 1 M stock.
d. Dilute 1 part stock 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 [1, 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 (KPL Buffered Substrate Solution) is employed as substrate. Enzymatic hydrolysis, in the presence of hexazotized triaminotrimethyltriphenylmethane (KPL 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].

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REFERENCES

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The product listed herein is for research use only and is not intended for use in human or clinical diagnosis.