HistoMark® Double Staining Procedures

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HistoMark® Double Staining Procedures

Researchers often need the ability to visualize multiple proteins in one tissue sample. KPL's HistoMark® products make this possible. With contrasting stains and counterstains, different targets are easily visible. The following double staining procedures outline some of the ways in which KPL's HistoMark products may be used for this purpose.

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Methods for Reducing Background Staining

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1. Dilute the primary antibody further, or reduce the antibody incubation time.

The following problems are often caused by the use of excess antibody:
- excessively dark staining,
- staining occurs throughout the section rather than in expected areas of antigen localization,
- staining appears to fade, float, or leach off the section during or after substrate reaction,
- color develops immediately after addition of substrate,
- particles of dye are scattered across the section after staining.

2. Reduce substrate staining time. In some instances prolonged staining also inhibits nuclear counterstaining.

3. Some samples contain endogenous peroxidase or phosphatase enzyme. Treatments to reduce endogenous enzyme activity include methanol/H_2O_2 to block endogenous peroxidase, and Bouin's Solution to block endogenous phosphatase (see Solutions and Calculations section).

4. Raise the pH, or increase the ionic strength of diluent buffers. Sodium Chloride (0.1 - 0.5M) added to the buffer diluent reduces nonspecific binding on endothelia and collagen fibers.

5. Apply blocking protein prior to the application of primary antibody. The blocking protein should be of the same species used in the link or enzyme labeled antibody diluent, and should not be used in the primary antibody diluent.

6. The addition of ethylene glycol, Tween 20 or other detergent to the antibody diluent and washes will help remove unbound antibodies and artifacts which contribute to non-specific binding, as well as remove excess dye from the section.

7. Try an alternative enzyme system, such as alkaline phosphatase, horseradish peroxidase, or β-galactosidase.

8. Factors related to tissue preparation and fixation:

a. Inadequate penetration of fixative can produce nonspecific staining.
b. Over fixation can destroy antigens contained within the tissue.
c. Tissue which has dried out can exhibit artifact and nonspecific staining.
d. Incomplete removal of paraffin from tissue sections will result in artifacts.
e. Poor dehydration causes smudging of the stain.
f. Thick sections may prevent adequate penetration of primary antibody, producing false reactions or uneven staining over the tissue surface.

Solutions and Calculations

1. Buffers and Solutions

A. Tris Buffer Concentrate (1 M)

1. Dissolve 121 g of Tris Base in 500 ml of reagent quality water (deionized, distilled water or equivalent).
2. Adjust pH to 7.6 with approximately 200 to 300 mL of 2 M HCl.
3. Q.S. to 1 liter with reagent quality water.
4. Dilute 1:10 with reagent quality water to obtain a 100 mM working solution.

B. Peroxidase Blocking Solutions

1. **Methanolic H_2O_2** (1 mL 3% H_2O_2 + 4 mL absolute methanol)
   Treat sample for twenty minutes*. Not recommended if cell surface markers are to be stained; methanolic treatment may also cause frozen sections to detach from slide.
2. **Periodic Acid Solution**
   1. Dissolve 0.28 grams of Periodic Acid in 50 mL of reagent quality water or equivalent.
   2. Add sufficient distilled water to obtain a final volume of 100 mL.
3. **Sodium Azide and H_2O_2**[1]
4. **Periodic Acid and Sodium Borohydride**[2,3]

C. Alkaline Phosphatase Blocking Solutions

1. **1 M Citric Acid (free acid)**
   1. Dissolve 192 grams of Citric Acid (free acid) in 500 mL of reagent quality water or equivalent.
   2. Q.S. to 1 liter with reagent quality water.
2. **Bouin's Solution**
   1. Mix 75 mL of picric acid (saturated aqueous solution), 25 mL formalin (37-40%), and 5 mL glacial acetic acid.
3. **Levamisole**

2. Antibody Dilution Calculation

$$\frac{\text{Starting Antibody Conc. x Volume of Antibody Required}}{\text{Total Volume*}} = \text{Desired Concentration}$$

**Total Volume = volume of antibody required + volume of antibody diluent*

Example:

$$\frac{100\ \mu g/mL \times y\ mL}{2\ mL} = 5 mg/mL$$

100 µg/mL = concentration listed by manufacturer (0.1 mg/ml)
y mL = milliliters of antibody required
2 mL = total volume of diluted antibody
5 µg/mL = desired concentration of antibody

References

1. Li C-Y et al. *J Histochem* 35:1456-1460, 1987.
2. Isobe Y., Chen S.T., Nakane P., Brown W.R. Studies on translocation of immunoglobulins across intestinal epithelium. I. Improvements in the peroxidase-labeled antibody method for application to study of human intestinal mucosa. *Acta Histochem Cytochem* 1977; 10:161-171.
3. Brown W.R., Isobe Y., Nakane P.K. Studies on translocation of immunoglobulins across intestinal epithelium. II. Immunoelectron microscopic localization of immunoglobulins and secretory component in human intestinal mucosa. *Gastroenterology*. 1976; 985-995.

Double Alkaline Phosphatase Labeling

Using KPL's HistoMark RED and HistoMark BLUE Substrate Systems

Direct Assay

1. Hydrate fixed sample slides with 0.1M Tris Buffer pH 7.6.*
2. Shake off excess buffer and treat sample with first alkaline phosphatase labeled antibody for 10 - 20 minutes.
3. Wash off antibody solution with Tris Buffer and then soak sample in Tris Buffer for 5 - 10 minutes.
4. Shake off excess buffer and react with alkaline phosphatase substrate staining system. (HistoMark RED, Cat. No. 55-69-00)
5. Wash in reagent quality water for 10 - 15 minutes.
6. Treat with 1M Citric Acid for 15 minutes or Bouins Solution for 1 - 2 minutes to eliminate any residual phosphatase activity*.
7. Wash in reagent quality water to remove excess Citric Acid or Bouins Solution for 1 - 2 minutes.
8. Soak in Tris Buffer for approximately 10 minutes.
9. Shake off excess buffer and treat sample with second phosphatase-labeled antibody for 10 - 20 minutes.
10. Wash in Tris Buffer as in step 3.
11. Shake off excess buffer and react with appropriate contrasting alkaline phosphatase substrate staining system. (HistoMark BLUE, Cat. No. 55-70-00)
12. Wash in reagent quality water for 10 - 15 minutes.
13. Air dry and mount using a water-based mounting medium.

DO NOT USE XYLENE BASED MOUNTING MEDIA!!

*See Page 2, Solutions and Calculations

Notes

Double Alkaline Phosphatase Labeling

Using KPL's HistoMark RED and HistoMark BLUE Substrate Systems

Indirect Assay

1. Hydrate fixed sample slides with 0.1M Tris Buffer pH 7.6*.
2. Shake off excess buffer and treat sample with the first primary antibody.
3. Wash off excess antibody solution with Tris Buffer and then soak sample in Tris Buffer for 5 - 10 minutes.
4. Shake off excess buffer and treat sample with alkaline phosphatase labeled antibody directed against the first primary antibody for 10 - 20 minutes.
5. Wash in Tris Buffer as in step 3.
6. Shake off excess buffer and react with alkaline phosphatase substrate staining system. (HistoMark RED, Cat. No. 55-69-00)
7. Wash in reagent quality water for 10 - 15 minutes.
8. Treat with 1M Citric Acid for 15 minutes or Bouins Solution for 1 - 2 minutes to eliminate any residual phosphatase activity*.
9. Wash in reagent water to remove excess Citric Acid or Bouins Solution for 1 - 2 minutes.
10. Soak in Tris Buffer for approximately 10 minutes.
11. Shake off excess buffer and treat sample with second primary antibody.
12. Wash in Tris Buffer as in step 3.
13. Shake off excess buffer and treat sample with alkaline phosphatase labeled antibody directed against the second primary antibody for 10 - 20 minutes.
14. Wash in Tris Buffer as in step 3.
15. Shake off excess buffer and react with appropriate contrasting alkaline phosphatase substrate staining system (HistoMark BLUE, Cat. No. 55-70-00)
16. Wash in reagent quality water for 10 - 15 minutes.
17. Air dry and mount using a water-based mounting medium.

DO NOT USE XYLENE BASED MOUNTING MEDIA!!

Notes

Double Peroxidase (HRP) Labeling

Using KPL's HistoMark ORANGE and HistoMark BLACK Substrate Systems

Direct Assay

1. Rehydrate fixed sample slides with 0.1M Tris Buffer pH 7.6*.

2. Block for endogenous peroxidase if desired for 10 - 20 minutes.

3. Wash thoroughly in reagent quality water.

4. Soak in Tris Buffer for 5 - 10 minutes.

5. Shake off excess buffer and treat sample with Peroxidase (HRP) labeled antibody for 10 - 20 minutes.

6. Wash off antibody solution with Tris Buffer and soak sample in Tris Buffer for 5 - 10 minutes.

7. Shake off excess buffer and react with peroxidase substrate staining system. (HistoMark ORANGE, Cat. No. 54-74-00)

8. Wash in reagent quality water for 10 - 15 minutes.

9. Block to eliminate any residual peroxidase activity for 10 - 20 minutes.

10. Wash in reagent quality water 1 - 2 minutes.

11. Soak in Tris Buffer for approximately 10 minutes.

12. Shake off excess buffer and treat sample with second Peroxidase (HRP)-labeled antibody for 10 - 20 minutes.

13. Wash in Buffer as in step 6.

14. Shake off excess buffer and react with contrasting peroxidase substrate staining system. (HistoMark BLACK, Cat. No. 54-75-00).

15. Wash in reagent quality water for 10 - 15 minutes.

16. Counter stain if desired and mount appropriately.

*SeePage 2, Solutions and Calculations

Notes

Double Peroxidase (HRP) Labeling

Using KPL's HistoMark ORANGE and HistoMark BLACK Substrate Systems

Indirect Assay

1. Hydrate fixed sample slides with 0.1M Tris Buffer pH 7.6*.

2. Block for endogenous peroxidase if desired*.

3. Wash thoroughly in reagent quality water.

4. Soak in Tris Buffer for 5 - 10 minutes.

5. Shake off excess buffer and treat sample with primary antibody.

6. Wash off excess antibody solution with Tris Buffer and soak sample in Tris Buffer for 5 - 10 minutes.

7. Shake off excess buffer and treat sample with Peroxidase (HRP) labeled antibody directed against the primary antibody for 10 - 20 minutes.

8. Wash in Tris Buffer as in step 6.

9. Shake off excess buffer and react with peroxidase substrate staining system. (HistoMark ORANGE, Cat. No. 54-74-00)

10. Wash in reagent quality water for 10 - 15 minutes.

11. Block to eliminate any residual peroxidase activity*.

12. Wash in reagent quality water 1 to 2 minutes.

13. Soak in Tris Buffer for approximately 10 minutes.

14. Shake off excess buffer and treat sample with second primary antibody.

15. Wash as in step 6.

16. Shake off excess buffer and treat sample with Peroxidase (HRP) labeled antibody directed against the second primary antibody for 10 - 20 minutes.

17. Wash as in step 6.

18. Shake off excess buffer and react with contrasting peroxidase substrate staining system. (HistoMark BLACK, Cat. No. 54-75-00).

19. Wash in reagent quality water for 10 - 15 minutes.

20. Counterstain if desired and mount appropriately.

*See page 2, Solutions and Calculations

Notes

Double Peroxidase (HRP) Labeling

using KPL's DAB and TrueBlue® Peroxidase Substrate Systems

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1. Rehydrate fixed sample slides.
2. Block for endogenous peroxidase as necessary (see page 1, Methods for Reducing Background Staining).
3. Wash in distilled water for 5 - 10 minutes.
4. Soak in PBS or 0.1 M Tris-HCl (pH 7.6) for 5 - 10 minutes.
5. Shake off excess buffer and treat sample with primary antibody diluted in Tris-HCl for 15 - 20 minutes.
6. Wash off excess antibody solution with Tris-HCl; soak sample in Tris-HCl for two washes of 5 - 10 minutes each.
7. Shake off excess buffer and treat sample with Peroxidase (HRP) labeled secondary antibody (directed against host species of the primary antibody), diluted to approximately 2 µg/mL in PBS or Tris-HCl, for 15 - 20 minutes.
8. Wash sample as in step 6.
9. Shake off excess buffer and react with DAB (Catalog # 54-11-00 or 54-10-00) for 10 minutes.
10. Wash in distilled water for 10 - 15 minutes.
11. Soak in PBS or Tris-HCl for about 10 minutes.
12. Shake off excess buffer and treat sample with second primary antibody diluted in Tris-HCl for 10 - 15 minutes.
13. Wash as in step 6.
14. Shake off excess buffer and treat sample with HRP labeled antibody (directed against the second primary antibody), diluted to approximately 2 µg/mL in PBS or Tris-HCl, for 15 - 20 minutes.
15. Wash as in step 6.
16. Shake off excess buffer and react with TrueBlue Peroxidase Substrate (Catalog # 71-00-64) for 10 minutes.
17. Wash in distilled water for one minute.
18. Counterstain with Contrast RED (Catalog # 71-00-05) if desired for 1 - 3 minutes.
19. Dehydrate through alcohols (3 minutes each in 20%, 40%, 80%, 100% and 100% EtOH).
20. **Air dry** and mount slides using organic mounting media; KPL recommends Eukitt's Mounting Reagent or Fisher Permount®. **Fading of the substrate reaction may occur if aqueous mounting media are used.**

Double Labeling: Peroxidase(HRP)/Phosphatase(AP)
Using KPL's HistoMark® BLACK and HistoMark RED Substrate Systems

Direct Assay

1. Rehydrate fixed sample slides to 0.1M Tris Buffer pH 7.6*.
2. Block for endogenous peroxidase if desired*.
3. Wash thoroughly in reagent quality water.
4. Soak in Tris Buffer for 5 - 10 minutes.
5. Shake off excess buffer and treat sample with Peroxidase (HRP) labeled primary antibody.
6. Wash off antibody solution with Tris Buffer and soak sample in Tris Buffer 5 - 10 minutes.
7. Shake off excess buffer and react with peroxidase substrate staining system. (HistoMark Black, Cat. No. 54-75-00)
8. Wash in reagent quality water 10 - 15 minutes.
9. Soak in Tris Buffer for approximately 10 minutes.
10. Shake off excess buffer and treat sample with Alkaline Phosphatase (AP) labeled primary antibody.
11. Wash in Tris Buffer as in step 6.
12. Shake off excess buffer and react with contrasting alkaline phosphatase substrate staining system. (HistoMark RED, Cat. No. 55-69-00)
13. Wash in reagent quality water for 10 - 15 minutes.
14. Counterstain if desired and mount appropriately.

* See page 2, Solutions and Calculations

Notes

Double Labeling: Peroxidase (HRP)/Phosphatase (AP)
Using KPL's HistoMark BLACK and RED Substrate Systems

Indirect Assay

1. Rehydrate fixed sample slides with 0.1M Tris Buffer pH 7.6*.
2. Block for endogenous peroxidase if desired for 10 - 20 minutes.
3. Wash thoroughly in reagent quality water.
4. Soak in Tris Buffer for 5 - 10 minutes.
5. Shake off excess buffer and treat sample with primary antibody.
6. Wash off excess antibody solution with Tris Buffer and soak sample in Tris Buffer for 5 - 10 minutes.
7. Shake off excess buffer and treat sample with Peroxidase (HRP) labeled antibody directed against the primary antibody for 10 - 20 minutes.
8. Wash in Tris Buffer as in step 6.
9. Shake off excess buffer and react with peroxidase substrate staining system. (HistoMark BLACK, Cat. No. 54-75-00).
10. Wash in reagent quality water 10 - 15 minutes.
11. Soak in Tris Buffer for approximately 10 minutes.
12. Shake off excess buffer and treat sample with second primary antibody.
13. Wash in Tris Buffer as in step 6.
14. Shake off excess buffer and treat sample with Alkaline Phosphatase (AP) labeled antibody directed against the second primary antibody.
15. Wash in Tris Buffer as in step 6.
16. Shake off excess buffer and react with contrasting alkaline phosphatase substrate staining system. (HistoMark RED, Cat. No. 55-69-00)
17. Wash in reagent quality water for 10 - 15 minutes.
18. Counterstain if desired and mount appropriately.

* See page 2 Solutions and Calculations

Notes

Double Labeling: Peroxidase (HRP)/Phosphatase (AP)
using KPL's TrueBlue and HistoMark RED Substrate Systems

Notes

1. Rehydrate fixed sample slides.
2. Block for endogenous phosphatase as necessary (see Methods for Reducing Background Staining).
3. Wash in distilled water for 5-10 minutes.
4. Soak in PBS or 0.1 M Tris-HCl (pH 7.6) for 5 - 10 minutes.
5. Shake off excess buffer and treat sample with primary antibody diluted in PBS or Tris-HCl for 15 - 20 minutes.
6. Wash off excess antibody solution with Tris-HCl; soak sample in Tris-HCl for two washes of 5 -10 minutes each.
7. Shake off excess buffer and treat sample with Phosphatase (AP) labeled secondary antibody (directed against host species of the primary antibody), diluted to approximately 2 µg/mL in PBS or Tris-HCl, for 15 - 20 minutes.
8. Wash sample as in step 6.
9. Shake off excess buffer and react with HistoMark RED Phosphatase Substrate System (Catalog # 55-69-00) for 10 minutes.
10. Wash in distilled water for 10 - 15 minutes.
11. Soak in PBS or Tris-HCl for approximately 10 minutes.
12. Shake off excess buffer and treat sample with second primary antibody diluted inTris-HCL for 10 - 15 minutes.
13. Wash as in step 6.
14. Shake off excess buffer and treat sample with HRP labeled antibody (directed against the second primary antibody), diluted to approximately 2 µg/mL in PBS or Tris-HCl, for 15 - 20 minutes.
15. Wash as in step 6.
16. Shake off excess buffer and react with TrueBlue Peroxidase Substrate (Catalog # 71-00-64) for 10 minutes.
17. Wash in distilled water for one minute.
18. Dehydrate through alcohols (3 minutes each in 20%, 40%, 80%, 100% and 100% EtOH).
19. Air dry and mount slides using organic mounting media; KPL recommends Eukitt's Mounting Reagent or Fisher Permount®. Fading of the substrate reaction may occur if aqueous mounting media are used.

Double Labeling: Peroxidase(HRP)/β-Galactosidase(β-Gal)
Using KPL's HistoMark BLACK and HistoMark X-Gal Substrate Systems

Direct Assay

1. Rehydrate fixed sample slides with 0.1 M Tris-HCl pH 7.6*.

2. Block for endogenous peroxidase if desired for 10 - 20 minutes.

3. Wash thoroughly in reagent quality water.

4. Soak in Tris Buffer for 5 - 10 minutes.

5. Shake off excess buffer and treat sample with Peroxidase (HRP) labeled primary antibody for 10 - 20 minutes.

6. Wash off antibody solution with Tris Buffer and soak sample in Tris Buffer for 5 - 10 minutes.

7. Shake off excess buffer and react with peroxidase substrate staining system. (HistoMark BLACK, Cat. No. 54-75-00)

8. Wash in reagent quality water for 10 - 15 minutes.

9. Soak in Tris Buffer for approximately 10 minutes.

10. Shake off excess buffer and treat sample with β-Galactosidase (β-Gal) labeled primary antibody.

11. Wash in Tris Buffer as in step 6.

12. Shake off excess buffer and react with contrasting β-Galactosidase substrate staining system. (HistoMark X-Gal Substrate Set, Cat. No. 54-13-00)

13. Wash in reagent quality water for 10 - 15 minutes.

14. Counterstain with either Contrast RED (Cat. # 71-00-05) or Contrast GREEN (Cat. # 71-00-11) if desired and mount appropriately.

* See page 2 Solutions and Calculations

Notes

Double Labeling: Peroxidase(HRP)/ β-Galactosidase(β-Gal)

Using KPL's HistoMark BLACK and HistoMark X-Gal Substrate Systems

Indirect Assay

1. Rehydrate fixed sample slides in 0.1 M Tris Buffer pH 7.6*.
2. Block for endogenous peroxidase if desired for 10 - 20 minutes.
3. Wash thoroughly in reagent quality water.
4. Soak in Tris Buffer for 5 - 10 minutes.
5. Shake off excess buffer and treat sample with primary antibody.
6. Wash off excess antibody solution with Tris Buffer and soak sample in Tris Buffer for 5 - 10 minutes.
7. Shake off excess buffer and treat sample with Peroxidase (HRP) labeled antibody directed against the primary antibody for 10 - 20 minutes.
8. Wash in Tris Buffer as in step 6.
9. Shake off excess buffer and react with peroxidase substrate staining system. (HistoMark BLACK, Cat. No. 54-75-00)
10. Wash in reagent quality water for 10 - 15 minutes.
11. Soak in Tris Buffer for approximately 10 minutes.
12. Shake off excess buffer and treat sample with second primary antibody.
13. Wash in Tris Buffer as in step 6.
14. Shake off excess buffer and treat sample with β-Galactosidase (β-Gal) labeled antibody directed against the second primary antibody.
15. Wash in Tris Buffer as in step 6.
16. Shake off excess buffer and react with contrasting β–Galactosidase substrate staining system. (HistoMark X-Gal Substrate Set, Cat. No. 54-13-00).
17. Wash in reagent quality water for 10 - 15 minutes.
18. Counterstain if desired and mount appropriately.

* See page 2, Solutions and Calculations

Notes

Double Labeling:Phosphatase(AP)/β-Galactosidase(β-Gal)
Using KPL's HistoMark RED and HistoMark X-Gal Substrate Systems

Direct Assay

1. Rehydrate fixed sample slides in Tris Buffer pH 7.6*.
2. Treat for endogenous phosphatase if necessary for 10 - 20 minutes.
3. Shake off excess buffer and treat sample with alkaline phosphatase labeled antibody for 10 - 20 minutes.
4. Wash off antibody solution with Tris Buffer and soak sample in Tris Buffer for 5 - 10 minutes.
5. Shake off excess buffer and react with alkaline phosphatase substrate staining system. (HistoMark RED, Cat. No. 55-69-00)
6. Wash in reagent quality water for 10 - 15 minutes.
7. Soak in Tris Buffer for approximately 10 minutes.
8. Shake off excess buffer and treat sample with β–Galactosidase (β–Gal) labeled antibody.
9. Wash in Tris Buffer as in step 3.
10. Shake off excess buffer and react with β-Galactosidase substrate staining system. (HistoMark X-Gal Substrate Set, Cat. No. 54-13-00)
11. Wash in reagent quality water for 10 - 15 minutes.
12. Counterstain if desired and mount appropriately.

* See page 2, Solutions and Calculations

Notes

Double Labeling:Phosphatase(AP)/β-Galactosidase(β-Gal)

Using KPL's HistoMark RED and HistoMark X-Gal Substrate Systems

Indirect Assay

1. Hydrate fixed sample slides in 0.1 M Tris Buffer pH 7.6*.
2. Treat for endogenous phosphatase if necessary* for 10 - 20 minutes.
3. Shake off excess buffer and treat sample with primary antibody.
4. Wash off excess antibody solution with Tris Buffer and soak sample in Tris Buffer for 5 - 10 minutes.
5. Shake off excess buffer and treat sample with alkaline-phosphatase labeled antibody directed against the primary antibody for 10 - 20 minutes.
6. Wash in Tris Buffer as in step 3.
7. Shake off excess buffer and react with alkaline phosphatase substrate staining system. (HistoMark RED, Cat. No. 55-69-00).
8. Wash in reagent quality water for 10 - 15 minutes.
9. Soak in Tris Buffer for approximately 10 minutes.
10. Shake off excess buffer and treat sample with second primary antibody.
11. Wash in Tris Buffer as in step 3.
12. Shake off excess buffer and treat sample with β-Galactosidase (β–Gal) labeled antibody directed against the second primary antibody.
13. Wash in Tris Buffer as in step 3.
14. Shake off excess buffer and react with β-Galactosidase substrate staining system. (HistoMark X-Gal Substrate Set, Cat. No. 54-13-00)
15. Wash in reagent quality water for 10 - 15 minutes.
16. Counterstain if desired and mount appropriately.

* See page 2, Solutions and Calculations

Notes

STREPTAVIDIN β-GALACTOSIDASE LABELING

Using KPL's β-Gal Universal Kit and HistoMark X-Gal Substrate System

Rehydration of Slides

1. Rehydrate fixed sample slides in Tris buffer pH 7.6*.

Apply Serum Block

2. Shake off buffer and wipe off excess surrounding section.
3. Completely cover section with normal goat serum.
4. Incubate 15 minutes at room temperature in a humidity chamber.

Apply Primary Antibody

5. Shake off serum and wipe off excess surrounding section.
6. Completely cover section with primary antibody.
7. Incubate for 15 minutes at room temperature in a humidity chamber.
8. Rinse off primary antibody with wash buffer and soak in same buffer for five minutes.

Apply Biotinylated Link Antibody

9. Shake off buffer and wipe off excess surrounding section.
10. Completely cover section with biotinylated link antibody.
11. Incubate for 15 minutes at room temperature in a humidity chamber.
12. Rinse off biotinylated link antibody with wash buffer and soak in same buffer for 5 minutes.

Apply Streptavidin-β-Galactosidase

13. Shake off buffer and wipe off excess surrounding sections.
14. Completely cover section with streptavidin-β–galactosidase.
15. Incubate for 15 minutes at room temperature in a humidity chamber.
16. Rinse off streptavidin-β–galactosidase with wash buffer and soak in same buffer for 5 minutes.

Apply X-GAL Substrate

17. Shake off buffer and wipe off excess surrounding sections.
18. Completely cover tissues with X-GAL substrate. (50 µl X-GAL substrate solution in 2 ml iron buffer)
19. Incubate for 15 - 30 minutes at room temperature.
20. Rinse slides thoroughly in reagent quality water.
21. If desired, tissues may be counterstained for 2 - 4 minutes in Contrast Red.
22. Dehydrate by rinsing briefly (10 dips) in 80% alcohol, 100% alcohol and xylene or xylene substitutes.
23. Mount in xylene-based mounting media, or use aqueous mounting media if tissues are thoroughly air dried.

*See Page 2, Solutions and Calculations

Streptavidin Peroxidase Labeling Procedure

Using KPL's TrueBlue® Peroxidase Substrate System

1. Rehydrate fixed sample slides in water.

2. Block 4 minutes for endogenous peroxidase.

3. Wash 5 minutes in reagent quality water.

4. Soak in 0.1 M Tris-HCl Buffer (pH 7.6) for 10 minutes.

5. Block for 10 minutes at room temperature in Normal Goat Serum.

6. Shake off excess buffer and treat sample with diluted primary antibody for 15-20 minutes. **TrueBlue Peroxidase Substrate is 10 to 50 times more sensitive than most DAB preparations**; t**he primary antibody must be diluted in PBS or Tris-HCl to a concentration 10 to 50 times lower than the normal working concentration with DAB to avoid overstaining.**

7. Wash off excess antibody solution with Tris Buffer for 10 minutes.

8. Treat sample with biotin-labeled antibody directed against the primary antibody for 15 - 20 minutes.

9. Wash as in Step 7.

10. Shake off excess buffer and treat sample with Peroxidase (HRP) labeled Streptavidin for 15 - 20 minutes.

11. Wash as in step 7.

12. Shake off excess buffer and react with TrueBlue Peroxidase Substrate for 10 minutes.

13. Wash 1 - 5 minutes in distilled water.

14. Counterstain 1 - 3 minutes with Contrast RED if desired.

15. Wash 5 minutes in distilled water.

16. Dehydrate through alcohols (3 minutes each in 20%, 40%, 80%, 100% and 100% EtOH).

17. **Air dry** and mount slides using organic mounting media; **fading of the substrate reaction may occur if aqueous mounting media is used.**

Notes

Double Peroxidase (HRP) labeling of Trypsin-sensitive Antigens Using KPL's StableDAB™ and TrueBlue® Peroxidase Substrate Systems

Notes

1. Deparaffinize and rehydrate fixed tissue sections through a graded alcohol series.
2. Block endogenous peroxidase activity for 20 - 30 minutes. (See page 1,Methods for Reducing Background Staining.)
3. Rinse in distilled water for 5 - 10 minutes.
4. Soak in PBS, TBS or 0.1 M Tris-HCl (pH 7.6) for 5 - 10 minutes.
5. Block nonspecific protein binding sites by treating the section with normal goat serum (NGS) or bovine serum albumin (BSA) diluted in wash buffer for 10 minutes.
6. Shake off excess blocking reagent (do not wash section) and treat with primary antibody directed against the trypsin-sensitive antigen (dilute in NGS or BSA).
7. Rinse off excess antibody solution and wash the section in two changes of wash buffer for 5 minutes each.
8. Treat the sample with biotin-labeled link antibody directed against the primary antibody host species for 15 - 20 minutes. If using HRP-labeled secondary antibody, proceed to Step 10.
9. Wash as in Step 7.
10. Treat the sample with HRP-streptavidin or HRP-labeled secondary antibody diluted in NGS or BSA for 15 - 20 minutes.
11. Wash as in Step 7.
12. Cover section with 1X StableDAB and incubate for 10 minutes at room temperature.
13. Rinse off excess substrate with distilled water.
14. Soak section in distilled water for 5 - 10 minutes.
15. Block residual peroxidase activity for 20 to 30 minutes. (See page 1, Methods for Reducing Background Staining.)
16. Using the second primary antibody, repeat Steps 3 to 10.
17. Cover section with TrueBlue and incubate for 10 minutes at room temperature.
18. Rinse section thoroughly in distilled water.
19. Dehydrate through a graded alcohol series to xylene; or air dry, rinse in xylene and mount with a xylene-based mounting medium.

Immunohistochemical Staining of Thick Sections

(Floating Sections 10 to 100 Microns)

Depending upon the thickness of the tissue and tissue type, sections can be kept up to 1 year if refrigerated and stored in buffer.

1. Remove sections from buffer and block using an appropriate enzyme block (either phosphatase or peroxidase). Blocking for both enzymes allows greater flexibility in future steps. Blocking should be carried out for 1 hour at room temperature.

2. Remove sections from blocking solution and soak in distilled water: first rinse - 10 minutes; second rinse - 20 to 30 minutes.

3. Return sections to appropriate buffer for 10 to 30 minutes.

4. Depending on the species of origin of the secondary antibody to be used, blocking the section with normal goat serum (NGS) or normal rabbit (NRS) is now recommended. Add 1 to 2 drops of a 1%(v/v) detergent solution (NP40, Igepal or detergent of choice) to the NGS or NRS. Blocking sections 30 minutes to 1 hour at room temperature or overnight at 4°C is recommended.

5. Dilute primary antibody in buffer or dilute buffer and serum solution to the desired concentration. Add 1 to 2 drops of detergent as above. At this point, incubation overnight at 4° C is recommended, In some cases, this incubation can be extended from 1 to 5 days.

6. Wash sections in appropriate buffer: first rinse- 10 minutes, second rinse- 20 to 30 minutes. Additional buffer washes can be incorporated if needed.

7. For thick sections biotin/streptavidin works very well. Indirect procedures using only a labeled secondary antibody can be used. Once again it is suggested that 1 or 2 drops of detergent be used in incubations with the labeled antibody to facilitate penetration into the section. Reaction times will vary from 1 to 4 hours at room temperature, or from 8 hours to 2 days at 4°C.

Notes

Ordering Information

HistoMark Immunohistochemical Staining Systems

For use with peroxidase

Each kit contains Substrate, Enzyme Block and Counterstain.

Catalog No.	Description	Size
54-75-00	HistoMark BLACK	500 mL
54-74-00	HistoMark ORANGE	500 mL
54-78-00	HistoMark TrueBlue	500 slides

For use with phosphatase

Each kit contains Substrate and Counterstain.

55-69-00	HistoMark RED	1000 slides
55-70-00	HistoMark BLUE	1000

For use with β-galactosidase

54-13-00	HistoMark X-Gal Substrate Set	200 slides

HistoMark Universal Streptavidin Kits

Each kit contains serum block, biotinylated secondary antibody, and labeled streptavidin.

HistoMark Streptavidin Peroxidase Kits for Use with

71-00-18	Mouse Primary Antibody	500 slides
71-00-19	Rabbit Primary Antibody	500
71-00-20	Rat Primary Antibody	500
71-00-26	Goat Primary Antibody	500

HistoMark Streptavidin Phosphatase Kits for Use with

71-00-39	Mouse Primary Antibody	500 slides
71-00-40	Rabbit Primary Antibody	500
71-00-41	Rat Primary Antibody	500

Peroxidase Substrate - Substrate Only

50-78-02	TrueBlue Peroxidase Substrate	200 mL
71-00-64	TrueBlue Peroxidase Substrate	50 mL
71-00-67	TrueBlue Peroxidase Substrate	10 mL
54-11-00	StableDAB®	500 slides
54-11-02	StableDAB	25 slides
54-10-00	DAB Reagent Set	500 slides

Counterstains

Catalog No.	Description	Size
71-01-01	Orcein	50 mL
71-02-01	Eosin	10 mL
71-00-11	Contrast GREEN	50 mL
71-00-06	Contrast BLUE	50 mL
71-00-05	Contrast RED	50 mL

Blocking Solutions & Other Products

71-00-10	Blocking Solution Concentrate	10 mL
71-00-61	Universal Block for Immunohistochemistry	100 mL
71-00-16	Fluorescent Mounting Media	15 mL

Components Available Separately

71-00-29	Goat Anti-Mouse IgG (H+L)	50 mL
71-00-30	Goat Anti-Rabbit IgG (H+L), HSA	50 mL
71-00-31	Goat Anti-Rat IgG (H+L), MSA	50 mL
71-00-37	Rabbit Anti-Goat IgG (H+L)	50 mL
71-00-38	HRP-Streptavidin	50 mL
71-00-45	AP-Streptavidin	50 mL
71-00-27	10% Normal Goat Serum	50 mL
71-00-28	10% Normal Rabbit Serum	50 mL
71-18-01	10% Normal Mouse Serum	10 mL
71-00-01	Activator Solution	10 mL
71-00-02	PhthaloRED	10 mL
71-00-03	PhthaloBLUE	10 mL
71-00-04	Buffered Substrate Solution	50 mL
71-00-09	Peroxide Solution	10 mL

HSA = Human Serum Adsorbed
MSA = Mouse Serum Adsorbed

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To order or for Technical Support, please contact your KPL representative at 800.638.3167 or 301.948.7755. By fax at 301.948.0169.

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