KPL TrueBlue™ Substrate

Catalog No.	Size
5510-0052 (71-00-67)	10 mL
5510-0049 (71-00-64)	50 mL
5510-0051 (71-00-66)	1 Liter
5510-0030 (50-78-02)	200 mL

DESCRIPTION

KPL TrueBlue™ Peroxidase Substrate is a highly sensitive chromagenic substrate for visualization of horseradish peroxidase-labeled reporter reagents. It provides a brilliant blue specific stain which provides excellent contrast with red counterstains. KPL TrueBlue is a buffered solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and H_2O_2. KPL TrueBlue is free of known carcinogens. KPL TrueBlue is optimized for use with cellular preparations and can be used for Immunoblotting procedures, but not for microwell ELISA or other applications requiring a soluble reaction product.

CONTENTS

5510-0030 (50-78-02) contains 2 x 100 mL.
Sufficient reagent to stain approximately 500-1000 slides.

551-0049 (71-00-64) contains 1 x 50 mL.
Sufficient reagent to stain approximately 125 - 250 slides.

5510-0052 (71-00-67) contains 1 x 10 mL.
Sufficient reagent to stain approximately 25 - 50 slides.

SUGGESTED REAGENTS NOT INCLUDED

1. Primary antibody.
2. HRP-labeled reagents.
3. Isopropyl alcohol.
4. Serum Block: 10% normal serum from the species the secondary antibody was made in (See RELATED PRODUCTS).
5. Endogenous Peroxidase Block: KPL Blocking Solution Concentrate, KPL Universal Block (See RELATED PRODUCTS) or 0.3% H_2O_2 in 100% MeOH.
6. Counterstain: KPL recommends KPL Contrast RED, KPL Orcein or Eosin (See RELATED PRODUCTS).
7. Organic Mounting Media: KPL recommends Permount from Fisher Scientific.
8. 0.1M Tris-HCl or PBS (See SOLUTION PREPARATION).
9. DAB for double staining (See RELATED PRODUCTS).

FORM/STORAGE/STABILITY

KPL TrueBlue is a single component, ready to use, liquid substrate. No mixing is required. Store at room temperature (22 - 28°C). TrueBlue is stable for up to 30 months from the date of manufacture.
PLEASE NOTE: Optimal performance in plaque assays will occur within 1 year from date of manufacture.

KPL TrueBlue may appear clear to light blue. Product stability and performance are not affected by variations in solution color. Discard solution if it becomes turbid.

APPLICATIONS/USE

The application of antibodies and other proteins covalently coupled to horseradish peroxidase (HRP) in immunohistochemistry is well documented (5 - 9). In the presence of hydrogen peroxide (H_2O_2), HRP catalyzes the oxidation of TMB, forming a blue chromogenic product visible by light microscopy. Rye et. al. have shown that the reaction of TMB with HRP is a sensitive method for demonstration of retrograde tracing in neural tissue(10). Lu and Ho obtained excellent staining with TMB-molybdate in anterograde labeling of neural tissue at the ultrastructure level (11). When used in double labeling for localization of innervation of feline cerebral arteries, TMB and DAB peroxidase substrates provided high resolution morphological results (12). Previous methods to stabilize the blue reaction color required multiple staining steps, but KPL TrueBlue provides a permanent blue color when used as recommended. Like DAB, the KPL TrueBlue reaction product is insoluble in alcohols and Xylene.

SENSITIVITY

KPL studies show improved sensitivity when KPL TrueBlue is used as an alternative to DAB or AEC in peroxidase-based immunoassays (Table 1).

Due to this increase in sensitivity, protocols optimized for DAB must be adjusted when incorporating KPL TrueBlue by lowering antibody concentration. The use of excess antibody may cause overly rapid color development, which prevents proper attachment of the substrate and can result in either high background or fading.

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Table 1: Endpoint sensitivity comparison of KPL TrueBlue, DAB and AEC.
Model: Cytomegalovirus Antibody to Nuclear Antigen (MAB8135) with Cytomegalovirus-infected Fibroblasts.

Antibody Dilution	TrueBlue	DAB	AEC
1/25	++++	+++	++
1/250	++++	++	+
1/2,500	++++	+	-
1/25,000	+++	-	-
1/250,000	++	-	-
1/500,000	+	-	-
1/1,000,000	-	-	-
Endpoint	1/500,000	1/2,500	1/250

Key:
+ + + + Highly overstained
+ + + Strongly stained
+ + Moderately stained
+ Visibly stained
- Not visibly stained

PREPARATION/OPTIMIZATION

KPL TrueBlue is a ready to use, one component substrate, requiring no mixing or dilution. Do not dilute the substrate.

KPL recommends the following optimization protocol for initial evaluation of KPL TrueBlue compared to DAB:

1. Run one control with DAB, using standard antibody dilutions.
2. Run three test samples with KPL TrueBlue, using primary antibody dilutions of 1/10, 1/100 and 1/500 times the normal working concentration with DAB. Signal intensity equivalent to the DAB control should be seen with one of the KPL TrueBlue samples at a lower primary antibody concentration.

It may also be necessary to dilute the HRP-labeled antibody or HRP Streptavidin in order to obtain optimal results.

SINGLE STAINING 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.
2. To block endogenous peroxidase activity, immerse samples in 0.3% H_2O_2/100% MeOH 20 - 30 minutes or in the working solution of KPL Blocking Solution as follows:
   a. Frozen Sections 45 seconds
   b. Paraffin Sections 4 minutes
   c. Cytospin Preps 45 seconds
   d. Blood Films 45 seconds
   e. Touch/Squash Preps 1 minute
   f. Floating or Whole Sections 5 minutes

If not blocking for endogenous activity, proceed to Step 5.

3. Rinse five minutes in reagent quality water.
4. Soak in 0.1M Tris-HCl or PBS 10 minutes.
5. Block with Serum Block 10 minutes.
6. Treat sample with primary antibody diluted in Tris-HCl or PBS, 15 - 20 minutes.

NOTE: Dilute primary antibody to a concentration at least 10 - 50 times lower than the standard concentration for use with DAB.

7. Wash sample with Tris-HCl or PBS 10 minutes.
8. Incubate 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. Shake off excess buffer and incubate sample with HRP Streptavidin or HRP-labeled secondary antibody diluted in Tris-HCl or PBS, 15 - 20 minutes.
11. Wash as in Step 7.
12. Shake off excess buffer and react sample with KPL TrueBlue Peroxidase Substrate 10 minutes.

NOTE: Color development in less than 10 minutes indicates excess antibody or HRP Streptavidin; fading or background may result.

13. Wash sample in reagent quality water 1 - 5 minutes.

NOTE: Washing with PBS or other buffer will result in fading of the blue color.

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14. Air dry thoroughly.
15. Counterstain with KPL Orcein, KPL Contrast RED or Eosin (See RELATED PRODUCTS) 1 - 3 minutes if desired. Wash again with reagent quality water 5 minutes.
16. Dehydrate through graded alcohol (3 minutes each in 20%, 40%, 80% and 100% EtOH). NOTE: Floating sections or whole mounts may be fixed to slides by drying under low heat followed by a 1 minute rinse in 95% EtOH.
17. Air dry thoroughly.
18. Mount slides in organic mounting media.

NOTE: Fading of the substrate reaction may occur with the use of aqueous mounting media or clearing agents.

DOUBLE STAINING PROTOCOL

KPL TrueBlue provides excellent contrast with DAB and other substrates when used for sequential localization of antigens(2). Refer to KPL Technical Manual ML-168, HistoMark Double Staining Procedures, for additional dual labeling protocols and suggestions.

1. Follow steps 1 - 11 as described under Single Staining Procedure, using the first primary antibody at standard concentration.
2. Shake off excess buffer and react sample with KPL DAB or KPL StableDAB Peroxidase Substrate (See RELATED PRODUCTS) 10 minutes.
3. Wash in distilled water 10 - 15 minutes.
4. Soak in Tris-HCl or PBS 10 minutes.
5. Shake off excess buffer and react sample in the second primary antibody, diluted at least 10 - 50 times lower than the standard concentration for use with KPL DAB.
6. Follow steps 7 - 18 as described under Single Staining Procedure.

NOTES:

- Always optimize first and second detection sequences separately prior to performing a double stain.
- Because of its high sensitivity, KPL TrueBlue should be used to detect the least abundant marker.
- DAB should be used for the initial detection sequence and KPL TrueBlue Peroxidase Substrate for the second detection, because solvents contained in many DAB preparations may dissolve the TMB product.
- When one sequence requires detection of a surface marker, that sequence should be performed last. Attachment of antibody and substrate to the surface may inhibit penetration of a second marker.

RESULTS/SOLUTION PREPARATION

Areas of positive activity should appear light blue to purple, with little to no staining in areas where antigen is not present. For long-term preservation of results, store slides in the dark.

- Always include positive and negative controls.
- To decrease background staining, include detergents (NP-40 or Triton X) in the antibody diluent and wash, or use hypertonic wash buffer(4).
- If ANY of the following results are seen, antibody concentration (primary, secondary or HRP-Streptavidin) MUST be reduced:
  - Color fades or floats off section during wash or dehydration
  - Overall high background.
  - Excessively dark or clumped staining
  - Particles of dye scattered over the section

SOLUTION PREPARATION

0.1M Tris-HCl:

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

0.01M Phosphate Buffered Saline (PBS):

5. Dissolve 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 500 mL reagent quality water.
6. Adjust pH to 7.4 with 2M HCl.
7. QS to 1 Liter with reagent quality water.

PRODUCT SAFETY AND HANDLING

See SDS (Safety Data Sheet) for this product.

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RELATED PRODUCTS	CAT. NO.
KPL Blocking Solution Concentrate	5560-0006 (71-00-10)
KPL Universal Block	5560-0009 (71-00-61)
KPL Normal Mouse Serum	5560-0010 (71-18-01)
KPL Normal Goat Serum	5560-0007 (71-00-27)
KPL Normal Rabbit Serum	5560-0008 (71-00-28)
KPL Orcein	5930-0004 (71-01-01)
KPL Contrast RED	5540-0001 (71-00-05)
KPL DAB Reagent Set	5510-0031 (54-10-00)
KPL StableDAB®	5510-0032 (54-11-00)

TROUBLESHOOTING GUIDE

PROBLEM	POSSIBLE CAUSE	CORRECTIVE ACTION
Background	Excess antibody; reaction too fast. Endogenous peroxidase activity	• Dilute primary/secondary antibody or HRP Streptavidin. Reduce incubation times. DO NOT dilute KPL TrueBlue! • KPL Blocking Solution, KPL Universal Block, 0.3% H_2O_2 in 100% MeOH(13) or 0.03% NaN_3(14).
Floating Precipitate	Excess antibody; reaction too fast.	• Dilute primary/secondary antibody or HRP Streptavidin. • To recover staining, shake off substrate and apply fresh substrate for 10 minutes.
Fading	Excess antibody; reaction too fast.	• Dilute primary/secondary antibody or HRP Streptavidin. • To recover staining, shake off substrate and apply fresh substrate for 10 minutes.
Fading during wash	Excess antibody. Inappropriate wash buffer.	• Dilute primary/secondary antibody or HRP Streptavidin. • Use Tris-HCl or PBS for washes prior to KPL TrueBlue. Use only H_2O for washing after TrueBlue staining.
Fading during dehydration	Excess antibody. Drying process.	• Dilute primary/secondary antibody or HRP Streptavidin. • Omit alcohol or use graded acetone. • Air dry or heat fix.
Fading after clearing.	Clearing agent.	• Use very pure Xylene, HistoClear or omit.
Fading after mounting.	Mounting media.	• Use only non-aqueous mounting media. SeraCare recommends Permount.

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PROBLEM	POSSIBLE CAUSE	CORRECTIVE ACTION
Fading after storage.	Storage conditions.	• Store slide in the dark.
Weak staining.	Insufficient binding of antibodies/streptavidin.	• Increase incubation times.
	Excessive washing.	• Reduce wash time.
	Poor contrast.	• Counterstain with KPL Orcein, KPL Contrast RED or Eosin (See Related Products). • Prolonged substrate incubation may inhibit counterstaining.
Purple or other color.	Excess counterstain.	• Shorten counterstain incubation time.

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