

KPL TrueBlue Substrate

<u>Catalog No.</u>	<u>Size</u>
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 H₂O₂. 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% H₂O₂ 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 (H₂O₂), 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	++++		

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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 Na₂HPO₄ and 0.24 g KH₂PO₄ 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	primary/secondary antibody or HRP Streptavidin. Reduce incubation times. DO NOT dilute KPL TrueBlue! DI E ; Solution, KPL Universal Block, 0.3% H ₂ O ₂ in 100% MeOH(13) or 0.03% NaN ₃ (14).
Floating Precipitate	Excess antibody; reaction too fast.	primary/secondary antibody or HRP Streptavidin. M staining, shake off substrate and apply fresh substrate for 10 minutes.

PROBLEM	POSSIBLE CAUSE	CORRECTIVE ACTION
Fading	Excess antibody; reaction too fast.	primary/secondary antibody or HRP Streptavidin. M staining, shake off substrate and apply fresh substrate for 10 minutes.
Fading during wash	Excess antibody. Inappropriate wash buffer.	primary/secondary antibody or HRP Streptavidin. N M -HCl or PBS for washes prior to KPL TrueBlue. Use only H ₂ O for washing after TrueBlue staining.
Fading during dehydration	Excess antibody. Drying process.	primary/secondary antibody or HRP Streptavidin. H Z a use graded acetone. : a Z '
Fading after clearing.	Clearing agent.	N Xylene, HistoClear or omit.
Fading after mounting.	Mounting media.	N only non-aqueous mounting media. SeraCare recommends Permount.

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PROBLEM	POSSIBLE CAUSE	CORRECTIVE ACTION
Fading after storage.	Storage conditions.	L the dark.
Weak staining.	Insufficient binding of antibodies/streptavidin.	B Z incubation times.
	Excessive washing.	K Z a time.
	Poor contrast.	Z with KPL Orcein, KPL Contrast RED or Eosin (See Related Products). I substrate incubation may inhibit counterstaining.
Purple or other color.	Excess counterstain.	L a counterstain incubation time.

REFERENCES

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