For sensitive immunohistochemical staining

KPL HistoMark®
TrueBlue™
Peroxidase System

Catalog Number
5510-0035 (54-78-00)

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DESCRIPTION

KPL HistoMark® TrueBlue is a highly sensitive substrate system for visualization of horseradish peroxidase (HRP)-labeled reporter reagents. It provides a brilliant blue specific stain with red nuclear counterstain. KPL TrueBlue is a buffered solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and H_2O_2. It provides 10 50 times the sensitivity compared to standard DAB systems. TrueBlue is free of known carcinogens; a Safety Data Sheet is supplied. KPL TrueBlue is optimized for use with cellular preparations and can be used for immunoblotting procedures and plaque assays, but not for Microwell ELISA or other applications requiring a soluble reaction product.

PRINCIPLE

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 permanent blue color when used as recommended. Like DAB, the KPL TrueBlue reaction product is insoluble in alcohols and xylene. Endogenous peroxidase activity is eliminated by controlled oxidation prior to application of antibodies [15].

KIT COMPONENTS	CAT. NO.	VOLUME
KPL TrueBlue Peroxidase Substrate	5510-0050 (71-00-65)	100 mL
KPL Blocking Solution	5560-0006 (71-00-10)	10 mL
KPL Orcein	5930-0003 (71-01-00)	50 mL

Sufficient reagents are supplied to process approximately 500 slides.

FORM/STORAGE/STABILITY

- Reagents are stable for a minimum of one year from date of receipt when stored at room temperature (24 28°C). Discard solutions that become turbid.
- KPL TrueBlue is a single component, ready to use, liquid substrate. No mixing is required.
- KPL TrueBlue may appear clear, light blue to light yellow/orange after extended storage.
- Product stability and performance are not affected by variations in solution color.
- KPL Orcein is deep red in color. Over time a precipitate may form which does not affect product performance. If desired, Orcein may be filtered through Whatman paper or a syringe filter prior to use.
- KPL Blocking Solution Concentrate may be stored at room temperature or refrigerated at 2-8°C.

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.
5. DAB for double staining.
6. Organic Mounting Media.
7. 0.1 M Tris-HCl or PBS (See BUFFER PREPARATION).

SENSITIVITY

SeraCare 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.

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

- KPL TrueBlue Substrate Solution: supplied at use dilution.
- KPL Orcein Solution: supplied at use dilution.
- KPL Blocking Solution: dilute 1/10 with reagent quality water (i.e. 1 mL of KPL Blocking Solution Concentrate + 9 mL water). Diluted solution may be stored tightly capped at 2 8°C for up to one week.

SUBSTRATE OPTIMIZATION

SeraCare 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.
3. 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.

PROTOCOLS

Single Staining Protocol

1. Place slides in a Xylene bath and incubate for 5 minutes. Change baths and repeat once.
2. 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.
3. To block endogenous peroxidase activity, immerse samples in 0.3% H_2O_2/100% MeOH for 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 Preparations - 45 seconds
   d. Blood Films - 45 seconds
   e. Touch or Squash Preparations - 1 minute
   f. Floating or Whole Sections - 5 minutes

   If not blocking for endogenous activity, proceed to Step 5.
4. Rinse five minutes in reagent quality water.
5. Soak in 0.1 M Tris-HCl or PBS for 10 minutes.
6. Block with Serum Block 10 minutes.
7. Treat sample with primary antibody diluted in Tris-HCl or PBS for 15 - 20 minutes.

   Note 1: Dilute primary antibody to a concentration at least 10 - 50 times lower than the standard concentration for use with DAB.

   Note 2: Extended incubation may improve sensitivity.
8. Wash sample with Tris-HCl or PBS for 10 minutes.

9. 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.
10. Wash as in Step 8.
11. Shake off excess buffer and incubate sample with HRP-Streptavidin or HRP-labeled secondary antibody diluted in Tris-HCl or PBS, 15 - 20 minutes.
12. Wash as in Step 8.
13. 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 HRPStreptavidin; fading or background may result.
14. Wash sample in reagent quality water 1 - 5 minutes. Note: Washing with PBS or other buffer will result in fading of the blue color.
15. Counterstain with KPL Orcein, KPL Contrast RED or Eosin for 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 the Technical Manual, KPL HistoMark Double Staining Procedures, for additional dual labeling protocols and suggestions.

1. Follow Steps 1 12 as described under Single Staining Procedure, using the first primary antibody at standard concentration.
2. Shake off excess buffer and react sample with DAB or KPL *Stable*DAB Peroxidase Substrate 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 DAB.
6. Follow steps 7 - 17 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.

PROBLEM	POSSIBLE CAUSE	CORRECTIVE ACTION
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.	Dilute primary/secondary antibody or HRP Streptavidin.
	Inappropriate wash buffer.	Use Tris-HCl or PBS for washes prior to KPL TrueBlue. Use only H_2O for washing after KPL TrueBlue staining.
Fading during dehydration	Excess antibody.	Dilute primary/secondary antibody or HRP Streptavidin.
Fading during dehydration.	Drying process.	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.
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 Prolonged substrate incubation may inhibit counterstaining.
Purple or other color.	Excess counterstain	Shorten counterstain incubation time.

PRODUCT SAFETY AND HANDLING

See SDS (Safety Data Sheet) for this product.

RELATED PRODUCTS	CAT. NO.
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 Contrast RED	5540-0001 (71-00-05)
KPL DAB Reagent Set	5510-0031 (54-10-00)
KPL *Stable*DAB®	5510-0032 (54-11-00)

REORDERING INFORMATION

The components of the KPL HistoMark TrueBlue Substrate System may be purchased separately using the following ordering information.

PRODUCT	CAT. NO.
KPL TrueBlue Peroxidase Substrate	5510-0049 (71-00-64)
KPL Orcein Counterstain	5930-0004 (71-01-01)
KPL Blocking Solution	5560-0006 (71-00-10)

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.