RealBlue Peroxidase Substrate Kit

Catalog Number KA0565

500 Slides

Version: 04

Intended for research use only

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Introduction

Intended Use

RealBlue Peroxidase Substrate Kit 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. RealBlue 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. RealBlue is free of known carcinogens. RealBlue 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.

Principle of the Assay

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 innervations 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 RealBlue provides permanent blue color when used as recommended. Like DAB, the RealBlue reaction product is insoluble in alcohols and Xylene. Endogenous peroxidase activity is eliminated by controlled oxidation prior to application of antibodies (15)

General Information

Materials Supplied

List of component

Component	Amount
RealBlue Peroxidase Substrate	100 mL
Blocking Solution Concentrate (10X)	10 mL
Orcein	50 mL

Storage Instruction

- ✓ Reagents are stable for a minimum of one year from date of receipt when stored at room temperature (24 – 28℃). Discard solutions that become turbid.
- ✓ Blocking Solution Concentrate may be stored at room temperature or refrigerated at 2 –8℃. However, refrigeration will provide better long-term shelf life.
- ✓ RealBlue is a single component, ready to use, liquid substrate with a clear to light blue appearance. No mixing is required. Product stability and performance are not affected by variations in solution color.
- ✓ 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.

Materials Required but Not Supplied

- ✓ Primary antibody.
- ✓ Biotinyltaed secondary antibody, HRP-Streptavidin and serum block
- ✓ Isopropyl alcohol.
- ✓ Serum Block: 10% normal serum from the species the secondary antibody was made in.
- ✓ DAB for double staining.
- ✓ Organic Mounting Media: Recommends Permount from Fisher Scientific.
- ✓ 0.1 M Tris-HCl or PBS (See Preparation of reagent).

Assay Protocol

Reagent Preparation

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

- 0.1M Tris-HCl:

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

- 0.01M Phosphate Buffered Saline (PBS):

a. Dissolve 8.0 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4 and 0.24 g KH2PO4 in 500 mL reagent quality water.
b. Adjust pH to 7.4 with 2M HCl.
c. QS to 1 Liter with reagent quality water.

- Substrate Optimization

  Abnova recommends the following optimization protocol for initial evaluation of RealBlue compared to DAB:

  - ✓ Run one control with DAB using standard antibody dilutions.
  - ✓ Run three test samples with RealBlue 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 RealBlue 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.

Assay Procedure

- 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 Abnova 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 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 RealBlue Peroxidase Substrate 10 minutes.
    Note: Color development in less than 10 minutes indicates excess antibody or HRP-Streptavidin; 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 Orcein, 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

RealBlue provides excellent contrast with DAB and other substrates when used for sequential localization of antigens (2). 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 DAB or StableDAB 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, RealBlue should be used to detect the least abundant marker.*
- ✓ *DAB should be used for the initial detection sequence and RealBlue 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.*

Data Analysis

Results

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 diluents 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

Performance Characteristics

- sensitivity

Abnova studies show improved sensitivity when RealBlue 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 RealBlue 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 RealBlue, DAB and AEC.

Model: Cytomegalovirus Antibody to Nuclear Antigen (MAB8135) with Cytomegalovirusinfected Fibroblasts.

Antibody Dilution	RealBlue	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

Resources

Troubleshooting

Problem	Possible Cause	Corrective action
Background	Excess antibody; reaction too fast.	Dilute primary/secondary antibody or HRP Streptavidin. Reduce incubation times. DO NOT dilute RealBlue!
	Endogenous peroxidise activity.	Abnova Blocking Solution, 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.	Dilute primary/secondary antibody or HRP Streptavidin.
	Inappropriate wash buffer.	Use Tris-HCl or PBS for washes prior to RealBlue. Use only H_2O for washing after RealBlue 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. Abnova 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 Orcein, Contrast RED or Eosin Prolonged substrate incubation may inhibit

		counterstaining.
Purple or other color.	Excess counterstain.	Shorten counterstain incubation time.

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