Immunohistochemistry Accessory Kit

For Use with Primary Antibodies made in Rabbit
Cat No. IHC-101
User Manual

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Introduction

Bethyl Laboratories' Immunohistochemistry Accessory Kit provides the reagents and method for sensitive, reliable and economical immunohistochemical staining. This Kit is designed for immunostaining formalin-fixed, paraffin-embedded tissue sections with primary antibodies made in rabbit. The Kit contains all the reagents required for the immunostaining procedure and will stain 250 slides. For convenience, the Kit includes Ready-To-Use reagents in dropper bottles and additional dropper bottles for preparation of working solutions from concentrated reagents.

The anti-Rabbit IHC Antibody improves immunohistochemical applications by reducing the processing time relative to other widely used detection systems. The use of the anti-Rabbit IHC Antibody results in sensitivity and specificity comparable to or better than the commonly used biotin-streptavidin or biotin-avidin detection systems. The anti-Rabbit IHC Antibody is designed to eliminate the streptavidin or avidin-HRP step required when using biotinylated secondary antibodies. Since there is no biotin in the system, there is no need to block for endogenous biotin.

Storage/Stability

Store reagents at 2 - 8° C. All reagents are stable for a minimum of one year from date of receipt.

Kit Components

The Immunohistochemistry Accessory Kit components, quantities, and storage conditions are as follows:

Component	Quantity	Storage
Concentrated Epitope Retrieval Buffer-Reduced pH	100 ml	2 - 8° C
Ready-To-Use IHC Blocking Solution	60 ml	2 - 8° C
Ready-To-Use IHC Antibody Diluent	100 ml	2 - 8° C
Concentrated anti-Rabbit IHC Antibody	1.5 ml	2 - 8° C
Concentrated IHC Wash Solution	50 ml	2 - 8° C
Concentrated DAB Substrate: DAB Solution A (buffer) DAB Solution B (DAB solution) DAB Solution C (Peroxide solution)	2.5 ml each	2 - 8° C
Ready-To-Use IHC Hematoxylin	60 ml	2 - 8° C
Ready-To-Use IHC Bluing Solution	60 ml	2 - 8° C

Other Reagents Needed

- Xylene
- Reagent Alcohol
- Hydrogen Peroxide
- Methanol
- 1N HCl
- 1N NaOH
- Hydrophobic marking pen
- Mounting media and coverslips

Protocol for Staining Formalin-Fixed, Paraffin-Embedded Tissue Sections

Buffer and Reagent Preparation

Peroxidase Quenching Solution

To prepare 200 ml of Quenching Solution:

30% Hydrogen Peroxide	3 ml
Methanol	200 ml
Total volume	203 ml

*Use within 4 hours of preparation.

Epitope Retrieval Buffer, Reduced pH

To prepare 200 ml of Epitope Retrieval Buffer Working Solution:

Concentrated Epitope Retrieval Buffer, Reduced pH	4 ml
Distilled Water	196 ml
Total volume	200 ml

pH should be 5.8 – 6.2. Adjust pH, if necessary, with 1N HCl or 1N NaOH.

OR

To prepare 1 liter of Epitope Retrieval Buffer Working Solution:

Concentrated Epitope Retrieval Buffer, Reduced pH	20 ml
Distilled Water	980 ml
Total volume	1000 ml

pH should be 5.8 – 6.2. Adjust pH, if necessary, with 1N HCl or 1N NaOH.

*Store at to 2 - 8^{o} C. Discard after 3 months.

IHC Wash Solution

To prepare 1 liter of Working IHC Wash Solution:

Concentrated IHC Wash Solution	5 ml
Distilled Water	995 ml
Total volume	1000 ml

*Store at 2 – 8° C

Anti-Rabbit IHC Antibody

Using the dropper bottle provided, prepare Working anti-Rabbit IHC Antibody for 10 slides:

Concentrated anti-Rabbit IHC Antibody	1 drop
Ready-to-Use Antibody Diluent	2 ml
Total volume	2 ml

*Rinse dropper bottle with di-H_2O after each use.

DAB Substrate

Using the dropper bottle provided, prepare Working DAB Substrate:

DAB Solution A	1 drop
DAB Solution B	1 drop
DAB Solution C	1 drop
Distilled Water	2.5 ml
Total volume	2.5 ml

Prepare just prior to use.

*Rinse dropper bottle with di-H_2O after each use:

Deparaffination

A. Treat slides with Xylene: 3 changes for 5 minutes each. Drain slides for 10 seconds between changes.
B. Treat slides with 100% Reagent Alcohol: 3 changes for 5 minutes each. Drain slides for 10 seconds between changes.

Quench Endogenous Peroxidase

A. Place slides in peroxidase quenching solution (*not provided)*: 15-30 minutes.
B. Place slides in distilled water: 2 changes for 2 minutes each.

Retrieve Epitopes

A. Preheat Epitope Retrieval Buffer. Place 200 ml of Epitope Retrieval Buffer Working Solution into container, cover and place into steamer (e.g. Cuisinart CRC-800). Heat to 90-96° C.
B. Place rack of slides into hot Working Epitope Retrieval Buffer for 20 minutes. Cover.
C. Carefully remove container with slides from steamer and cool on bench, uncovered, for 20 minutes.
D. Slowly add distilled water to further cool for 5 minutes. Rinse slides with distilled water. 2 changes for 2 minutes each.

Immunostaining Procedure

A. Remove each slide from rack and circle tissue section with a hydrophobic barrier pen (e.g. Liquid Blocker – Super Pap Pen).
B. Flood slide with Working IHC Wash Solution. ***Do not allow tissue sections to dry for the rest of the procedure.***
C. Drain wash solution and apply 4 drops of Ready-To-Use IHC Blocking Reagent to each slide and incubate for 15 minutes.
D. Drain Blocking Reagent (*do not wash off the Blocking Reagent*), apply 200 µl of Working Primary Antibody solution to each slide, and incubate for 1 hour.

    a. To prepare Working Primary Antibody: For Bethyl IHC antibodies, add 2-10 µl of antibody to 1 ml of Ready-To-Use IHC Antibody Diluent, mix. Suggested starting dilutions are 1:100-1:500. *Optimal working dilutions for other antibodies should be determined experimentally by the investigator.*

E. Wash slides with Working IHC Wash Solution: 3 changes for 5 minutes each.
F. Drain wash solution, apply 4 drops of Working anti-Rabbit IHC Antibody to each slide and incubate for 1 hour.
G. Wash slides with Working IHC Wash Solution: 3 changes for 5 minutes each.
H. Drain wash solution, apply 4 drops of Working DAB Substrate to each slide and develop for 5-10 minutes. *Check development with microscope.*
I. Wash slides with Working IHC Wash Solution: 3 changes for 5 minutes each.
J. Drain wash solution, apply 4 drops of Ready-To-Use IHC Hematoxylin to each slide and stain for 1-3 minutes. *Increase time if darker counterstaining is desired.*
K. Wash slides with Working IHC Wash Solution: 2-3 changes for 2 minutes each.
L. Drain wash solution and apply 4 drops of Ready-To-Use IHC Bluing Solution to each slide for 1-2 minutes.
M. Rinse slides in distilled water.
N. Soak slides in 70% reagent alcohol: 3 minutes with intermittent agitation.
O. Soak slides in 95% reagent alcohol: 2 changes for 3 minutes each with intermittent agitation.
P. Soak slides in 100% reagent alcohol: 3 changes for 3 minutes each with intermittent agitation. Drain slides for 10 seconds between each change.
Q. Soak slides in Xylene: 3 changes for 3 minutes each with intermittent agitation. Drain slides for 10 seconds between each change.
R. Apply 2-3 drops of non-aqueous mounting media to each slide and mount coverslip.
S. Lay slides on a flat surface to dry prior to viewing under microscope.

Notes

- Use treated slides (e.g. HistoBond) to assure adherence of FFPE sections to slide.
- Prior to deparaffinization, heat slides overnight in a 60 C oven.
- All steps in which Xylene is used should be performed in a fume hood.
- For Epitope Retrieval, a microwave or pressure cooker may be substituted for the steamer method. Adjust times as necessary depending on conditions.
- For the initial IHC run with a new primary antibody, test tissues with and without Epitope Retrieval. In some instances, Epitope Retrieval may not be necessary.
- The Ready-To-Use IHC Antibody Diluent provided in this kit is recommended for Bethyl Laboratories primary and secondary antibodies.
- 200 µl is the recommended maximum volume to apply to a slide for full coverage. Using more than 200 µl may allow solutions to wick off the slide and create drying artifacts. For small tissue sections less than 200 µl may be used.
- 5 minutes of development with DAB Substrate should be sufficient. Do not develop for more than 10 minutes. If 5 minutes of development causes background staining, further dilution of the primary antibody may be necessary.
- IHC Hematoxylin was developed to produce a light nuclear counterstain so as not to obscure the DAB staining. Counterstain for 1-1 ½ minutes for nuclear antigens. Counterstain for 2-3 minutes for cytoplasmic and membranous antigens. If darker counterstaining is desired increase time (up to 10 minutes).

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Replacement Reagent List

Description	Size	Catalog No.
Concentrated Epitope Retrieval Buffer-Reduced pH	100 ml	IHC-101A
Ready-To-Use IHC Blocking Solution	60 ml	IHC-101B
Ready-To-Use IHC Antibody Diluent	100 ml	IHC-101C
Concentrated anti-Rabbit IHC Antibody	1.5 ml	IHC-101D
Concentrated IHC Wash Solution	50 ml	IHC-101E
Concentrated DAB Substrate	250 slides	IHC-101F
Ready-To-Use IHC Hematoxylin	60 ml	IHC-101G
Ready-To-Use IHC Bluing Solution	60 ml	IHC-101H
Concentrated Epitope Retrieval Buffer-High pH	100 ml	IHC-101J

Immunohistochemistry Accessory Kit Quick Reference Guide

REAGENTS	**PROCEDURES** (Perform all steps at room temperature)	**INCUBATIION**
Peroxidase Quenching Solution: (Not provided). For paraffin-embedded tissues. Add 3 ml of 30% Hydrogen Peroxide to 200 ml of Methanol. Mix well.	Place slides in peroxidase quenching solution. Incubate. Place slides in distilled water: 2 changes for 2 minutes each.	15 – 30 min.
Epitope Retrieval Buffer: Add 4 ml of Concentrated Epitope Retrieval Buffer to 196 ml of distilled water. pH should be 5.8 – 6.2. *(Epitope Retrieval may not be required for your antibody.*	Preheat 200 ml of Epitope Retrieval Buffer to 90-96 C. Place rack of slides into hot Epitope Retrieval Buffer. Cover. Incubate. Remove slides. Cool uncovered for 20 min. Add distilled water for 5 min. Rinse with distilled water 2 changes for 2 minutes each.	20 min.
Hydrophobic Barrier Pen: (Not provided)	Circle each tissue section with the hydrophobic barrier pen	
Working Wash Solution: Add 5 ml of concentrated Wash Solution to 995 ml of distilled water.	Flood each slide with Working Wash Solution. Drain.	5 min.
IHC Blocking Reagent: (ready-to-use)	Apply 4 drops of Ready-To-Use Blocking Reagent to each slide. Incubate. Drain. Do not wash.	15 mn.
Primary Antibody: (Not provided) Suggested starting dilutions are 1:100-1:500 for Bethyl IHC antibodies. Optimal working dilutions for other antibodies should be determined experimentally by the investigator	Apply 200 ul of diluted Primary Antibody to each slide. Incubate. Wash with Working Wash Solution: 3 changes for 5 minutes each. Drain.	
anti-Rabbit IHC Antibody: Add 1 drop of concentrated anti-Rabbit IHC antibody to 2 ml of Ready-To-Use Antibody Diluent for every 10 slides being stained.	Apply 4 drops of Working anti-Rabbit IHC Antibody to each slide. Incubate. Wash with Working Wash Solution: 3 changes for 5 minutes each. Drain.	1 hour
DAB Substrate: To 2.5 ml of distilled water, add 1 drop of DAB Solution A, mix; add 1 drop of DAB Solution B, mix; add 1 drop of DAB Solution C, mix.	Apply 4 drops of Working DAB Substrate to each slide. Incubate. Wash with Wash Solution: 3 changes for 5 minutes. Drain.	5 – 10 min.
IHC Hematoxylin: (ready-to-use)	Apply 4 drops of IHC Hematoxylin to each slide. Incubate. Wash with Working Wash Solution: 2 - 3 changes for 2 minutes each. Drain	1 – 3 min.
IHC Bluing Solution: (ready-to-use)	Apply 4 drops of Ready-To-Use IHC Bluing Solution to each slide. Incubate. Rinse with distilled water.	1 – 2 min.
	Dehydrate, Clear and Mount	