

ab210061
DoubleStain IHC Kit:
M&R on human tissue
(HRP/Green & AP/Red)

Instructions for use:

For the detection of mouse and rabbit primary antibodies
on human tissue.

This product is for research use only and is not intended
for diagnostic use.

Table of Contents

INTRODUCTION	1
1. BACKGROUND	1
GENERAL INFORMATION	2
2. PRECAUTIONS	2
3. STORAGE AND STABILITY	2
4. LIMITATIONS	3
5. MATERIALS SUPPLIED	3
6. MATERIALS REQUIRED, NOT SUPPLIED	4
7. TECHNICAL HINTS	5
PREPARATION	6
8. RECOMMENDED PREPARATION	6
PROCEDURE	7
9. PROCEDURE	7
RESOURCES	11
10. TROUBLESHOOTING TIPS	11
11. GENERAL IHC TROUBLESHOOTING	11

1. BACKGROUND

Abcam's DoubleStain IHC Kit: M&R on human tissue (HRP/Green & AP/Red) (ab210061) is designed to detect two distinct antigens on human tissue or cell samples using a simplified protocol with user-supplied mouse and rabbit antibodies. This kit has been tested in paraffin embedded tissues. However, this kit can be used to stain frozen specimen and/or freshly prepared monolayer cell smears.

Double staining is a common method used in immunohistochemistry to evaluate two distinct antigens in a single tissue. This kit contains a HRP-Polymer anti-Mouse IgG, AP-Polymer anti-Rabbit IgG, and two distinct chromogens, Emerald (green) and Permanent Red (red). User may apply the two enzyme conjugates onto the specimen at the same time and mix them on the slide. Simplified steps offer user much faster and quicker protocol than a sequential procedure. A second advantage is it allows the researcher to visualize when two proteins are co-localized because of the color change when the chromogens overlap that can be semi-quantitative. For example, if the area of co-localization stains blue, the antigen indicated by Emerald is expressed at higher concentration in the cell and if the color is purple, the antigen indicated by Permanent-Red is expressed at higher concentrations. DoubleStain IHC Kit: M&R on human tissue (HRP/Green & AP/Red) is a non-biotin system that avoids endogenous biotin non-specific binding.

2. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

3. STORAGE AND STABILITY

Store kit at 4°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

GENERAL INFORMATION

4. LIMITATIONS

- Kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

5. MATERIALS SUPPLIED

Item	Amount			Storage Condition Before Preparation	Storage Condition After Preparation
	6 mL kit	18 mL kit	60 mL kit		
Mouse HRP polymer	6 mL	18 mL	60 mL	4°C	4°C
Rabbit AP polymer	6 mL	18 mL	60 mL	4°C	4°C
Permanent Red Substrate	15 mL	36 mL	120 mL	4°C	4°C
Permanent Red Activator (5X)	3 mL	7.2 mL	24 mL	4°C	4°C
Permanent Red Chromogen (100X)	150 µL	360 µL	1.2 mL	4°C	4°C
Emerald Chromogen	6 mL	18 mL	60 mL	4°C	4°C
Organic Mounting Medium	6 mL	18 mL	N/A*	4°C	4°C

*Organic Mounting Medium not supplied with 60 mL kit.

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- Rabbit and mouse primary antibodies
- Peroxidase blocking buffer (ab64218)
- Alkaline phosphatase blocking buffer
- Distilled water
- 100% ethanol
- 100% xylene
- Wash buffer: PBS-T - 10 mM PBS, 0.05% Tween 20 pH 7.4
- Wash buffer: TBS-T – 50 mM Tris HCl, 150 mM NaCl, 0.05% Tween 20, pH7.6
- Hematoxylin (ab220365)
- Antigen retrieval buffers for HIER (not always required)
- Goat serum (ab7481, not always required)

7. TECHNICAL HINTS

- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

8. RECOMMENDED PREPARATION

- Briefly centrifuge small vials at low speed prior to opening
- 8.1. Fixation: to ensure the quality of the staining and obtain reproducible performance, user needs to supply appropriately fixed tissue and well prepared slides.
 - 8.2. Tissues must be adhered to the slide properly to avoid the sample falling off.
 - 8.3. Paraffin embedded sections must be deparaffinized with xylene and rehydrated with a graded series of ethanol before staining.
 - 8.4. Cell smear samples should be as close to a monolayer as possible to obtain satisfactory results.
 - 8.5. Three control slides will aid the interpretation of the result: positive tissue control, reagent control (slides treated with isotype controls) and negative control.
 - 8.6. DO NOT let specimen or tissue dry out once IHC staining has started.
 - 8.7. Use fresh hematoxylin and only expose for recommended time.
 - 8.8. It may be necessary to decrease hematoxylin staining in order to distinguish antigen staining better in multicolor experiments.
 - 8.9. The fixation method, tissue slide thickness, antigen retrieval protocol and primary antibody dilution and incubation times affect results significantly. The user needs to consider all factors and determine optimal conditions when interpreting results.
 - 8.10. We recommend TBS-T as the wash buffer as phosphate in the PBS may inhibit the activity of the alkaline phosphatase detection enzyme.

9. PROCEDURE

- Equilibrate all materials and prepared reagents to correct temperature prior to use.
- Prepare all reagents, and samples as directed in the previous sections.
- Unless otherwise specified all steps are performed at room temperature.

9.1. Tissue Preparation

- 9.1.1. Incubate slides in peroxidase blocking reagent (0.3% H₂O₂ solution or ab64218) and alkaline phosphatase blocking reagent (*i.e.* 1 mM levamisole) for 10 minutes.
- 9.1.2. Rinse the slides at least twice using distilled water.
- 9.1.3. Heat Induced Epitope Retrieval (HIER) may be required for primary antibody - follow manufacturer's suggestions.
- 9.1.4. Wash three times with PBS-T or TBS-T for 2 minutes/wash.
- 9.1.5. Blocking is not required for paraffin sections. For frozen sections, blocking with 10% goat serum (ab7481) may be required depending on the fixative used.

9.2. Antibody and Permanent Red Staining

9.2.1. Apply 2 drops (100 μ L) or enough volume of both the mouse and the rabbit primary antibody to cover the tissue completely. Mix well on the slide. Incubate in a moist chamber for 30-60 minutes.

NOTE: *User needs to optimize dilution and incubation times prior to double staining as both Permanent Red and Emerald chromogens produce strong staining.*

9.2.2. Wash three times with PBS-T or TBS-T for 2 minutes/wash.

9.2.3. Thoroughly mix Mouse HRP polymer and Rabbit AP polymer at a ratio of 1:1. Only make enough mixture for the experiment performed. Mixture is not stable for long term storage.

9.2.4. Add 1 to 2 drops (50-100 μ L) of the mixture to cover each section.

9.2.5. Incubate in a moist chamber for 30 minutes.

9.2.6. Wash three times with TBS-T only for 2 minutes/wash.

9.2.7. Shake Permanent Red Activator thoroughly before use.

9.2.8. To make Permanent Red working solution, add 200 μ L of Permanent Red Activator (5X) into 1 mL of Permanent Red Substrate and mix well. Add 10 μ L of Permanent Red Chromogen (100X) into the Permanent Red working solution and mix well. *Note: for fewer slides, halve the volumes stated above).*

The Permanent Red working solution is not stable. It must be used (freshly) immediately after it is made.

9.2.9. Apply 2 drops (100 μ L) or enough volume of Permanent Red working solution to completely cover the tissue.

9.2.10. Incubate for 10 minutes, and observe appropriate color development. To increase the AP signal, aspirate off chromogen and apply 2-3 drops of Permanent Red working solution to completely cover the tissue and incubate for an additional 5-10 minutes.

PROCEDURE

9.2.11. Rinse well with distilled water.

9.3. Counterstaining

Must be done before Emerald Chromogen step

NOTE: *If two antigens are co-localized in the nucleus, less counterstain is required in order to optimize visualization of the staining pattern. For antigens that are co-localized in cytoplasm or membrane or the 3 antigens are localized in different cells, you can counter stain using the standard protocol timings.*

9.3.1. Dip the tissue sections in diluted hematoxylin for 5 seconds for nuclear co-localization or 30 seconds for cytoplasmic or membrane co-localization. **DO NOT** over stain with hematoxylin.

9.3.2. Rinse thoroughly with tap water for 1 minute.

9.3.3. Put slides in PBS for 5-10 seconds to develop blue color. **DO NOT** overdevelop the blue stain.

9.3.4. Rinse well in distilled or tap water for 1 minute.

9.3.5. Wash slides three times with PBS-T or TBS-T for 2 minutes/wash.

9.4. Emerald Staining

NOTE: As Emerald Chromogen is water soluble, the counterstain is performed first. Do not leave slides sitting in water. Always stain Emerald chromogen AFTER GBI-Permanent Red stain (because GBI-Permanent Red removes the Emerald stain) and after hematoxylin.

9.4.1. Apply 1 to 2 drops (50-100 μL) of Emerald Chromogen to cover the tissue completely.

9.4.2. Incubate in a moist chamber for 5 minutes.

9.4.3. Wash slides in tap water for 1 minute.

9.4.4. Rinse with distilled water.

9.4.5. Wipe off excess water and air dry slides.

9.4.6. Dehydrate with 85% ethanol for 20 seconds.

9.4.7. Dehydrate with 95% ethanol for 20 seconds.

9.4.8. Dehydrate with 100% ethanol for 20 seconds.

9.4.9. Dehydrate with 100% ethanol for 20 seconds.

9.4.10. Dehydrate with 100% ethanol for 20 seconds.

9.4.11. Dehydrate with xylene for 20 seconds.

NOTE: Do not dehydrate with xylene for longer than 20 seconds as this will erase the Permanent Red stain.

9.5. Mounting

9.5.1. Apply 1 drop (50 μL) of Organic Mounting Medium to cover the tissue section and apply glass coverslip.

Note: Leave the mounting media at room temperature for 10-15mins before each use. Immediately after use, store the mounting media again at 4°C.

9.5.2. Apply enough force to the coverslip to squeeze out any extra mounting medium and bubbles for optimal clarity and to prevent leaching of Permanent Red chromogen.

10. TROUBLESHOOTING TIPS

Cause	Solution
Uneven stain on 2 primary antibodies	Adjust the titer of each antibody. The amount of each protein expressed on tissue may be different. Ensure that slides are not left in water for too long to prevent Emerald chromogen from washing away. Ensure that slides are not left in xylene for too long to prevent Permanent Red chromogen from washing away.
Emerald chromogen is blue not green when it is not co-localized with Permanent Red.	If Emerald chromogen is blue, the primary antibody is probably not dilute enough for the protocol. Re-titer primary antibodies individually first.
No stain on 1 or 2 antibodies	Ensure that no steps were missed or reversed.
Green background on the slide	Titer primary antibody.
Permanent Red is leaching	Use fresh 100% ethanol and xylene. Ensure that slide is not immersed in xylene for more than 20 seconds
Artifacts on slides	Ensure that slides are completely dried before mounting. Use fresh 100% ethanol and xylene.

11. GENERAL IHC TROUBLESHOOTING

Problem	Cause	Solution
No Staining	The primary antibody and the secondary detection polymer are not compatible.	Use a primary antibody that was raised in a species that can be detected by the polymer detection system (e.g. goat primary antibody with goat AP polymer).
	Not enough primary antibody is bound to the protein of interest.	Use less dilute antibody, Incubate longer (e.g. overnight) at 4°C.
	The antibody may not be suitable for IHC procedures which reveal the protein in its native (3D form).	Test the antibody in a native (non-denatured) WB to make sure it is not damaged.

Problem	Cause	Solution
	The protein is not present in the tissue of interest.	Run a positive control recommended by the supplier of the antibody.
	Deparaffinization may be insufficient.	Deparaffinize sections longer, change the xylene.
No Staining (cont.)	The primary/secondary antibody/amplification kit may have lost its activity due to improper storage, improper dilution or extensive freezing/thawing.	Run positive controls to ensure that the primary/secondary antibody is working properly.
	The protein of interest is not abundantly present in the tissue.	Use an amplification step to maximize the signal.
	Fixation procedures (using formalin and paraformaldehyde fixatives) may be modifying the epitope the antibody recognizes.	Use antigen retrieval methods to unmask the epitope, fix for less time.
	The protein is located in the nucleus and the antibody (nuclear protein) cannot penetrate the nucleus.	Add a permeabilizing agent to the blocking buffer and antibody dilution buffer.
	The PBS buffer is contaminated with bacteria that damage the phosphate groups on the target protein.	Add 0.01% azide in the PBS antibody storage buffer or use fresh sterile PBS.
High Background	Blocking of non-specific binding might be absent or insufficient.	Increase the blocking incubation period and consider changing blocking agent. Abcam recommends 10% normal serum 1hr for sections or 1-5% BSA for 30 min for cells in culture.
	Incubation temperature may be too high.	Incubate sections or cells at 4°C.
	The primary antibody concentration may be too high.	Titrate the antibody to the optimal concentration, incubate for longer but in more dilute antibody (a slow but targeted binding is best).
	The secondary detection polymer may be binding non-specifically (damaged).	Run a secondary polymer negative control without primary antibody.

Problem	Cause	Solution
	Tissue not washed enough, fixative still present.	Wash extensively in PBS between all steps.
High Background (cont.)	Endogenous peroxidases are active.	Use enzyme inhibitors i.e. Levamisol (2 mM) for alkaline phosphatase or H ₂ O ₂ (0.3% v/v) for peroxidase.
	Fixation procedures (using formalin and paraformaldehyde fixatives) are too strong and modified the epitope the antibody recognizes.	Change antigen retrieval method, decrease the incubation time with the antigen unmasking solution.
	Too much substrate was applied (enzymatic detection).	Reduce substrate incubation time.
	The chromogen reacts with the PBS present in the cells/tissue (enzymatic detection).	Use Tris buffer to wash sections prior to incubating with the substrate, then wash sections/cells in Tris buffer.
	Permeabilization has damaged the membrane and removed the membrane protein (membrane protein).	Remove permeabilizing agent from your buffers.
Non-specific staining	Primary/secondary polymer concentration may be too high.	Try decreasing the antibody concentration and/or the incubation period. Compare signal intensity against cells that do not express the target.
	Endogenous peroxidases are active.	Use enzyme inhibitors i.e. Levamisol (2 mM) for alkaline phosphatase or H ₂ O ₂ (0.3% v/v) for peroxidase.
	The primary antibody is raised against the same species as the tissue stained (e.g. mouse primary antibody tested on mouse tissue). When the secondary antibody is applied it binds to all the tissue as it is raised against that species.	Use a primary antibody raised against a different species than your tissue.
	The sections/cells have dried out.	Keep sections/cells at high humidity and do not let them dry out.



Technical Support

Copyright © 2024 Abcam. All Rights Reserved. The Abcam logo is a registered trademark. All information / detail is correct at time of going to print.

For all technical or commercial enquiries please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)