ab210059
DoubleStain IHC Kit: M&R on human tissue (DAB & 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.

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1. BACKGROUND

Abcam's DoubleStain IHC Kit: M&R on human tissue (DAB & AP/Red) (ab210059) 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, an AP-Polymer anti-Rabbit IgG, and two distinct chromogens. The DAB chromogen (brown color) is used with the HRP-Polymer anti-Mouse IgG and Permanent Red (red color) is used with the AP-Polymer anti-Rabbit IgG. DoubleStain IHC Kit: M&R on human tissue (DAB & AP/Red) is a non-biotin system that avoids the extra steps involved in blocking non-specific binding due to endogenous biotin.

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 handle 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 4ºC 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.

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
DAB substrate	12 mL	36 mL	120 mL	4°C	4°C
DAB Chromogen (20x)	1.5 mL	2 mL	6 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
Aqueous Mounting Medium	6 mL	18 mL	60 mL	4°C	4°C

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 *i.e.* levamisole
- 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, pH 7.6
- Hematoxylin (ab220365)
- Antigen retrieval buffers for HIER (not always required)
- Goat serum (ab7481, not always required)
- Xylene-based mounting medium or ab104141 (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, the 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 10- 30 seconds.

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.

8.11. Permanent Red is insoluble in organic solvent and can also be coverslipped. However, the dehydration steps much be shorter for optimal tissue structure and chromogen signal maintenance.

8.12. The mounting medium is water-based and is used as the permanent mounting medium for alcohol-soluble chromogens such as Permanent Red, AEC and BCIP. Use of a coverslip is not essential. If you require a coverslip, the dehydration steps must be shorter for optimal tissue structure and chromogen signal maintenance.

NOTE: Wipe off extra water and air dry the slides for a few seconds before following the dehydration and clearing steps.

a. 1x 80% ethanol 20 seconds;

b. 1x 95% ethanol 20 seconds;

c. 3x 100% ethanol 20 seconds each;

d. 1x 100% xylene 20 seconds;

e. Add 1 drop of xylene-based mounting media or ab104141 and add coverslip. Press to push the air bubble out.

CAUTION: DO NOT dehydrate in xylene longer than 20 seconds! It will erase the Permanent Red staining.

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 (3% H_2O_2 solution or ab64218) and alkaline phosphatase blocking reagent (*i.e.* 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. locking is not required for paraffin sections. For frozen sections, blocking may or may not be required. Blocking agent e.g. 10% goat serum (ab7481) may be required depending on the fixative used.

9.2. Staining Protocol

9.2.1. Apply 2 drops (100 µL) or enough volume of both the mouse and 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*

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

9.2.3. Apply 1 drop (50-100 µL) of Rabbit AP Polymer and 1 drop of Mouse HRP polymer to cover each section and mix well on the slide. Alternatively, mix 50 µL of Rabbit AP Polymer and 50 µL of Mouse HRP polymer and add to the slide.

9.2.4. Incubate in a moist chamber for 30 minutes.

9.2.5. Wash three times with PBS-T for 2 minutes/wash.

9.2.6. Prepare DAB Working Solution: add 1 drop of DAB Chromogen to 1 mL DAB Substrate and mix well. Add 2 drops of DAB Chromogen if higher sensitivity and contrast are required. Protect from light at 4°C and use within 7 hours.

9.2.7. Apply 2 drops or enough volume of DAB Working Solution to completely cover tissue.

9.2.8. Incubate for 5 minutes.

9.2.9. Rinse well with distilled water

9.2.10. Wash three times with TBS-T only, 2 minutes/wash.

9.2.11. Prepare Permanent Red Working Solution: Shake the Activator Solution well before use. Add 4 drops (200 µL) of Permanent Red Activator to 1 mL of Permanent Red Substrate and mix well. Add 10 µL of Permanent Red Chromogen to this mixture and mix well.

NOTE: *For fewer slides use half of the quantities given above.*

9.2.12. Apply 2 drops (100 µL) or enough volume of the Permanent Red Working Solution to completely cover the tissue.

9.2.13. Incubate for 10 minutes and check for appropriate color development.

Note: To increase the AP signal, make fresh working solution again, tap off previous chromogen, apply 2-3 drops (100 µL) immediately and incubate for an additional 10 minutes.

9.2.14. Rinse well with distilled water.

9.3. **Counterstaining/Mounting**

9.3.1. Counterstain with 2 drops (100 µL) or enough volume of hematoxylin to completely cover tissue. Incubate for 10-15 seconds.

9.3.2. Rinse thoroughly with tap water for 2-3 minutes.

9.3.3. Place slides in PBS until blue color shows (30 – 60 seconds)

9.3.4. Rinse well in distilled water

9.3.5. While tissue is wet, apply 2 drops (100 µL) or enough volume of Aqueous Mounting Medium to cover tissue. Rotate the slides to allow the mounting medium to spread evenly.
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.

9.3.6. Place slides horizontally in an oven at 40-50°C for at least 30 minutes or leave it at room temperature until slides are thoroughly dried.

10. GENERAL IHC TROUBLESHOOTING TIPS

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.
	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.
	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.

Problem	Cause	Solution
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.
	Tissue not washed enough, fixative still present.	Wash extensively in PBS between all steps.
	Endogenous peroxidases are active.	Use enzyme inhibitors i.e. Levamisol (2 mM) for alkaline phosphatase or H_2O_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.
	Pemeabilization has damaged the membrane and removed the membrane protein (membrane protein).	Remove permeabilizing agent from your buffers.

Problem	Cause	Solution
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_2O_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.

11. NOTES

RESOURCES

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Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)