ab183275 –DoubleStain IHC Kit: M&M on Rodent Tissue (Green/HRP & AP/Red)

Instructions for Use

For the detection of Mouse Primary antibodies on Rodent Tissue.

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

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1. Introduction

Abcam's Doublestain IHC Kit (ab183275) is designed to use with two user supplied mouse antibodies to detect two distinct antigens on mouse and rat tissue or cell samples. The advantage of this kit series is that it will allow you to visualize when two proteins are co localized by producing a third color blue purple. Specimens can be frozen or paraffin embedded, or freshly prepared monolayer cell smears.

2. Principle of Assay

Double staining is a common method used in immunohistochemistry that allows for detection of two distinct antigens in a single tissue. This kit uses an HRP or AP polymer based technology combined with a proprietary blocking buffer system that achieves ultra-sensitivity with no background or cross reactivity. Abcam's Doublestain IHC Kit (ab183275) supplies the user with a primer system to enhance the two polymer enzyme conjugates; Mouse HRP Polymer and Mouse AP Polymer with two distinct substrates/chromogens, Permanent Red and Emerald. Permanent Red reacts with Mouse AP Polymer conjugate to produce a red color. Emerald chromogen reacts with Mouse HRP Polymer conjugate to produce a green color. However when the chromogens are produced in the same place the color appears blue to purple in color. Abcam's Doublestain IHC Kit (ab183275) is a non-biotin system that avoids the extra steps involved in blocking non-specific binding due to endogenous biotin.

3. Kit Contents

Item	Quantity (12 mL) (60 slides)	Quantity (36 mL) (180 slides)	Quantity (120 mL) (600 slides)
Mouse AP Polymer	6 mL	18 mL	60 mL
Mouse HRP Polymer	6 mL	18 mL	60 mL
Mouse Primer	6 mL	18 mL	60 mL
Blocker A	6 mL	18 mL	60 mL
Blocker B	6 mL	18 mL	60 mL
Antibody Blocker (40x)	30 mL	50 mL	125 mL
Permanent Red Chromogen (100x)	70 µL	180 µL	0.6 mL
Non-aqueous Mounting medium	6 mL	2 x 9 mL	-
Permanent Red Substrate	7 mL	1 x 18 mL	60 mL
Permanent Red Activator (5x)	1.4 mL	3.6 mL	12 mL
Emerald Chromogen	6 mL	18 mL	60 mL

4. Storage and Handling

Store at 2-8°C. Do not freeze. The reagents must be returned to the storage conditions after use.

5. Additional Materials Required

- Mouse primary antibodies
- Wash buffer: PBS-T - 0.01M pH7.4 PBS with 0.05% Tween20
- Wash buffer: TBS-T - 50mM Tris HCl, 150mM NaCl, 0.05% Tween20, pH 7.6
- Peroxidase and alkaline phosphatase blocking buffers
- 100% Ethanol
- 100% Xylene
- Hematoxylin (ab128990)
- Optional: Rat on Rat Blocking Buffer (ab186026)

6. Recommendations

Read all protocol steps before starting staining experiment and follow each step carefully in the order given.

1. *The volumes provided in this kit are sufficient for the number of slides indicated if 100 µL are used.*

2. *Fixation: To ensure the quality of the staining and to obtain reproducible performance the user needs to supply appropriately fixed tissue and well prepared slides*

3. *Tissues must be adhered to the slide properly to ensure maximum quality staining*

4. *Paraffin embedded sections must be deparaffinised with xylene and rehydrated with a graded series of ethanol before staining.*

5. *Cell smear samples should be made up to as much of a monolayer as possible to obtain satisfactory results.*

6. *Three control slides will aid the interpretation of the result: positive and negative tissue controls, reagent control (slides treated with Isotype control reagent).*

7. *During IHC staining: DO NOT let specimens or tissues dry from this point on.*

8. *pH plays an important role for that reason use fresh hemotoxylin and only expose for 10- 30 seconds.*

9. *The more colors you use in multi-staining the more pertinent if becomes to keep the hemotoxylin as weak as possible to distinguish antigen staining better.*

10. *The fixation, tissue slide thickness, antigen retrieval and primary antibody dilution and incubation time affect results significantly. The Investigator needs to consider all factors and determine optimal conditions when interpreting results.*

11. *We recommend TBS-T to be used as the wash buffer to get the highest sensitivity and clean background. Phosphate in the PBS may inhibit the activity of the alkaline phosphatase.*

7. Protocol

Unless otherwise stated all steps are performed at room temperature.

Tissue Preparation (Up to 135 minutes)

1. Incubate slides in peroxidase blocking reagent (3% H_2O_2 solution) and alkaline phosphatase blocking reagent (i.e. levamisole) for 10 minutes.

2. Rinse the slides using 2 changes of distilled water.

3. Heat induced Epitope Retrieval may be required for primary antibody as suggested by manufacturer

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

5. If there are no background issues please proceed to the Staining Protocol. If background is an issue you may want to include a blocking step here using a suitable blocking buffer (eg ab186026).

Staining Protocol (Up to 210 minutes)

1. Apply 2 drops or enough volume of the first mouse primary antibody to cover the tissue completely. Incubate in a moist chamber for 30-60 minutes.
   Note: *Investigator needs to optimize dilution prior to double staining*

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

3. Apply 1-2 drops (50-100 µL) of Mouse Primer to cover each section.

4. Incubate in a moist chamber for 10 minutes.

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

6. Apply 1-2 drops (50-100 µL) of Mouse AP Polymer to cover each section.

7. Incubate in a moist chamber for 10 minutes.

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

9. **Preparation of Permanent Red Working Solution:** Add 200 µL of Permanent Red Activator to 1 mL of Permanent Red Substrate and mix well. Then add 10 µL of Permanent Red Chromogen to this mixture and mix well.
   Note: For fewer slides use half of the quantities given above

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

11. Incubate for 10 minutes observing appropriate color development.

12. Rinse well with distilled water.

13. **Optional Antibody Blocker (40x) Step:** This step will block antibodies of the previous step so no cross reaction will occur at the end of the protocol. However, please skip this step if antigen retrieval is used for the second mouse primary antibody.

14. Use hot plate or water bath to heat dilute a 1x solution made up of 1 part Antibody Blocker (40x) in 39 parts distilled water to 80-95 °C. Make enough volume to cover the tissue.

15. For paraffin embedded tissue put slides in heated Antibody Blocker for 10 minutes at 95-100 °C. For frozen embedded

tissue put slides in heated Antibody Blocker for 10 minutes at 80 °C.

16. Cool slides to 55 °C.

17. Rinse slides in multiple changes of distilled water.

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

19. Apply 2 drops (50-100 µL) or enough volume of Blocker A to cover the tissue completely. Mix well on the slide and incubate in a moist chamber for 5 minutes.

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

21. Apply 2 drops (50-100 µL) or enough volume of Blocker B to cover the tissue completely. Mix well on the slide and incubate in a moist chamber for 5 minutes.

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

23. Apply 2 drops or enough volume of the second mouse primary antibody mixture to cover the tissue completely. Incubate in a moist chamber for 30-60 minutes.
    Note: *Investigator needs to optimize dilution prior to double staining*

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

25. Apply 1-2 drops (50-100 µL) or enough to cover the tissue completely of Mouse HRP Polymer to cover each section.

26. Incubate in a moist chamber for 15 minutes.

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

Counterstaining/Mounting

1. Counterstain slides with enough hematoxylin to completely cover tissue; incubate for 5-10 seconds. OPTIONAL: Hematoxylin may be diluted if counterstaining too dark. DO NOT over stain with hematoxylin.

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

3. Place slides in PBS until blue color shows (5 seconds) DO NOT over blue.

4. Rinse well in distilled water

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

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

   Note: *Emerald Chromogen is water soluble, counter stain first. DO NOT leave slides sitting in water. Always stain with Emerald Chromogen after Permanent Red and hematoxylin staining as Permanent Red will remove Emerald.*

7. Incubate in a moist chamber for 5 minutes.

8. Wash slides in tap water for 1 minute.

9. Rinse with distilled water.

10. Please wipe off extra water and air dry slides before dehydration and clear.

    a. Dehydrate with 85% ethanol 20 seconds.
    b. Dehydrate with 95% ethanol 20 seconds.
    c. Dehydrate with 100% ethanol 20 seconds.
    d. Dehydrate with 100% ethanol 20 seconds.
    e. Dehydrate with 100% ethanol 20 seconds.
    f. Dehydrate with xylene 20 seconds.

    Caution: DO NOT dehydrate in xylene for longer than 20 seconds as it will erase the Permanent Red stain.

11. Apply 1-2 drops (50-100 µL) or enough volume of Non-aqueous Mounting Medium at room temperature to cover tissue and apply glass coverslip. Apply force to the coverslip to squeeze out any extra mounting solution and bubbles for optimal clarity. Removing excess also prevents the leach of Permanent Red stain.

    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.*

8. 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.

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.

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.

High Background (cont.)	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.
	Permeabilization 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.

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