ab183273 –DoubleStain IHC Kit: M&M on Rodent Tissue (DAB & AP/Red)

Instructions for Use

For the detection of Mouse Primary antibodies on Rodent Tissue Samples.

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

Version 5a Last updated 11 June 2025

Table of Contents

1. Introduction

Abcam's Doublestain IHC Kit (ab183273) is designed to use with two user-supplied mouse antibodies to detect two distinct antigens on mouse and rat tissue or cell samples. Specimens can be frozen, paraffin embedded, or freshly prepared monolayer cell smears.

2. Principle of Assay

Double staining is a common method used in immunohistochemistry that allows for the detection of two distinct antigens in a single tissue. This kit uses an HRP and 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 supplies the user with primer system to enhance the two polymer enzyme conjugates Mouse HRP Polymer and Mouse AP Polymer with two distinct substrates/chromogens: Permanent Red and DAB. Permanent Red reacts with Mouse AP Polymer conjugate to produce a red color. DAB chromogen reacts with Mouse HRP Polymer conjugate to produce a brown color. Abcam's Doublestain IHC Kit 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 (6 mL) (60 slides)	Quantity (18 mL) (180 slides)	Quantity (60 mL) (600 slides)
Mouse AP Polymer	6 mL	18 mL	60 mL
Mouse HRP Polymer	6 mL	18 mL	60 mL
Antibody Blocker (40x)	30 mL	50 mL	100 mL
Mouse Primer	6 mL	18 mL	60 mL
Permanent Red Chromogen (100x)	150 µL	360 µL	700 µL
Aqueous Mounting Medium	6 mL	18 mL	60 mL
Permanent Red Substrate	15 mL	36 mL	120 mL

Item	Quantity (12 mL) (60 slides)	Quantity (36 mL) (180 slides)	Quantity (120 mL) (600 slides)
Permanent Red Activator (5x)	3 mL	7.2 mL	14 mL
DAB Chromogen (20x)	1.5 mL	2 mL	3.5 mL
DAB Substrate	12 mL	30 mL	120 mL
Blocker A	12 mL	36 mL	120 mL
Blocker B	12 mL	36 mL	120 mL
Mouse Antibody Enhancer	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 immediately 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
- Optional: Rat on Rat Blocking Buffer

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 the best 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 hematoxylin and only expose for 10- 30 seconds.*

9. *The more colors you use in multi-staining the more pertinent if becomes to keep the hematoxylin 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.*

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. It does not need a coverslip, however, 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;

    Do not dehydrate in xylene longer than 20 seconds as it will erase Permanent Red stain

7. Protocol

Unless otherwise stated all steps are performed at room temperature.

Tissue Preparation (Up to 130 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.

    a. Apply 2 drops or enough volume of Blocker A to cover the tissue completely and incubate in moist chamber for 30 minutes.

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

c. Apply 2 drops or enough volume of Blocker B to cover the tissue completely and incubate in moist chamber for 5 minutes.

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

Staining Protocol (~215 minutes)

1. Apply 2 drops or enough volume of the first 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*

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

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

4. Incubate in 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 HRP Polymer to cover each section.

7. Incubate in a moist chamber for 10 minutes.

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

9. **DAB Staining:** Although the DAB step can be done at the end of the protocol, we find the DAB Chromogen acts as additional shielding between the first mouse and second mouse antibodies. We recommend you do this step now.

10. Add 1 drop of DAB Chromogen to 1 mL DAB Substrate. Mix well. Protect from light and use within 7 hours.

11. Apply 2 drops (50-100 µL) DAB Chromogen mixture to completely cover tissue. Incubate for 5 minutes.

12. Rinse well with distilled water.

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

14. **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.

15. 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.

16. 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.

17. Cool slides to 55 °C.

18. Rinse slides in multiple changes of distilled water.

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

20. 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 moist chamber for 30 minutes.

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

22. 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 moist chamber for 5 minutes.

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

24. 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*

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

26. Add 2 drops, or enough to cover tissue completely, of Mouse Antibody Enhancer to cover each section.

27. Incubate for 15 minutes in moist chamber.

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

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

30. Incubate in moist chamber for 15 minutes.

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

    Note: To intensify Permanent Red signal rinse with 1x 0.1M Tris pH 8.5 to 9.0

32. **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 12 µL of Permanent Red Chromogen to this mixture and mix well.
    Note: For fewer slides use half of the quantities given above

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

34. Incubate for 10 minutes observing appropriate color development.

    Note: To increase AP signal, aspirate or tap off chromogen and apply 2-3 drops (100µL) again of the Permanent Red working solution to completely cover the tissue for additional 5 to 10 minutes.

35. Rinse well with distilled water.

Counterstaining/Mounting

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

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

3. Place slides in PBS or Tris pH 7.4 to 8.4 until blue color appears.

4. Rinse well in distilled water.

5. Apply 2 drops (100µL) or enough aqueous mounting medium to cover tissue when tissue is wet. Rotate the slides to allow the medium spread evenly. DO NOT coverslip.

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

   The hardened mounting medium forms a polymer barrier impervious to organic solvent. Do not use oil directly on the top of the dried mounting medium.

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.

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)