ab183278 –DoubleStain IHC Kit: M&Rt on Mouse Tissue (BCIP & AEC)

Instructions for Use

For the detection of Mouse and Rat Primary antibodies on Mouse Tissue.

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

Version 2 Last updated 4 August 2016

Table of Contents

1. Introduction

Abcam's DoubleStain IHC Kit (ab183278) is designed to use with user supplied mouse and rat primary antibody to detect two distinct antigens on mouse tissue or cell samples. This kit has been tested on paraffin embedded tissue, however it can also be used on frozen specimens or freshly prepared monolayer cell smears. This kit is designed not to give background with most mouse strains however there may be some, especially when using frozen tissue, that require additional blocking.

2. Principle of Assay

Double staining is one of most common methods used in immunohistostaining allowing the detection of two distinct antigens in a single tissue. Abcam's DoubleStain IHC Kit (ab183278) supplies the user with two polymer enzyme conjugates: Mouse HRP Polymer and Rat AP Polymer and two distinct substrates/chromogens, AEC (Red color, used with the Mouse HRP Polymer) and BCIP/NBT (Purple color, used with the Rat AP Polymer). A Primer step is used to increase specificity of antibody staining. Both enzyme conjugates are applied to the specimen at the same time and mixed on the slide. This kit offers simplified steps making for a quicker and easier protocol than those used in a sequential procedure. Abcam's DoubleStain IHC Kit (ab183278) is a non-biotin system that avoids endogenous biotin non-specific binding.

3. Kit Contents

Item	Quantity (12 mL) (60 slides)	Quantity (36 mL) (180 slides)	Quantity (120 mL) (600 slides)
Rat AP Polymer	6 mL	18 mL	60 mL
Mouse HRP (AEC) Polymer	6 mL	18 mL	60 mL
Mouse Primer	12 mL	2 x 18 mL	120 mL
Hydrogen Peroxide (20x)	1 mL	2 mL	6 mL
Aqueous Mounting Medium	15 mL	2 x 18 mL	120 mL
Ready to Use BCIP/NBT	15 mL	2 x 18 mL	120 mL
AEC Chromogen (20x)	2 mL	4 mL	12 mL
AEC Substrate (20x)	1 mL	2 mL	6 mL
MRt Block A	6 mL	18 mL	60 mL
MRt Block B	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 and Rat 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

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.*

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

    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;

7. Protocol

Unless otherwise stated all steps are performed at room temperature.

Tissue Preparation (Up to 100 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.

Staining Protocol (~125 minutes)

1. Apply 2 drops or enough volume of both mouse and rat primary antibodies to cover the tissue completely.
   Note: *Investigator needs to optimize dilution prior to double staining*

2. Incubate in a moist chamber for 30-60 minutes.

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

4. Apply 2 drops (100 µL) or enough to cover each section of Mouse Primer.

5. Incubate in a moist chamber for 10-15 minutes.

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

7. Add 2 drops (100µL) or enough volume of MRt Block A to cover the tissue section and incubate.

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

9. Add 2 drops (100µL) or enough volume of MRt Block B to cover the tissue section and Incubate. Do not exceed 5min.

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

11. **Preparation of Polymer mixture:** Make sufficient polymer mixture by adding Mouse HRP Polymer and Rat AP Polymer at a 1:1 ratio, mix well. Do not mix more than you need for the experiment at the polymer mixture is not stable for long term storage.

12. Apply 1-2 drops (50-100 µL) of the Polymer Mixture to completely cover tissue.

13. Incubate in a moist chamber for 30 minutes.

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

15. Apply 2 drops (50-100 µL) of Ready to Use BCIP/NBT to completely cover tissue.

16. Incubate for 5-10 minutes.

17. Rinse well with distilled water.

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

19. **Preparation of AEC Working Solution:** Add 50 µL of AEC Substrate (20x) to 1 mL of distilled water and mix well. Then add 100 µL of AEC Chromogen and 50 µL of Hydrogen Peroxide (20x) to this mixture and mix well. Keep away from light and use within 1 hour.

20. Apply 2 drops (100 µL) or enough volume of the AEC Working Solution to completely cover the tissue.

21. Incubate for 5-15 minutes observing appropriate color development.

22. Rinse well with distilled water.

Note: AEC is alcoho*l soluble; *do not dehydrate.

Counterstaining/Mounting

1. Apply 2 drops (100 µL) or enough volume of hematoxylin to completely cover tissue.

2. Incubate for 10-15 seconds.

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

4. Place slides in PBS until blue color appears (30-60 seconds).

5. Rinse well in distilled water.

6. Apply 2 drops (100 µL) or enough volume of Aqueous Mounting Medium to cover tissue. Rotate the slides to allow even spread.

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

Note: If you need to coverslip, after the Aqueous Mounting Medium has dried dip the slide in xylene (1-2 seconds) and then apply an organic mounting solution and place coverslip on the slide and allow to dry completely.

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.

UK, EU and ROW
Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria
Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany
Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain
Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada
Email: ca.technical@abcam.com | Tel: 877-749-8807

China and Asia Pacific
Email: hk.technical@abcam.com | Tel: 108008523689 (中國聯通)

Japan
Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

www.abcam.com | www.abcam.cn | www.abcam.co.jp