ab183297 –TripleStain IHC Kit: M&R&Rt on rodent tissue (DAB, AP/Red & Green/HRP)

Instructions for Use

For the detection of Rat, Rabbit and Mouse Primary antibodies on Human or Mouse tissue or cell samples.

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

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1. Introduction

The TripleStain IHC kit (ab183297) is designed to be used with user supplied mouse, rat and rabbit primary antibodies to detect three distinct antigens on mouse tissue and cell samples or human tissue. This kit has been tested on paraffin embedded tissue, however it can also be used on frozen tissue or cell smears.

2. Principle of Assay

Triple staining is a common method used in immunohistochemistry for the detection of three distinct antigens and their co-expression in a single tissue. Abcam's TripleStain IHC Kit (ab183297) supplies polymer enzyme conjugates: mouse HRP polymer, rat AP polymer and rabbit HRP polymer with three substrates/chromogens; DAB (brown), Emerald (green), and Permanent Red (Red). The TripleStain Kit is a non-biotin system avoiding non-specific background due to endogenous biotin binding. This kit has been optimized to have no cross detection when detecting two primary antibodies from the mouse and rat host species using a unique blocking system. Simplified steps allow the user to complete triple staining within 5 hours (without antigen retrieval) or 6-7 hours (with antigen retrieval). This protocol also includes a method to dehydrate, clear and permanently mount slides with coverslip.

3. Kit Contents

Item	Quantity (24 mL) (120 slides)	Quantity (72 mL) (360 slides)	Quantity (120 mL) (1200 slides)
Rat primer	6 mL	18 mL	60 mL
Rat AP Polymer	6 mL	18 mL	60 mL
Rabbit HRP Polymer	6 mL	18 mL	60 mL
DAB Substrate	15 mL	2 x 18 mL	120 mL
DAB Chromogen (20x)	1.5 mL	2 mL	6 mL
Permanent Red Activator (5x)	3 mL	7.2 mL	2 x 12 mL
Permanent Red Chromogen (100x)	150 µL	360 µL	1.2 mL
Permanent Red Substrate	15 mL	2 x 18 mL	120 mL
Antibody Blocker (40x)	30 mL	50 mL	100 mL
Mouse HRP Polymer	6 mL	18 mL	60 mL
Emerald Chromogen	6 mL	18 mL	60 mL
Non-aqueous Mounting Medium	6 mL	18 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, Rat and Rabbit 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)

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-T may inhibit the activity of the alkaline phosphatise.*

7. Protocol

Unless otherwise stated all steps are performed at room temperature.

Tissue Preparation (Up to 70 minutes)

1. Incubate slides in peroxidase blocking reagent and alkaline phosphatase blocking reagent for 10 minutes.

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

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

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

Staining Protocol (Up to 230 minutes)

1. Apply 2 drops or enough volume of mouse primary antibody mixture to cover the tissue completely.
   Note: *Investigator needs to optimize dilution prior to triple staining.*

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

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

3. Apply 1-2 drops (50-100 µL) of Mouse HRP Polymer to cover the tissue completely.

4. Incubate in a moist chamber for 15 minutes.

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

6. **Preparation of DAB working solution:** Make enough DAB working solution by adding 1 drop of DAB Chromogen in 1mL of DAB Substrate. Mix well. Store at 4ºC and use within 7 hours.

7. Apply 1-2 drops (50-100 µL) of DAB Working Solution to cover the tissue completely

8. Incubate for 5 minutes.

9. Rinse slides in multiple changes of distilled water (3 times for 2 minutes per wash) or under running tap water for 1 minute.

10. **Preparation of Antibody Blocker:** Dilute 1 part Antibody Blocker with 39 parts distilled water and use a hot plate or water bath to heat to 80°C. Make enough volume to cover the tissue in beaker.

    Note: This step will block antibodies of previous step so no cross reaction will occur in this protocol. HIER can be done immediately after Antibody Blocker step if the primary antibodies requires antigen retrieval. For frozen tissues, a lower temperature of 65ºC must be used during the Antibody Blocker step to prevent dissociation of the tissue from the slide.

11. Put slides in heated Antibody Blocker for 10 minutes at 80°C (or 65°C).

12. Remove slides and cool for 5 seconds

13. Rinse slides in multiple changes of distilled water.

14. **Optional Step:** If antigen retrieval is required follow instructions on antibody manufacturer's datasheet

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

16. Apply 2 drops or enough volume of the rat and rabbit primary antibody mixture to cover the tissue completely.

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

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

19. Apply 1 to 2 drops (50-100µL) of Rat Primer to cover the tissue completely.
    Note: This step is required for activation of RAT AP Polymer, DO NOT skip.

20. Incubate in a moist chamber for 10 minutes.

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

22. **Preparation of Polymer Mixture**: Mix Rat AP polymer and Rabbit HRP Polymer at a 1:1 ratio, mix well.

Note: Do not mix more than needed for the experiment as the polymer mixture may not be as stable as non-mixed polymers.

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

24. Incubate in a moist chamber for 30 minutes

25. Wash with 1xTBS-T 3 times for 2 minutes each. .

26. **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

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

28. Incubate for 10 minutes observing appropriate color development.

29. Rinse well with distilled water

Counterstaining/Mounting

30. Counterstain by dipping in diluted hematoxylin for 5 seconds for nuclear colocalization or 30 seconds for cytoplasmic or membrane colocalization. DO NOT over stain with hematoxylin

If two antigens are colocalized in the nucleus less counterstaining is required to optimize the visualization in the nuceus; however normal counterstain protocol times can be used if colocalization occurs in the cytoplasm or membrane or all three antigens are localized in different cells.

31. Rinse thoroughly with tap water for 1 minute

32. Place slides in PBS until blue color shows (5-10 seconds) – DO NOT over blue.

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

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

35. 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.

36. Incubate in a moist chamber for 5 minutes

37. Wash slides in tap water for 30 seconds.

38. Wipe off extra water and air dry slides before dehydrating as follows
    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.

39. Apply 1 drop (50 µL) of the non-aqueous mounting medium to cover the tissue section and apply a glass cover slip.

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

40. Apply force to the coverslip to squeeze out any extra mounting solution and bubbles for optimal clarity. Removing excess also to prevent leaching of Permanent Red stain.

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.
	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.

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)