

**ab183285 –DoubleStain
IHC Kit: R&Rt on
Human/Mouse Tissue
(Green/HRP & AP/Red)**

Instructions for Use

For the detection of Rat and Rabbit Primary antibodies on Human/Mouse 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 (ab183285) is designed for use with user supplied rabbit and rat primary antibodies to detect two distinct antigens on human and mouse tissue or cell samples. This kit has been tested on paraffin embedded tissue, however, it can also be used to stain frozen specimens or freshly prepared monolayer cell smears.

2. Principle of Assay

Double staining is a common method used in immunohistostaining, allowing for the detection of two distinct antigens in a single tissue. Abcam's Doublestain IHC Kit (ab183285) supplies the user with two polymer enzyme conjugates: Rat HRP Polymer (minimal cross reaction to mouse) and Rabbit AP Polymer with two distinct substrates/chromogens, Emerald and Permanent Red. Emerald chromogen reacts with the Rat HRP polymer conjugate to produce a green color. Permanent Red reacts with the Rabbit AP polymer to produce a red color. When two proteins are co-localized a blue/purple color will develop depending on which antigen is stronger. If only the rat antigen is present only the Emerald chromogen will be present and if the rabbit antigen is present only the Permanent Red chromogen will be present. Abcam's Doublestain IHC Kit (ab183285) is a non-biotin system avoiding 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)
Rabbit AP Polymer	6 mL	18 mL	60 mL
Rat HRP Polymer	6 mL	18 mL	60 mL
Permanent Red Chromogen (100x)	150 µL	360 µL	1.2 mL
Non-aqueous Mounting Medium	1 x 12 mL	4 x 9 mL	-
Permanent Red Substrate	15 mL	2 x 18 mL	120 mL
Permanent Red Activator (5x)	3 mL	7.2 mL	2 x 12 mL
Emerald Chromogen	15 mL	2 x 18 mL	120 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. See section 5 for storage of components after preparation.

5. Reagent preparation.

Non-aqueous Mounting Medium:

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

All other components are ready to use.

6. Additional Materials Required

- Rat and rabbit primary antibodies
- Wash buffer: PBS-T - 0.01M pH7.4 PBS with 0.05% Tween20
- Peroxidase and alkaline phosphatase blocking buffers
- 100% Ethanol
- 100% Xylene
- Hematoxylin (ab128990)

7. 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 μ L 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 it 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.*

8. Protocol

Unless otherwise stated all steps are performed at room temperature.

Tissue Preparation (Up to 70 minutes)

1. Incubate slides in peroxidase blocking reagent (3% H₂O₂ 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 for 2 minutes/wash.

Staining Protocol (Up to 100 minutes)

1. Apply 2 drops or enough volume of both rabbit 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 for 2 minutes/wash.

4. **Preparation of Polymer mixture:** Make sufficient polymer mixture by adding Rat HRP Polymer and Rabbit AP Polymer at a 1:1 ratio, mix well. Make only enough mixture for your experiment. The polymer mixture is not stable for long term storage.
5. Apply 1-2 drops (50-100 μ L) of the Polymer mixture to cover each section.
6. Incubate in a moist chamber for 30 minutes.
7. Wash three times with PBS-T for 2 minutes/wash.
8. **Preparation of Permanent Red Working Solution:** Add 200 μ L of Permanent Red Activator to 1 mL of Permanent Red Substrate and mix well. Then add 10 μ L of Permanent Red Chromogen to this mixture and mix well.
Note: For fewer slides use half of the quantities given above
9. Apply 2 drops (100 μ L) or enough volume of the Permanent Red Working Solution to completely cover the tissue.
10. Incubate for 10 minutes observing appropriate color development.
11. Rinse well with distilled water.

Counterstaining/Mounting

1. Dip the slide in diluted hematoxylin for 5 seconds for nuclear co-localization or 30 seconds for cytoplasmic or membrane co-localization. DO NOT over stain with hematoxylin.
2. Rinse thoroughly with tap water for 1 minute.
3. Place slides in PBS until blue color shows (5-10 seconds) DO NOT over blue.
4. Rinse well in distilled water for 1 minute.
5. Wash three times with PBS-T for 2 minutes/wash.
6. Apply 1-2 drops (50 – 100 μ L) 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. Wipe off extra water and air dry the slides for a few seconds before following the dehydration and clearing steps.
 - a. 1x 85% ethanol 20 seconds.
 - b. 1x 95% ethanol 20 seconds.
 - c. 3x 100% ethanol 20 seconds.
 - d. 1x 100% 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 μ L) or enough volume of Non-aqueous Mounting Medium to cover tissue and apply glass coverslip.

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.

12. Apply force to the coverslip to squeeze out any extra mounting solution and bubbles for optimal clarity. Removing the excess will also prevent the leach 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 ₂ O 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 ₂ O 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

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