ab183281 – DoubleStain IHC Kit: G&Rt on human/mouse tissue (BCIP & AEC)

Instructions for Use

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

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

Version 1a Last updated 8 May 2024

Table of Contents

1.	Introduction	3
2.	Principle of Assay	4
3.	Kit Contents	5
4.	Storage and Handling	6
5.	Additional Materials Required	6
6.	Recommendations	7
7.	Protocol	9
8.	General IHC Troubleshooting Tips	13

1. Introduction

The DoubleStain IHC kit (ab183281) is designed to be used with user supplied goat and rat primary antibodies to detect two distinct antigens on human or mouse tissue or cell samples. The kit has been tested on paraffin embedded human and mouse tissues, however, it can also be used on frozen specimen and freshly prepared monolayer cell smears.

2. Principle of Assay

Double staining is one of the most common methods used in immunohistostaining allowing the detection of two distinct antigens in a single tissue. Abcam's DoubleStain IHC Kit (ab183281) supplies the user with two polymer enzyme conjugates: HRP polymer anti-Goat IgG and AP polymer anti-Rat IgG with two distinct substrates/chromogens: BCIP/NBT (Purple) and AEC (Red). Users sequentially apply the two enzyme conjugates onto the specimen. When two proteins are present a purple/red color will develop depending on the presence and location of the antigens the two colors should be distinct. If only the anti-goat antigen is present only Red will be visible and if the anti-Rat antigen is present Purple will be present. The kit is non-biotin system avoiding endogenous biotin non-specific binding.

3. Kit Contents

Item	Quantity (6 mL) (60 slides)	Quantity (18 mL) (180 slides)	Quantity (60 mL) (600 slides)
Rat AP Polymer	6 mL	18 mL	60 mL
Rat Primer	6 mL	18 mL	60 mL
Double Stain Block	6 mL	18 mL	60 mL
Goat HRP (AEC) Polymer	6 mL	18 mL	60 mL
Ready to Use BCIP/NBT	6 mL	18 mL	60 mL
AEC Substrate (20x)	1 mL	2 mL	3 mL
AEC Chromogen (20x)	2 mL	4 mL	6 mL
Hydrogen Peroxide (20x)	1 mL	2 mL	3 mL
Aqueous Mounting Medium	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

- Goat and Rat primary antibodies
- Wash buffer: PBS-T - 0.01M pH7.4 PBS with 0.5% 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 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 may be required for primary antibody as suggested by manufacturer

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

Staining Protocol (Up to 135 minutes)

1. Apply 2 drops or enough volume of goat and rat primary antibody mixture 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 1-2 drops (50-100 µL) of Goat HRP (AEC) Polymer to cover each section.

5. Incubate in a moist chamber for 15 minutes.

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

7. **Preparation of AEC Working Solution:** Add 1 drop (50 µL) of AEC Substrate (20x) to 1mL distilled water and mix well. Then add 2 drops of AEC Chromogen (20x) and 1 drop of Hydrogen Peroxide (20x) and mix well. Keep away from light and use within 1 hour.

8. Apply 2 drops (100 µL) or enough volume of pre-mixed AEC solution to completely cover the tissue.

9. Incubate for 5-15min observing appropriate color development.

10. Rinse well with distilled water.
    (AEC is alcohol soluble; do not dehydrate).

11. Apply 1 to 2 drops (50-100 µL) of DoubleStain block to cover each section.

12. Incubate in a moist chamber for 10 minutes.

13. Blot off solution. DO NOT Rinse.

14. Add 2 drops (100 µL) or enough volume of Rat Primer to cover the tissue section.

15. Incubate for 10-15 minutes.

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

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

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

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

20. Apply 2 drops or enough volume of BCIP/NBT to completely cover tissue.

21. Incubate for 10 minutes.

22. Rinse thoroughly with distilled water.

Counterstaining/Mounting

23. Counterstain with 2 drops (100 µL) or enough volume of hematoxylin to completely cover tissue.

24. Incubate for 5 seconds. DO NOT over stain with hematoxylin.

25. Rinse thoroughly with tap water for 1 minute.

26. Put slides in PBS for 5 seconds to blue; DO NOT over blue.

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

28. 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.

29. 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.
	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)