# HiPer® Immunohistochemistry Teaching Kit

## Product Code: HTI023

## Number of experiments that can be performed: 5

## Duration of Experiment: 7 Hours

## Storage Instructions:

- The kit is stable for 6 months from the date of manufacture
- Store secondary antibody at -20°C
- Blocking solution, Primary antibody, Negative Control antibody, Antibody diluent buffer, Antigen retrieval buffer, DAB substrate & slides with paraffin sections should be stored at 2-8°C.
- Other kit contents can be stored at room temperature (15-25°C)



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WHO GMP CERTIFIED
ISO 13485-2016 CERTIFIED

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## Aim:

To stain tissue sections using antibody and to learn the technique of IHC.

## Introduction:

Immunohistochemistry (IHC), or immunohistochemical staining, is a technique which employs antibodies to detect antigens in cells within a tissue section. This application is used to locate specific antigens in tissue sections with labeled antibodies based on antigen-antibody interactions. The immune reactive products can be visualized by a marker including fluorescent dyes, enzymes, radioactive elements or colloidal gold. The IHC principle has been known since the 1930s, but it was not until 1942 that the first IHC study was reported, where scientists used FITC labeled antibodies to identify Pneumococcal antigens in infected murine tissue. Since then, advancements in protein conjugation, tissue fixation methods, detection labels and microscopy have allowed immunohistochemistry to become an essential tool in diagnostic and research laboratories.

**Applications:**

- IHC is used by clinicians to detect and diagnose abnormal cells found in disease states such as cancer.
- During basic research, to evaluate the location and co-localization of proteins within a cell, for instance in the nucleus, cytoplasm or membrane.

## Principle:

IHC staining is a highly sensitive and specific method in identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions. Antibodies are very selective in recognizing their antigens, which makes them a perfect tool for IHC. This technique utilizes formalin fixed, paraffin embedded (FFPE) tissue sections on a glass slide. Fixation of tissue is necessary to adequately preserve cellular components, including soluble and structural proteins. Paraffin has to be removed in order to allow the antibodies to penetrate the tissues cells and binds to the antigen. Deparaffinization is achieved by using Xylene. Tissue sections need to be hydrated for antigen antibody interaction therefore, there is need to gradually introduce water into the cells of the tissue sections through grades of alcohol. Most of the tissues express the enzyme Horse Radish Peroxidase endogenously Hence, it is important to block the endogenous activity of HRP using methanol and $H_2O_2$.

The term antigen retrieval is predominantly defined as high temperature heating method to recover the antigenicity of tissue sections that had been masked by formalin fixation. The blocking steps avoid any non specific binding of primary antibody. The site of antibody binding is identified by direct labelling of the antibody or by use of secondary labelling method. The most frequently used enzyme label is HRP. Due to its small size it gives good intracellular penetration and has fast conversion rate of chromogenic substrate. DAB (3,3'- diamino benzidine) which gives a brown color is often used with HRP as a substrate. For strongly expressed antigens, the DAB substrate gives a long lasting staining allowing the slides to be stored. Hematoxylin is used as a counterstain which gives blue color to the nucleus of the cells. The HRP labelled secondary antibody, through chemical reaction, converts colorless substrate into insoluble particles with color at the site of protein of interest which can be visualized using light microscopy.

## Kit Contents:

This kit can be used to stain tissue sections using antibody and to learn the technique of IHC

**Table 1: Enlists the materials provided in this kit with their quantity and recommended storage**

| Sr. No. | Product Code | Materials Provided | Quantity | Storage |
|---|---|---|---|---|
| | | | 5 expts | |
| 1 | TKC426 | Blocking Solution | 4.5ml | 2-8°C |
| 2 | TKC427 | Primary Antibody | 1.5ml | 2-8°C |
| 3 | TKC428 | Negative Control Antibody | 1.5ml | 2-8°C |
| 4 | TKC148 | Secondary Antibody | 12.5µl | -20°C |
| 5 | TKC429 | Antibody Diluent Buffer | 3ml | 2-8°C |
| 6 | TKC430 | 10XAntigen Retrieval Buffer | 60ml | 2-8°C |
| 7 | MB121 | 3,3',4,4'- Tetraaminobiphenyl tetrahydrocholoride (DAB) | 5X5mg | 2-8°C |
| 8 | TKC431 | 30% $H_2O_2$ | 1ml | RT |
| 9 | ML023 | 10X PBS | 2X150ml | RT |
| 10 | S058 | Hematoxylin | 3.5ml | RT |
| 11 | TKC432 | Mounting Solution | 0.3ml | RT |
| 12 | TKC433 | Slides with paraffin sections | 10No's | 2-8°C |

## Materials Required But Not Provided:

**Glass wares:** Measuring cylinder, Slide staining jar/ container **(CG144/CG145),**
**Reagents:** Distilled water, Xylene, Methanol, Ethanol.
**Other requirements:** Light Microscope, Micropipettes, Tips, Cover slips, Moist chamber (Box with Wet blotting paper), Water bath at 95°C, 15ml Amber bottle for DAB Substrate preparation.

## Storage:

HiPer® Immunohistochemistry Teaching Kit is stable for 6 months from the date of manufacture without showing any reduction in performance. On receipt, store secondary antibody at -20°C. Blocking solution, Primary antibody, Negative Control antibody, Antibody diluent buffer, Antigen retrieval buffer, DAB substrate & slides with paraffin sections should be stored at 2-8°C. Other kit contents can be stored at room temperature (15-25°C).

## Important Instructions:

1. Read the entire procedure carefully before starting the experiment.
2. **Preparation of secondary antibody** – For 0.3 ml of diluted secondary antibody, add 1.5 µl of antibody to 298.5 µl of antibody diluent buffer. Mix by inversion.
3. **Preparations of DAB substrate –** For 10 ml of DAB substrate, dissolve 5 mg of DAB powder in 10 ml of 1XPBS and add 5µl of 30% $H_2O_2$.
4. **Preparation of 1XPBS (600ml) -** Dilute 60ml of 10XPBS with 540ml of Autoclaved MilliQ.
5. **Preparation of 3% $H_2O_2$ in Methanol to block endogenous peroxidase activity -** Add 0.1 ml of 30% $H_2O_2$ to 0.9 ml of Methanol.
6. **Preparation of 1X Antigen Retrieval buffer –** for 100 ml of 1X buffer, dilute 10 ml of 10 X antigen retrieval buffer with 90 ml of Autoclaved MilliQ.

## Procedure:

### Deparaffinization / Rehydration :

1. Take two slides – one for Positive control and another for negative control.

2. Deparaffinize the sections by placing the slide in a slide staining jar with Xylene for 5 minutes.

3. Transfer the slides to a second Coplin jar of Xylene and keep the slides for 5 minutes.

   **NOTE: If coplin jar is not available, carry out the experiment in glass jar. DONOT USE PLASTIC CONTAINERS.**

4. Rehydrate the sections by keeping the slides in various grades of alcohol i.e, in 100% Ethanol for 2 times, 3 minutes each & then once through 95%, 70% and 50% of ethanol for 3 minutes.

   **NOTE: Use separate glass jar for each step of rehydration.**

### Blocking of endogenous peroxidase activity:

5. Place the slides in a moist chamber. Block endogenous peroxidase in the tissue sections by covering each part of the sections with 100µl (Add more if required to cover the entire section) of freshly prepared mixture of methanol and 3% $H_2O_2$ & Incubate for 10 minutes at RT.

   **NOTE: For preparation of Methanol- $H_2O_2$ mixture, refer Important Instructions.**

6. Rinse the slides in 1X PBS for 2 times, 5 minutes each.

## Antigen retrieval:

7. Perform antigen retrieval to unmask the antigenic epitope. Arrange the slides in a staining container. Pour 50ml of 1X Antigen Retrieval buffer, pH 6.0 into the staining container and incubate at 95-100°C in a water bath for 10 minutes.

   **NOTE: For preparation of 1X Antigen Retrieval buffer, refer Important Instructions.**

8. Remove the staining container to room temperature and allow the slides to cool for 20 minutes.

9. Rinse the slide in 1X PBS for 2 times, 5 minutes each.

## Staining:

10. Add 200µl of blocking buffer onto the sections of the slides and incubate in a moist chamber at room temperature for 1 hour.

11. Drain off the blocking buffer from the slides.

12. Apply 100µl (Add more if required to cover the entire section) of primary antibody to the positive control slide and 100µl (Add more if required to cover the entire section) of negative control antibody to negative control slides. Incubate in a moist chamber at room temperature for 1 hour.

13. Wash the slides in 1X PBS for 2 times, 5 minutes each.

14. Apply 100µl (Add more if required to cover the entire section) of diluted secondary antibody to the sections on both the slides and incubate in a moist chamber at room temperature for 1 hour.

15. Wash the slides in 1X PBS for 2 times, 5 minutes each.

16. Apply 100µl (Add more if required to cover the entire section) of DAB substrate solution (freshly prepared just before use) to the sections on the slides to reveal the color of antibody staining. Allow the color development for 10 minutes until the desired color intensity is reached.

    **NOTE: For the preparation of DAB Substrate, refer Important instructions.**
    **Caution:** DAB is a suspect carcinogen. Handle with care. Wear gloves, lab coat and eye protection.

17. Wash the slides in 1X PBS for 3 times, 2 minutes each.

18. Add Hematoxylin counterstain on sections of slides for 2 minutes.

19. Rinse the slides in running tap water for 5 minutes.

## Dehydrate Sections:

20. Dehydrate the tissue slides through 4 changes of alcohol (95%, 95%, 100% and 100%), 5 minutes each.

    **NOTE: Use separate jar for each step of dehydration.**

21. Place the slides in a Coplin jar of Xylene 2 times, 1 minute each.

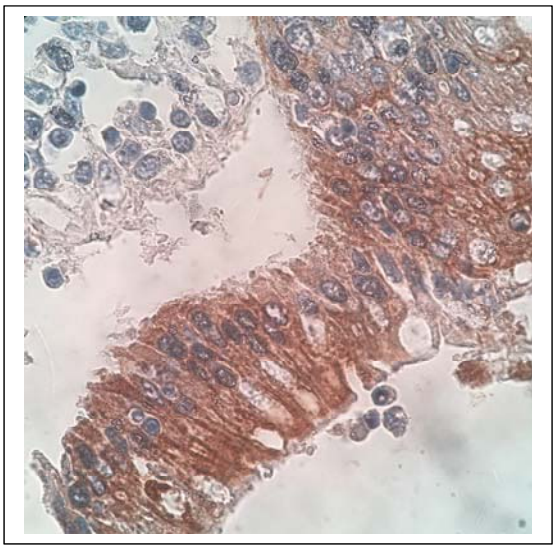
    **NOTE: If coplin jar is not available, carry out the experiment in glass jar. DONOT USE PLASTIC CONTAINERS.**

22. Mount the sections using mounting solution by slowly dropping a coverslip on it (no air bubbles should be present). The mounted slides can be stored at room temperature permanently.

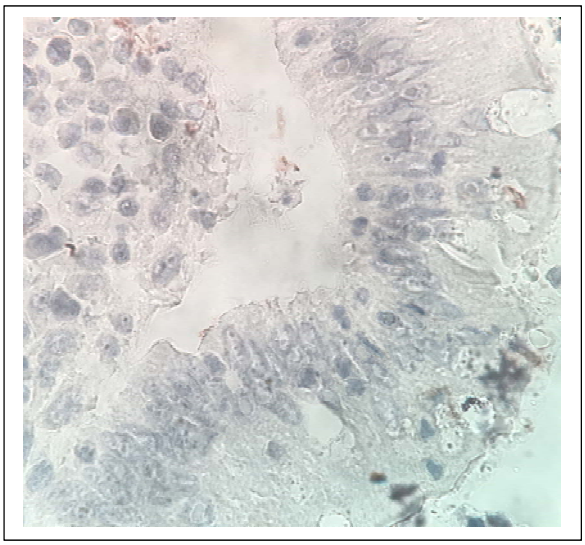
23. Observe the color of the antibody staining in the tissue sections under light microscopy.

## Observation and Result:

It was observed that cytoplasm & margins of the epithelial cells shows brown color staining with positive control whereas negative control does not show this pattern. Nuclei of the cells stains blue due to Hematoxylin.



**Positive Staining**



**Negative Staining**

**Fig 1: Staining pattern of Tissue sections using the technique of IHC**

## Interpretation:

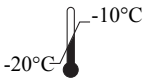
In the section stained with the primary antibody, the intracytoplasmic space and margins of epithelial cells are stained brown indicating the presence of the antigen. This staining pattern is not observed in the section stained with the negative control antibody. The primary antibody specifically localizes the antigen present in the intracytoplasmic space of epithelial cells.

## Troubleshooting Guide:

| Sr.No | Problem | Probable Cause | Solution |
|---|---|---|---|
| 1 | No signal | Omission of any step | Prepare a check-list for the steps followed |
| 2 | High background | 1. Insufficient washing<br>2. Blocking of non-specific binding might be absent or insufficient.<br>3. Sections dried out<br>4. Endogenous peroxidases are active. | 1. Wash plates thoroughly after each incubation steps.<br>2. Follow the incubation period and conditions properly<br>3. Avoid sections being dried out<br>4. Follow the endogenous peroxidase activity blocking step properly. |
| 3 | Overstainig | 1. Overincubation with antibodies or substrate.<br>2. Sections dried out | 1. Follow the incubation period and conditions as mentioned in the protocol.<br>2. Avoid sections being dried out |
| 4 | Poorly resolved or damaged tissue morphology | Antigen retrieval method may be performed too harshly. | Do not exceed the incubation time & temperature. |

## Technical Assistance:

At HiMedia we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance, mail at mb@himedialabs.com




Storage temperature

Do not use if package is damaged

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03/2025

PIHTI023_1/0322 HTI023-01

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