Rabbit Specific 2 step biotin-free HRP IHC Detection Kit – KOA0130

Rabbit Specific Two Step Biotin-Free Peroxidase Immunohistochemistry Detection Kit includes reagents specifically designed for the analysis of antigens in immunohistochemistry and other immunodetection assays. Using polymerization marking method, HRP is conjugated with secondary antibody to form a large molecule antibody-enzyme polymer. The secondary and third antibodies used in traditional methods can thus be replaced by this enzyme polymer. This enzyme polymer is specially fit for immunohistochemistry analysis due to its powerful nature of amplifying signal and permeating tissues and cells. This kit possesses superiority of high speed, high sensitivity, low background and ease-of-use. The provided protocol includes steps for immunohistochemical analysis of antigens with high sensitivity and low background using an unconjugated primary antibody, peroxidase enzyme polymer, and a 5% BSA blocking reagent.

I. KIT COMPONENTS

Rabbit Specific 2 step biotin-free HRP IHC Detection Kit			
Component	Catalog Number	Description	Size
Blocking Reagent	KOB0103	5% BSA Blocking Reagent	10 ml
Peroxidase Conjugated Secondary Antibody	KOM0302	Anti-Rabbit IgG (Goat) Polymerization Peroxidase Conjugated Antibody	10 ml
3% Hydrogen Peroxide	KOF0101	3% H_2O_2 solution	10 ml

II. STORAGE

Store at 4°C for one year. Avoid freezing.

III. REQUIRED EQUIPMENT AND REAGENTS

- APES or POLY-L-LYSINE
- 0.02M PBS (pH 7.2~7.6)
  - 8.5g sodium chloride, 2.8g anhydrous Na_2HPO_4 and 0.4g anhydrous NaH_2PO_4 in 1000ml of distilled water. (The weight should be adjusted accordingly if hydrous phosphates are used.)
- 0.01 M Citrate Buffer
  - 3g sodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) and 0.4g citric acid monohydrate ($C_6H_5Na_3O_7 \cdot H_2O$) in 1000ml of distilled water.
- DAB Chromogenic Kit, to be used with SABC-POD.
- 0.1% trypsinase or the compound digest solution

IV. IHC PROTOCOL

Note: Rabbit IgG refers to the animal origin of the primary antibody, not the origin of the specimen. This Kit must be used on primary antibodies from rabbit.

A. Options For Immunohistochemistry Staining Process

The best process among the following may have to be identified by the end user. The characteristics of the antigen/antibody used may be followed as a guideline.

- Heat repair antigen process
  Applies to immunohistochemical analysis of paraffin-embedded sections, to expose the antibody binding site on the antigens.

- Enzyme digestion process
  Applies to immunohistochemical analysis of paraffin-embedded sections, to expose the antibody binding site on the antigens.

- Non-digestion/non-repair process
  Applies to stable antigens using immunohistochemical analysis of paraffin-embedded sections.

- Blood smear, cultured cells and frozen section staining process
  Blood smear, cultured cells and frozen section staining process applies to immunocytochemistry of blood smear and cultured cells, and immunohistochemical analysis of frozen-embedded sections.

B. Assay Procedure

Heat repair antigen process

1. Cover the entire surface of a clean microslide with APES or POLY-L-LYSINE. Incubate for 1 minute and then rinse the microslide with water. Mount a tissue section (~5μm thick) onto the treated microslide and bake in an oven at 58-60˚C for 30-60 minutes to ensure strong adhesion of the tissue section.
2. Dewax the tissue section in dimethylbenzene for 10 minutes and rinse with water.
3. Incubate the tissue section for 5~10 minutes in 3% H_2O_2 solution to quench the endogenous peroxidase activity. Wash the tissue section with distilled water three times for 2 minutes each.
4. For heat-induced antigen retrieval, add a sufficient volume of 0.01M citrate buffer (pH 6.0) in a microwavable container and preheat the buffer to 90˚-100˚C in a microwave. Place the slides into the container with the preheated citrate buffer (the buffer should cover the slides by at least a few centimeters) and place the container inside the microwave. Set the microwave to full power and boil for 15-20 minutes. Remove the container from the microwave and allow the slides to cool at room temperature for 15-20 minutes.
5. Wash the slides two times for 2 minutes each with 0.01 M TBS (pH 7.2-7.4) with gentle agitation.
6. Add 5% BSA blocking solution to the tissue section and incubate at room temperature for 20 minutes. Discard the blocking reagent solution, but do not wash the tissue section.
7. Add appropriately diluted primary antibody (rabbit IgG) and incubate at 37˚C for 1 hour or at 20˚C for 2 hours or at 4˚C overnight.
8. Wash with 0.02M PBS (pH 7.2-7.6) 3 times for 2 minutes each.
   - The primary antibody concentration, incubation time and temperature directly affect the staining efficiency and background intensity. If the positive staining is too weak, the concentration of the primary antibody and the incubation time can be increased; if the background is too high, the primary antibody concentration and the incubation time can be decreased.
9. Add polymerization HRP marking anti-rabbit IgG secondary antibody to the tissue section and incubate at 37˚C for 30 minutes. Wash the slides with PBS or TBS 3 times for 2 minutes each.

10. Use a DAB chromogen Kit to stain the tissue section. Add Reagents, one drop each, into 1 ml of distilled water and mix thoroughly. Add this solution to the tissue section and incubate at room temperature. Control the time of incubation under a microscope. Usually 5-30 minutes is sufficient.

11. Wash the tissue section with distilled water.

12. Slightly counterstain the tissue section with hematoxylin or nuclear fast red and wash with distilled water to clean the hematoxylin. Dry the tissue section by baking, and put on a drop of resin seal. The tissue section is ready for observation under a microscope.

Blood smear, cultured cells or frozen sections staining process

1. Treat a microslide with POLY-L-LYSINE as described above.
   - Blood samples: Add anticoagulant to the samples and smear the blood samples onto the treated slide.
   - Cultured cells: Cultured cells can be smeared onto or directly cultivated on the treated slide.
   - Frozen tissue sections: Sections of frozen tissue may be placed onto the treated slide and air-dry at room temperature for 30 minutes until no liquid is visible.
2. Fix the sample with 4% paraformaldehyde or acteone for 10-20 minutes.
   - Dilute 30% H_2O_2 at 1:50 with pure methanol. Incubate the fixed sample for 30 minutes in the diluted H_2O_2 to quench the endogenous peroxidase activity. Wash the sample with distilled water once or twice for 2 minutes each.
   - *If the direct staining result of frozen sections is not satisfactory, the tissue sections may be repaired by following the step 4 under antigen retrieval process.*
3. Follow steps 5-12 in the immunohistochemistry paraffin tissue sections staining process.

***Note**:*

1. *If the staining background is too high, wash the section with 0.01-0.02% TWEEN 20-PBS (pH 7.2-7.6) 4 times and then with PBS twice after SABC reaction and before DAB staining, followed by DAB chromogenic Kit to stain the section.*
2. *0.01M citrate buffer (pH 6.0), PBS, or TBS buffer may be used to repair the section.*

V. TROUBLESHOOTING

Weak or No Signal

Possible Cause	Solution
Slides lose signal over time during storage	Prepare slides with freshly-sectioned tissues Store slides at 4°C Do not bake slides before storage
The antibody used is not suitable for IHC procedures which detect proteins in its native conformation	Check the antibody datasheet to make certain that it has been validated for IHC applications Check if the antibody is applicable for the right IHC samples (paraffin sections vs. frozen samples) Perform Western blot in both its native and denatured forms to ensure that the antibody detects the native form

Fixation procedures (using formalin/paraformaldehyde fixatives) have masked the epitope that the antibody recognizes	Use different antigen retrieval methods to unmask the epitope (HIER or PIER) Fix the sections in a shorter time
The primary and/or secondary antibody has lost its activity due to improper storage, dilution or excessive freezing and thawing	Run positive controls to ensure that the primary and/or secondary antibody is working properly Store the antibodies per manufacturer instructions Avoid contamination of antibodies and exposure to light
Insufficient deparaffinization	Increase the deparaffinization time Use fresh dimethylbenzene
The protein is located in the nucleus and the antibody cannot penetrate the nucleus	Add a permeabilizing agent (e.g. Triton X) to the blocking buffer and antibody dilution buffer
The PBS buffer is contaminated with bacteria that damage the phosphate groups on the protein of interest	Add 0.01% azide in the PBS antibody storage buffer Use fresh sterile PBS
The primary and the secondary antibodies are not compatible	Use a secondary antibody that was raised against the species in which the primary was raised (e.g. if the primary antibody was raised in mouse, an anti-mouse secondary antibody should be used) Check that the isotypes of the primary and secondary antibodies are compatible
The protein is not present in the tissue of interest or is not sufficiently expressed	Run positive controls to ensure that target protein is present in the tissue Include an amplification step in your protocol Use higher antibody concentration
Insufficient antibody to detect protein of interest	Use a higher antibody concentration Incubate for a longer time (e.g. overnight at 4°C)
Tissue has dried out	Cover the tissue sections in liquid at all time during the experiment

High Background

Possible Cause	Solution
The blocking buffer is incorrect	Make sure to use the blocking buffer recommended by the manufacturer
Blocking is insufficient (Do not over-block the tissue because antigenic sites may be masked)	Increase blocking time Change blocking reagent: (a) For tissue sections, use 10% normal serum (1 hour) (b) For cell cultures, use 1-5% BSA (0.5 hours)
The primary antibody concentration is too high	Titrate the antibody to determine the optimal concentration Incubate at 4°C
Non-specific binding by secondary antibody	Run a secondary control without primary antibody: If you see staining with your secondary only: (a) Change secondary antibody or (b) Use secondary antibody that has been pre-adsorbed Block sample with serum from the same species as the host in which the secondary antibody was raised
Endogenous peroxide or phosphatase is active	Quench the endogenous peroxidase or phosphatase activity by enzyme inhibitors: (a) Peroxidase: use H_2O_2 and methanol (v/v: 0.3%:

	99.7%) (b) Phosphatase: 2 mM Levamisol
Tissue section is too thick for reagent penetration	Prepare thinner section
Too much substrate was applied (enzymatic detection)	Dilute substrate Reduce substrate incubation time Choose substrate of higher S/N ratio e.g. Metal-enhanced DAB
Incubation temperature is too high	Incubate samples at 4°C
Primary antibody was raised in the same species as source of tissue (therefore, secondary antibody recognizes and binds non-specifically to the tissue)	Use primary antibody raised against a species which is different from the source of tissue Use biotinylated primary antibody and conjugated streptavidin for the detection system
Secondary antibody binds endogenous IgG	Include control slide stained without the primary antibody to confirm whether the secondary antibody is the source of the background
Fixation reagents are still present (Due to insufficient tissue washing)	Wash the tissues extensively with PBS buffer
Reaction between chromogens and PBS buffer in tissue or cell samples	Before incubating with the substrate, use Tris buffer to wash the samples
Membrane damage by permeabilization	Use a less stringent detergent such as Tween 20 (instead of Triton X) Remove permeabilizing agent from your buffers
Insufficient deparaffinization	Increase the deparaffinization time Use fresh dimethylbenzene
High levels of endogenous biotin in biotin-based detection systems for samples (e.g. liver and kidney tissues)	Perform biotin block after normal blocking procedure (before primary antibody incubation) Use polymer-based detection

RELATED PRODUCTS

Component	Catalog #	Size
10x TBS pH 7.5	MB-012	1000 ml
10x TTBS pH 7.5	MB-013	1000 ml
10X PBS pH 7.2	MB-008	1000 ml
10x PBST pH 7.2	MB-075-1000	1000 ml