Immunohistochemistry (IHC) Protocols

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-Instruction

Immunohistochemistry depends on the specific binding between antigen and antibody. It is a technology that uses color-labeled (such as fluorescein, enzyme, metal ions and isotope) secondary antibody to recognize the primary antibody. So we can confirm the expression and location of target protein in cells. For paraffin sections, we need to perform dewaxing and hydration at first, and then remove the possible interference of endogenous enzyme and biotin. Furthermore, antigen repair is needed to expose concealed epitopes and followed by blocking and incubation of antibody. Finally, we can observe the outcome of the experiment under microscope.

-Solution and reagents

2%APES- acetone (makes 300ml)	
APES	**6ml**
acetone	**300ml**
Mix the solution up.	
25×PBS (makes 5000ml)	
KCl	**25g**
Na_2HPO_4 $2H_2O$	**180g**
KH_2PO_4	**55g**
NaOH	**7g**
NaCL	**1000g**
Fully dissolve the compounds. Then add water to make up to 5000ml.	
PBST (makes 1000ml)	
1×PBS	**1000ml**
Tween-20	**5μl**
Mix up	
Citric acid sodium citrate buffer (makes 500ml)	
Trisodium citrate dihydrate	**14.7g**

Fully dissolve in 500ml ddH_2O	
Citric acid monohydrate	**4.2g**
Fully dissolve in 200ml ddH_2O	
Add 9ml citric acid monohydrate solution into 41ml trisodium citrate dihydrate solution and mix up	

-Dewaxing and hydration

Remove of paraffin wax in the sections and expose the tissue. Further, recover the water environment of tissue to help the detection.

1. Soak slices with xylene or instead with environmental friendly dewaxing agent at least for 60min to dewax the tissue. It is better to heat up the slice to melt paraffin before this process, which will be more effective to remove paraffin wax.

2. Place the staining rack in gradient alcohol, which is 100%, 100%, 95%, 85%, 75%, 60% in turn, for 5min respectively.

3. 0.3% TritonX-100 is used to permeate cell membrane which is helpful for molecules to get into the target protein. 5min is enough.

NOTE: a. Keep the sections moist all the time. Otherwise there will be a high background.

b. Xylene is toxic. You can use tissue transparent dewaxing agent instead.

c. Before dewaxing, the section can be heated to help achieve a better dewaxing effect.

d. 2% APES- acetone buffer is usually used to prevent stripping of tissue from the slice by soaking the slice in it for 1min and heating the slice at 60℃ for 15min.

e. The time of dewaxing process will vary from temperature and reagents. In summer, room temperature is relatively high, so you can shorten the time properly. Besides, IF the dewaxing agent has been used for some time, you'd better extend the time or replace with fresh reagents.

-Removal of endogenous enzyme interference and antigen repair

In the fixing process, formaldehyde can cross-link with the target antigen in the tissue to block the antigenic determinant. Therefore, it is necessary to open the cross-linking effect between antigen and formaldehyde to improve the tissue antigen detection rate. There several approaches to the point, including enzyme repair and hotfix. After experimental verification, hotfix works better than enzyme repair. Besides, antigen retrieval effect is also influenced by the repair buffer. There are three buffers that differ in pH, including citrate buffer (pH 6.0), EDTA buffer (pH 8.0), and TE buffer (pH 9.0). Generally, the higher

the pH value is, the stronger the repair forces. Endogenous enzymes, including peroxidase and biotin, can interact with chromogenic substrate DAB and biotin labeled secondary antibody resulting in false positive results. So we need to remove the endogenous enzyme. A long-term experiment confirms that 3% H_2O_2 which is diluted by ddH_2O or PBS or TBS or methanol can well eliminate the effect of endogenous enzyme for 10min.

1. 3% H2O2 is used to remove the interference of endogenous enzyme for 5min.
2. Soak slices in PBS pH 7.4.
3. Prepare 500ml citric acid sodium citrate buffer in a 1L beaker, and place it in a pressure cooker. Heat the buffer with a gasket in the water bath to 100℃. Then, the staining rack is placed in the boiled buffer. The beaker mouth is sealed with plastic wrap to prevent the water vapor from entering the buffer during heating. Continue heat the cooker with 1000W power to a high pressure state and keep for 2min. Then, heating is stopped immediately.
4. Wait until the pressure cooker cools naturally and you can open the lid. Take out the beaker and blow it to the room temperature by natural cooling.
5. Wash the slices with PBS for 5min by 3 times.

-Blocking

The process is to block the nonspecific binding of antibodies with the serum which is from same source of secondary antibody.

10% non-immunized goat serum is used to enclose tissues, each piece with 100ul, place the slice in wet box for 30min at RT.

-Antibody incubation

Validated primary antibody should be selected for the IHC experiment. The dilution ratio and incubation time of antibody should be optimized according to the actual situation. Low temperature and long incubation time are recommended to ensure a better binding between antibody and antigen.

1. Wipe off the blocking buffer.
2. Dilute antibody to a proper concentration and add to the tissues to incubate overnight at 4℃. We recommend 0.01μg/ml as a working concentration.
3. Wash the slices in PBST for 3×5min.
4. According to instructions, dilute the second antibody to working concentration and incubate for 1h at 37℃. We choose the Biotin-labeled antibody as a second antibody.

5. Wash the slices in PBST for 3×5min.

6. According to instructions, dilute the third antibody to working concentration and incubate for 1h at 37℃.

7. Wash the slices in PBST for 3×5min.

NOTE: a. In order to seek for an optimum antibody incubation concentration, a preliminary experiment should be done before the formal experiment by setting a gradient dilution.

b. We recommend a long time and a low temperature condition to ensure the full combination between antibody and antigen.

c. Slices must be washed gently and clean, especially as you incubate different antibody.

-Staining

Different detecting systems perform in this process. A labeled antibody reacting directly with the substrate is called one-step staining method. But it is expensive. Besides, you can stain the target protein by labeling the secondary antibody to amplify detection signal, which is called two-step method. There is also a three-step method that labels a third antibody to get a better amplification of detection signal. The label groups can be HRP, AP and Biotin. The group can be colored by reacting with different substrates. DAB is a common staining substrate. As follow, we perform the three-step method, in which the secondary antibody is labeled by Biotin and the third antibody is marked by HRP. Finally, the nucleus is stained with hematoxylin.

1. Wash the slices in PBS for 2×5min.

2. Prepare fresh DAB, and filter it before use.

3. Add DAB to the tissues and observe the staining under the microscope immediately.

4. When the tissue has an obvious staining, you have to wash the slices in the PBS in time.

5. Hematoxylin staining solution is used to dye nuclear to make the cell structure clearer.

NOTE: a. DAB and hematoxylin should be filtered before use.

b. H2O2 should be fresh when added into the DAB before use.

c. Pay close attention to the termination color time by observing the reaction between label groups and substrate below the microscope. The time is not more than 10min.

-Detection

1. Heat the slices at 60℃ to dry up, and then add proper neutral balsam to seal the tissue

2. Observe the staining under the microscope and take photos with the right field of view.