1.Specimen fixation

The purpose of fixation is not only to solidify protein in cells, to reduce or terminate the reaction of endogenous or exogenous intracellular decomposing enzymes, but also to prevent autolysis of tissue cells, so as to preserve the antigenicity of tissues or cells, so that antigens do not lose activity and or disperse. The tolerance of different antigens to the fixed solution is different, so the appropriate fixative should be chosen according to the antigens.

Now the commonly used fixers are neutral formaldehyde, 4% polyformaldehyde - phosphate buffer. Some fixed agents have better effect on special organization, for example, picric acid has a fixed effect for softening scalp and nails, Helly is better for fixing islet and pituitary, PLP is better for fixing liquid sugar and preserved tissue antigen.

2. Dehydration, paraffin embedded and cut into slices

Dehydration with gradient ethanol (low to high) and full dehydration, for some brittle tissues, such as liver, spleen, etc. It should reduce the retention time of high concentration alcohol, and transparent time should also be controlled.

For tissue impregnation, the paraffin wax is usually used at 56 ℃~58℃ melting point, and the best temperature of the wax impregnation is not more than 60 ℃, so as to prevent the loss of antigen.

Buried the tissue quickly so that the section has a complete cut. Check if the blade is notched in time to prevent the waxes from scratching.

3.Deparaffinizing and rehydration

Deparaffinize the section to the normal state and exposes the antigen to facilitate the binding with the antibody. If the dewaxing or rehydration not prone to focal reaction and immersion is not complete, it may cause nonspecific background staining.

4.Antigen retrieval

Since the tissues are immobilized in formaldehyde or polyformaldehyde, the crosslinking of proteins and the sealing of aldehyde groups have been taken to cover the antigenic determinants. Through the antigen retrieval, the antigen determination re-exposed to make the antigen be detected by specificity antibodies.

The commonly retrieval methods are divided into three types from strong to weak, high pressure heating repair, microwave repair, and pancreatin repair. High pressure heating repair is simple and easy to operate, and the effect is better than the others.

It is very important to control the retrieval temperature and time when use high pressure heating. The higher temperature, the shorter of repair time, the temperature is negatively related to the repair time. After the retrievalling, cooling the section at room temperature,

so that the protein refolds naturally. Use excessive antigen repair solution to prevent the high temperature liquid volatilization dry to avoid causing irreversible damage to the slices.

5.Inactivation

In the traditional ABC method and SP method, the immunohistochemical reaction is easily interfered by endogenous peroxidase and biotin, so the slices must be inactivated and blocked by hydrogen peroxide and ovalbumin.

It's generally inactivated endogenous peroxidase with 3% hydrogen peroxide for 10 min, and the use of methanol to dilute hydrogen peroxide is better for protecting antigens and fixed tissues.

6.Serum blocking

In order to prevent the non-specific combination of the primary antibody with the tissue, the BSA or goat serum can be used to block these loci. The blocking serum is usually from the same animal of secondary antibody and the blocking time is 10-30 min at room temperature.

7.Antibody incubation

Different antibody concentration, incubation time and temperature have a great influence on the dyeing results. At 4℃, the reaction is slow and the background is shallow. The reaction is faster at 37 ℃ so the incubation time must be shorter. Choosing room temperature incubation is helpful for the convenience of the experimental process, and it also applies to the detection of more samples. It's general to incubate secondary antibody at room temperature for 30 min.

8.slices wishing

In order to prevent nonspecific staining caused by residues such as primary antibody and secondary antibody, proper wishing is especially important. It is recommended to wash 30s for 3 times, and TBST can be used separately after primary antibody incubation. While wishing, we should pay attention to prevent the contamination from the cross reaction, gently rinse and prevent the removal of the tablet. The pH of TBS is suggested to be used as 7.6-8.0, with a concentration of 0.01 M.

9.DAB Developing

The background and the depth of the specific staining can be determined by the conditions of DAB incubation. The color time of DAB is not fixed, and the time is mainly controlled under the microscope, washing the slices when the specific color is stronger and the background color is shallow.

DAB incubate for a short time (a few seconds or ten seconds) appears with deep brown, it means that he antibody concentration is too high, need to dilute the antibody concentration; Deep background with DAB incubate for a short time, there may be nonspecific protein insufficiency, need to extend the blocking time; DAB incubate for a long time (more than ten minutes) to appear positive staining may be caused by low concentration of antibody or too long time with blocking time.

For a weaker DAB color, an enhancement method can be taken sometimes. Adding metal ions such as copper sulfate, gomori methenamine silver, cobalt chloride, nickel sulfate, nickel chloride and imidazole.

10.Re-dyeing

After immunohistochemical staining, the cell nucleus must be stained to foil the structure of the tissue. At present, Mayer's hematoxylin is commonly used for dyeing about 2 min. It can shorten the re-dyeing time when nucleoprotein is dyed by DAB, then ammonia or pH 8 TBS returns to blue.

11.Dehydration and mounting

In order to preserve the Immunohistochemistry result for a long time, neutral balsam was used as mounting. Prevent effects of bubble with glass mounting a. If the neutral balsam is sticky, xylene can be added to dilute to the neutral balsam which makes neutral balsam quickly spread while mounting. It’s recommended that mounting while there is still xylene residual and operates in the fuming cupboard which helps to remove the bubble clean and does not affect the follow-up mirror inspection.