Materials and Reagents used in this protocol:

Description:

Formalin or other aldehyde fixatives (Ex: Formaldehyde, glutaraldehyde…) forms protein crosslinking within and between proteins that might mask the antibody epitopes in tissue specimens, thereby leading to get weak or false negative staining for immunohistochemical staining. The citrate and EDTA based solution are designed to break the protein cross-links, thereby unmasking the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections and enhancing the staining intensity of antibodies.

Solutions:

Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0):

Tri-sodium citrate (dihydrate) --------- 2.94 g

Distilled water ---------- 1000 ml

Mix to dissolve. Adjust pH to 6.0 with 1N HCl and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for up to 3 months; for longer storage, it is recommended store the solution at 4°C.

Citrate Buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0):

Citric acid (anhydrous) ---------- 1.92 g

Distilled water ---------- 1000 ml

Mix to dissolve. Adjust pH to 6.0 with 1N NaOH and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for up to 3 months; for longer storage, it is recommended store the solution at 4°C.

EDTA Buffer (1mM EDTA, 0.05% Tween 20, pH 8.0):

EDTA ---------- 0.37 g

Distilled water ---------- 1000 ml

Mix to dissolve. Adjust pH to 8.0 with 1N NaOH and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for up to 3 months; for longer storage, it is recommended store the solution at 4°C.

Note: EDTA buffer is used to improve staining with low abundance epitopes and with antibodies that have weak affinity; it appears to enhance staining for many antibodies, although background staining is often increased (maybe due to endogenous biotin revealed after this pretreatment). So primary antibody can often be highly diluted.

Tris-EDTA Buffer (10mM Tris Base, 1mM EDTA, 0.05% Tween 20, pH 9.0):

Tris Base ---------- 1.21 g

EDTA ---------- 0.37 g

Distilled water ---------- 1000 ml

Mix to dissolve. Adjust pH to 9.0 with 1N NaOH and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for up to 3 months; for longer storage, it is recommended store the solution at 4°C.

Note: EDTA buffer is used to improve staining with low abundance epitopes and with antibodies that have weak affinity; it appears to enhance staining for many antibodies, although background staining is often increased (maybe due to endogenous biotin revealed after this pretreatment). So primary antibody can often be highly diluted.

Washing Buffer:

1XPBS:

NaCl ---------- 8 g

KCl ---------- 0.2 g

Na_2HPO_4 ---------- 1.44 g

KH_2PO_4 ---------- 0.24 g

Distilled Water---------- 800 ml

Adjust pH to 7.2 with HCl.

Adjust volume to 1 L with additional Distilled Water.

1 x PBST (PBS with 0.05% Tween 20):

Add 0.5 ml of Tween 20 in 1L 1X PBS and mix well.

1X TBS (50mM Tris Base, pH 7.6):

Tris-base ---------- 6.05 g

NaCl ---------- 8.76 g

Distilled water ---------- 800 ml

Adjust pH to 7.6 with HCl.

Adjust volume to 1 L with additional Distilled Water.

1 xTBST (TBS with 0.05% Tween 20):

Add 0.5 ml of Tween 20 in 1L 1X TBS and mix well.

Note: PBS buffer is used for routine IHC staining. TBS is used for IHC staining when background is high or when alkaline phosphatase conjugated antibodies are used. PBS is often used for diluting secondary antibodies or streptavidin-HRP conjugate. TBS is often used for diluting secondary antibodies or streptavidin-AP conjugate. PBST/TBST is often used for washing steps.

Peroxidase Blocking Solution (3% H_2O_2 in PBS):

30% H_2O_2---------- 2 ml

1XPBS ---------- 18 ml

Mix well and store at 4°C for up to 3 months.

This solution is recommended for paraffin sections

Blocking Buffer:

Normal Serum Blocking Buffer: 1-10% Normal serum from host species of secondary antibody (blocking) in TBST or PBST.

BSA Blocking Buffer: 1-5% BSA in TBST or PBST.

Mix well and store at 4°C.

Process:

Tissue Sections Preparation and Deparaffinization / Rehydration:

- Fix the tissue in 10% formalin at 4°C overnight.
- Embed fixed tissue in paraffin.
- Mount the tissue sections on slides.
- Heating the samples at 55°C for ten minutes to melt the paraffin.
- Clearing the paraffin with xylene for ten minutes; move slides to a fresh dish of xylene for an additional ten minutes.
- Wash slides in xylene for 5 min at room temperature (3X).
- Wash slides in 100% alcohol for 3 min at room temperature (2X).
- Wash slides in 95% alcohol for 3 min at room temperature (2X).
- Wash slides in 70% alcohol for 3 min at room temperature (1X).
- Wash slides in 50% alcohol for 3 min at room temperature (1X).
- Rinse slides with distilled H_2O for 5 min at room temperature.

Heat-induced epitope retrieval: Sodium Citrate/EDTA Antigen Retrieval:

- Place slides in a glass slide holder and fill in the rest of the rack with blank slides (10 totals) to ensure even heating.
- Place rack in 600 ml of Antigen Retrieval buffer in a glass 2 L-beaker. Mark a line at the top of the liquid on the beaker.
- Microwave at 850W for 20 min total, replacing evaporated water every 5 min. Do not allow the slides to dry out.

  (Alternatively:
  - Pre-heat steamer or water bath with staining dish or beaker containing Antigen Retrieval buffer until temperature reaches 95-100 °C.

- Immerse slides in the staining dish. Place the lid loosely on the staining dish and incubate for 20-40 minutes (optimal incubation time should be determined by user).

➢ Cool slides for 20 -30 min at RT.
➢ Wash slides in dH2O for 5 min twice.
➢ Wash slides in 1X PBST or TBST for 5 min.

Blocking

➢ Block endogenous peroxidases by soaking slides in a solution of 3% H_2O_2 for 15 minutes at room temperature. Wash slides in PBST or TBST for 5 min x 3 times.
➢ Immerse slides in a dish containing blocking buffer (normal serum from host species of secondary antibody to be used, diluted 1:10 in TBS). Incubate at 37°C for one hour.

Immunostaining:

➢ Remove blocking buffer. Cover the tissue sections with primary antibody diluted in blocking buffer. Incubate for 1 hour at 37°C.
➢ Remove primary antibody solution and wash slides in PBST or TBST for 5 min x 3 times.
➢ Cover the tissue sections with secondary antibody diluted in blocking buffer according to manufacturer's instructions. Incubate at 37°C for 30 min.
➢ Remove secondary antibody solution and wash slides in PBST or TBST for 5 min x 3 times.
➢ Add DAB to each slide and monitor staining closely.
➢ **As soon as the target signal developed,** immerse slides in ddH_2O, or rinse the slides in running tap water for 5 min.
➢ Stain slides for 10-30 seconds with counter stain reagent (Ex: hematoxylin (blue)).
➢ Wash slides in ddH_2O for 5 min x 2.

Dehydrate/ Mounting:

(This method should only be used if the chromogen substrate is alcohol insoluble (e.g. Vector Red, DAB))

➢ Wash slides twice in 80% alcohol for 1 min at room temperature.
➢ Wash slides twice in 95% alcohol for 1 min at room temperature.
➢ Wash slides 3X in xylene for 1 min at room temperature.
➢ Mount the sections with coverslip.