# Protocol: Immunohistochemical staining (IHC)

## MATERIALS/ REAGENTS/ BUFFERS

- Tissue sections
- Primary antibody
- Conjugated secondary antibody
- ABC reagents
- Xylene
- 100% ethanol
- 95% ethanol
- 3% hydrogen peroxide (H2O2)
- PBS
- TBS
- Antigen retrieval solution: Ex. Sodium Citrate Solution (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0)
- Pepsin
- Blocking buffer: 5-10% normal animal serum

## PROTOCOL

### I.Tissue fixation

1. Harvest tissue and rinse in Phosphate-buffered saline (PBS) to remove blood.
2. Cut tissue into 3 mm slices before transfer into formalin due to the slow rate of diffusion of neutral buffered formalin (NBF).
3. Place tissue in at least 20 volumes of fixative 10% NBF.
4. Incubate for necessary fixation time (fixation between 18-24 hr seems to be ideal for most applications).
5. Rinse tissue with PBS, store at 4°C in 75% ETOH in $H_2O$.

### II.Tissue processing

1. After fixation, rinse tissue with PBS until fixative is completely removed.
2. Dehydrate tissue using ethanol in the following sequence:
   50% Ethanol: 50 min
   75% Ethanol: 50 min
   90% Ethanol: 50 min
   95% Ethanol: 50 min
   95% Ethanol: 90 min
   100% Ethanol: twice, each for 50 min
   100% Ethanol: 90 min
3. Exchange ethanol with xylene in the following sequence
   Ethanol : Xylene (1:1): 50 min
   Xylene : 50 min
   Xylene : 50 min





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## II.Tissue processing

3. Exchange ethanol with xylene in the following sequence
   Ethanol : Xylene (1:1): 50 min
   Xylene : 50 min
   Xylene : 50 min
4. Exchange xylene with paraffin at 58°C.
   Xylene : Paraffin (1:1): 60 min
   Paraffin: 120 min
   Paraffin: 120 min

## III.Tissue embeding

1. Put small amount of molten paraffin in mold, dispensing from paraffin reservoir
2. Transfer tissue into mold by warm forceps, placing cut side down.
3. Transfer mold to cold plate, paraffin will solidify in a thin layer which holds the tissue in position.
4. Add hot paraffin to the mold from the paraffin dispenser. Be sure there is enough paraffin to cover the face of the plastic cassette.
5. Paraffin should solidify in 30 minutes.

## IV.Tissue Sectioning

1. Placed tissue blocks face down on an ice block or cold plate for 10-30 minutes.
2. Place a fresh blade on the microtome. Blades may be used to section up to 10 blocks, but replace if sectioning becomes problematic
3. Insert the block into the microtome chuck so the wax block faces the blade and is aligned in the vertical plane.
4. Set the dial to cut 10μm sections to order to plane the block; once it is cutting smoothly, set to 4μm sections .
5. Pick sections and float them on the surface of the cold water. Float the sections onto the surface of clean glass slides.
6. Place the slides with paraffin sections on the surface of the 56℃ water bath to bond the tissue to the glass.

## V.Immunohistochemical staining (IHC)

### Dewaxing & Rehydrate

1. Place the slides with 4μm paraffin sections on the warming block in a 60°C oven for 30 minutes.
2. Perform the following washes
   Xylene: twice, each for 5 min
   100% ethanol: twice, each for 5 min
   95% ethanol: 5 min
   75 % ethanol: 5 min
   50 % ethanol: 5 min
   Rinse slides with deionized water

### Antigen retrieval (Heat-Induced Epitope Retrieval )

1. Fill individual plastic container(s) with appropriate retrieval solution and add enough deionized water to pressure cooker chamber or vegetable steamer.
2. Bring slides to a boil in antigen retrieval solution for 15 min in the pressure cooker or 30 min in the vegetable steamer.
3. Transfer slides to room temperature Tris-Buffered Saline (TBS).
4. Tissues are now ready for blocking and primary antibody incubation.

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## IHC staining

1. Wash sections in TBS twice, each for 3 min.
2. If using an HRP conjugate for detection, blocking of endogenous peroxidase can be performed here by incubation of sections in 3% H2O2 for 10 min.
3. Wash section in TBS twice, each for 3 min.
4. Block each section with blocking buffer for 30 min at RT.
5. Remove blocking buffer and add primary antibody at proper dilution and incubate for overnight at 4°C.
6. Remove antibody solution and wash sections in TBS three times, each for 5 min.
7. Add secondary antibody, and incubate for 30 min at RT.
8. Prepare avidin-biotin-enzyme complex (ABC) reagent according to the manufacturer's instructions.
9. Remove secondary antibody solution and wash sections in TBS three times, each for 5 min.
10. Add ABC reagent to each section and incubate for 30 min at RT.
11. Remove ABC reagent and wash sections in TBS three times ,each for 5 min.
12. Add DAB to each section and monitor staining closely.
13. As soon as the sections develop, immerse slides in ddH20.
14. Counter-stain sections in hematoxylin.
15. Wash sections in ddH20 twice, each for 3 min.
16. Dehydrate sections
    75 % ethanol: 3 min
    95% ethanol: 3 min
    100% ethanol: 5 min
    Xylene: 5 min
17. Mount sections with coverslip.

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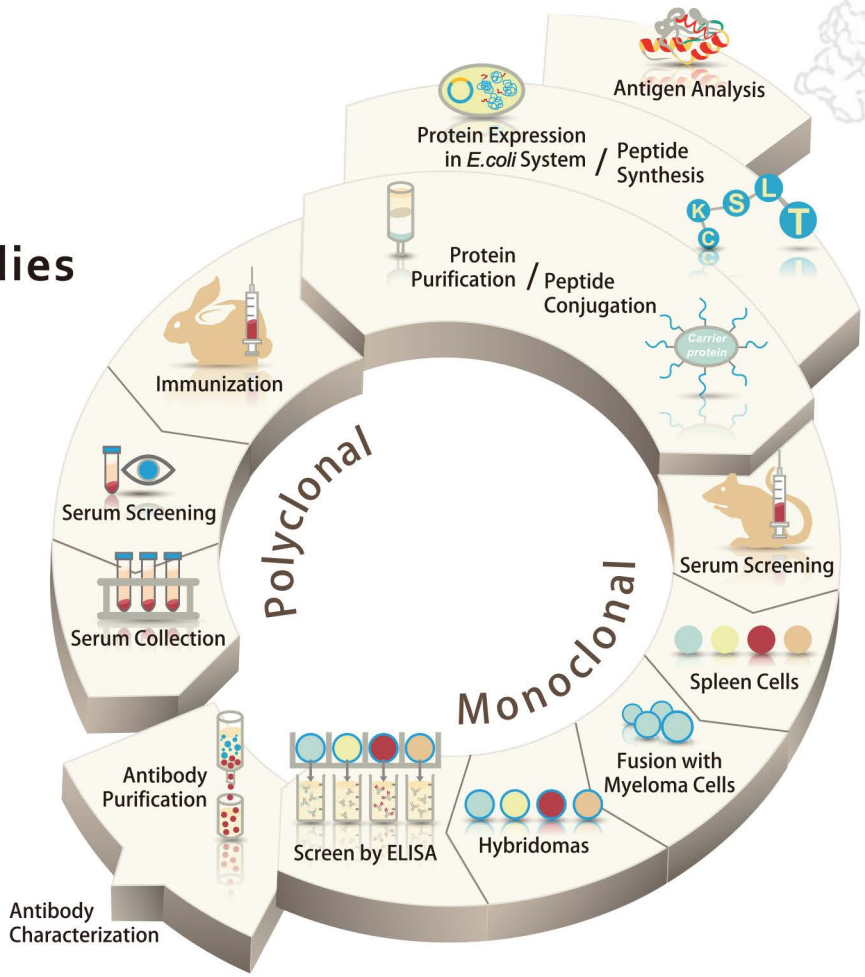


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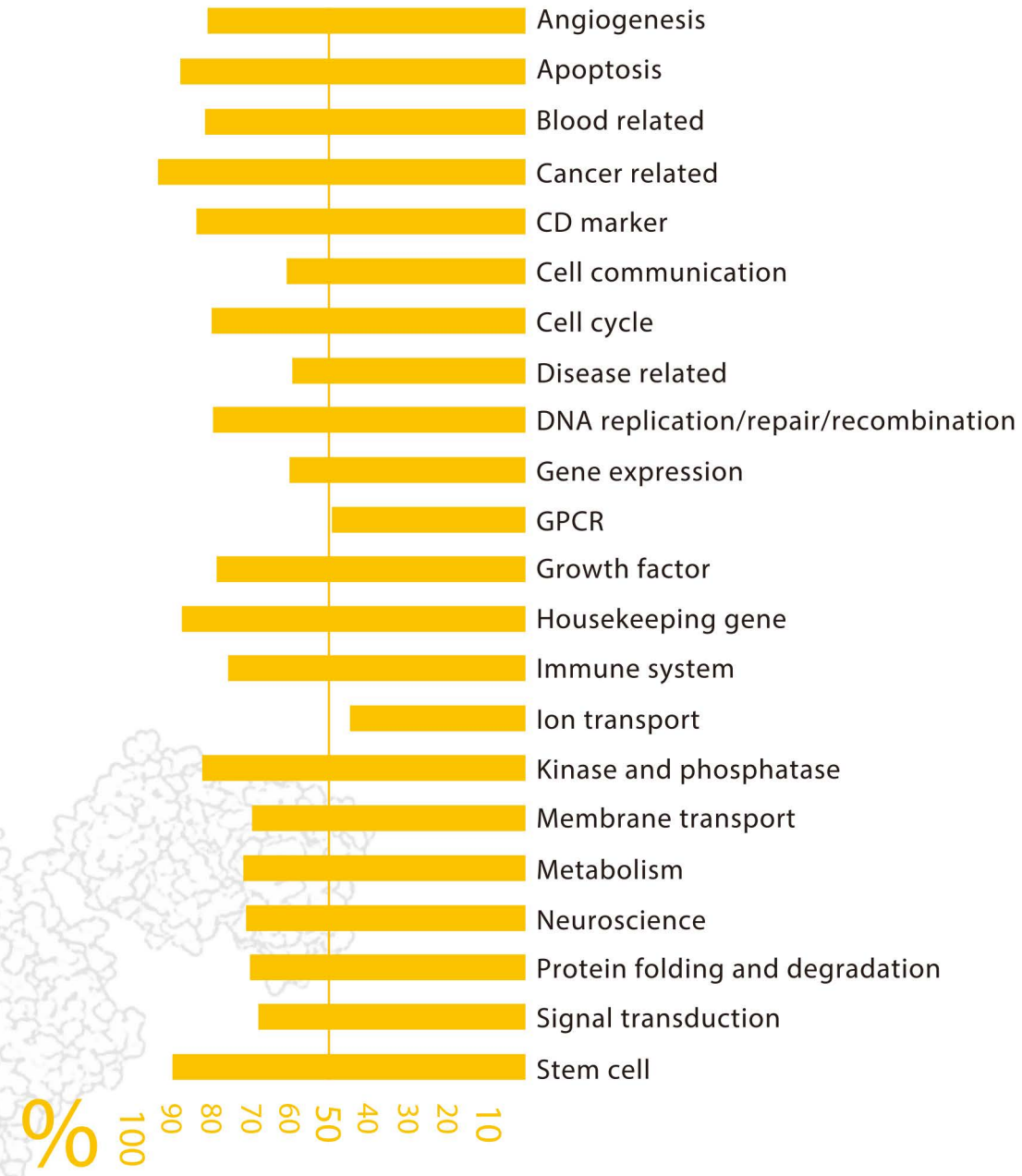


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