Immunohistochemistry on paraffin-embedded tissues (IHC-P)

1. Background and purpose of the procedure

To visualize protein localization within cells and tissue, using primary antibodies against protein and secondary antibodies attached to horseradish peroxidase and deposition of DAB.

2. Materials and equipment

- Paraffin-embedded tissue sections (4 to 5 µm thick)
- Xylene (Epredia, 6601)
- Ethanol Anhydrous (Fisher Chemical, Histological, A405P-4)
- ddH_2O
- Sodium citrate buffer for antigen retrieval (*see 3. Recipes*)
- Peroxidase block solution (*see 3. Recipes*)
- Tris-buffered saline wash solution (*see 3. Recipes*)
- Donor goat serum blocking solution (*see 3. Recipes*)
- Primary antibody specific for biomarker staining
- SignalStain Boost HRP anti-rabbit (Cell Signaling Technology, 8114)
- SignalStain Boost HRP anti-mouse (Cell Signaling Technology, 8125)
- SignalStain DAB substrate kit (Cell Signaling Technology, 8059)
- Mayer's (Lillie's modification) Hematoxylin stain (Volu Sol, VMH032)
- Ammonium hydroxide bluing solution (*see 3. Recipes*)
- Cytoseal 60 (Epredia, 831016)

- Absorbent paper
- Fume hood (for work with xylene baths)
- Oven at 60˚C for slide baking (if required)
- Deparaffinization and staining containers and slide holder racks
- Vegetable steamer
- Styrofoam container for cooling
- Pap-pen (Scientific Device Laboratory, Aqua-Hold Pap Pen 2, 980402)
- Slides incubation chamber
- Cover glass thickness No. 1 (Epredia, 22 x 40 mm, 102240)
- Pipets and tips
- Pipet gun and pipettes
- Tubes (1.5 mL, 15 mL, and 50 mL)
- Kimwipes (Kimberly-Clark Professional, 34155)

3. Recipes

a. Sodium citrate buffer solution 10X concentrate
100 mM Sodium citrate dihydrate (Fisher Chemical, Certified, S279-500) *[29.4 g for 1 L]*
ddH_2O qsp 1 L

Adjust pH to 6.0

b. Sodium citrate buffer for antigen retrieval
10% Sodium citrate buffer solution 10X concentrate *[100 mL for 1 L]*
0.05% Tween (Fisher Bioreagents, BP337-100) *[500 µL for 1 L]*
ddH_2O qsp 1 L

c. Peroxidase block solution
3% Hydrogen peroxyde (Fisher Chemical, 30% Certified ACS, H325-500) *[500 µL for 5 mL]*
ddH_2O qsp 5 mL

d. Tris-buffered saline (TBS) 10X concentrate
250 mM Tris-base (Fisher Bioreagents, Molecular Biology, BP152-500) *[24.2 g for 1 L]*
1.37 M Sodium chloride (Fisher Chemical, Certified ACS, S271-1) *[80 g for 1 L]*
ddH_2O qsp 1 L

Adjust pH to 7.6 using HCl (Fisher Chemical, Certified ACS Plus, A144-500)

e. Tris-buffered saline wash solution (TBS-T)
10% Tris-buffered saline 10X concentrate *[100 mL for 1 L]*
0.025% Tween 20 (Fisher Bioreagents, BP337-100) *[250 µL for 1 L]*
ddH_2O qsp 1 L

This solution can be stored for up to 6 weeks at room temperature.

f. Donor goat serum blocking solution
5% Donor goat serum (Novus Biological, heat inactivated, S13110HNOV) *[500 µL for 10 mL]*
TBS-T qsp 10 mL

This solution can be stored at 4˚C for maximum 5 days.

g. SignalStain DAB substrate working solution
1 volume of SignalStain DAB Chromogen Concentrate *[30 µL for 1 mL]*
33 volumes of SignalStain DAB Diluent *[970 µL for 1 mL]*

This solution can be stored at 4˚C for up to 14 days, or up to 5 days at room temperature.

h. Ammonium hydroxide bluing solution
37 mM ammonium hydroxide (Fisher Chemical, Certified ACS plus, A669-500) *[250 µL for 100 mL]*
ddH_2O qsp 100 mL

This solution can be conserved for 2 weeks at room temperature.

4. Method

A. Baking slides

a. If slides are not baked when received, warm up slides at room temperature for 45 min
b. Place slides in pre-heated oven
c. Incubate at 60˚C for 1 hr
d. Remove the slides from the oven and let the slides cool down at room temperature for 45 min

B. Deparaffinization and rehydration

Place the slides in a slide holder rack and perform the following washes:

Xylene ..... 12 min
Xylene ..... 5 min
Xylene/Ethanol (1:1) ..... 3 min
100% Ethanol ..... 3 min
100% Ethanol ..... 3 min
95% Ethanol ..... 3 min
70% Ethanol ..... 3 min
50% Ethanol ..... 3 min
Running dH_2O ..... Rinse
ddH_2O ..... Hold

C. Heat-induced epitope retrieval (HIER)

a. Place slides in plastic container filled with sodium citrate buffer for antigen retrieval and place in vegetable steamer (filled with 4 qtrs. of regular tap water) and set for 20 minutes. Steamer timer starts once the desired temperature is reached
b. After 20 minutes have elapsed, remove slide tray, and put the tray in the Styrofoam container for cooling. Run cold tap water into the Styrofoam container (not the slide container) for 10 minutes to cool down the slides
c. Rinse slides twice with dH_2O

D. Immunostaining

a. Delineate staining area using pap pen, be careful not to touch the tissue with the pap pen
b. Add peroxidase block solution on tissue *[75 to 300 µL depending on tissue area]*
c. Incubate for 10 min at room temperature
d. Rinse three times with dH_2O washes of 5 min
e. Add donor goat serum blocking solution *[75 to 300 µL depending on tissue area]*
f. Incubate for 30 min at room temperature
g. Remove blocking solution by inversion over absorbent paper
h. Add primary antibody diluted in donor goat serum blocking solution *[1:50 to 1:500 dilution depending on primary antibody, solution must cover the entire tissue]*
i. Incubate in a moisten slide incubation chamber overnight *[12 to 16 hrs]*
j. Remove primary antibody solution by inversion over absorbent paper
k. Rinse three times with TBS-T washes of 5 min

l. Add SignalStain Boost HRP secondary antibody solution *[anti-rabbit or anti-mouse according to the specie of the primary antibody used]*
m. Incubate at room temperature for 30 min to 1 hr
n. Remove secondary antibody solution by inversion over absorbent paper
o. Rinse three times with TBS-T washes of 5 min
p. Add SignalStain DAB substrate working solution
q. Stop DAB reaction after appropriate time by immersion of slide in dH_2O *[DAB staining usually takes 30 sec to 2 min]*
r. Rinse three times with dH_2O washes of 3 min

E. Counterstaining

a. Dip slide in Hematoxylin solution *[6-10 dips]*
b. Rinse slide under gentle dH_2O stream
c. Dip slide in ammonium hydroxide bluing solution *[6-10 dips]*
d. Rinse slide under gentle dH_2O stream

F. Dehydration and mounting

a. Place the slides in a slide holder rack and perform the following washes:

50% Ethanol	5 min
70% Ethanol	5 min
95% Ethanol	3 min
100% Ethanol	3 min
Xylene/Ethanol (1:1)	3 min
Xylene	3 min
Xylene (fresh)	Hold

b. Keep slides wet with xylene at all time
c. Add Cytoseal 60 to the tissue slide
d. Place coverslip over the tissue avoiding air bubbles
e. Let coverslipped slide cure for 12 hrs minimum
f. Wipe slide with xylene to remove excess Cytoseal before imaging

5. References

IHC-Paraffin protocol from Abcam

IHC staining method from University of Saskatchewan Health Sciences Histology Core Facility

Bayne LJ, Vonderheide RH. Immunohistochemical assessment of immune cells in mouse tumor tissue. Cold Spring Harb Protoc. 2013 Sep 1;2013(9):843-8. doi: 10.1101/pdb.prot077206. PMID: 24003205.

Rodig SJ. Counterstaining, Mounting, and Photographing Stained Cells. Cold Spring Harb Protoc. 2019 Apr 1;2019(4). doi: 10.1101/pdb.prot099770. PMID: 30936387.

6. Revision history

Revision #	Date	Prepared by
1.0	**2021-05-10**	**Elie Besserer-Offroy**
Summary of modifications		
Initial version of the protocol		