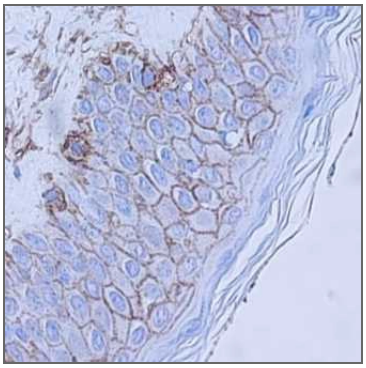

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Protocol status: Working
We use this protocol and it's working

Immunohistochemistry protocol optimized at iMM-JLA

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DISCLAIMER

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immunohistochemistry, histology, immunostaining, FFPE, frozen, Envision HRP

ABSTRACT

The immunohistochemistry (IHC) protocol is used to showcase the location of specific proteins in relation to the arquitecture of the tissue.
In this method, we use a primary antibody which binds to a specific antigen in the protein of interest. This antibody has a specific host and can be ready to use or undiluted. The secondary antibody is an anti-host IgG which will bind to the primary antibody [1]. It's also conjugated with an Horseradish Peroxidase (HRP).

The HRP will react with the Hydrogen Peroxide in the substract of the DAB solution, oxidizing the DAB, creating a brown deposit on the location of the protein of interest.

Depending on location of the protein, we can have staining in the nuclei, the cytoplasm or the cellular membrane.

Possible variables are:
the concentration of the antibody;
type of antigen retrieval;
the pH of the antigen retrieval solution (for Heat Induced Epitope Retrieval (HIER));
the time of incubation;
the tissue used [2].

You can use this protocol for Formalin-Fixed Paraffin-Embedded samples (FFPE) and Fixed Frozen samples (Gelatin and OCT).

MATERIALS

Coplin Jars
Incubation chamber
Slide Dipper
Slide dishes with Lids
Wash bottles with nozzle
Magnets
Magnetic mixer
Tweezers
Micropipettes p20, p200 and p1000 and respective tips
Hydrophobic PAP pen
Eppendorfs 1.5mL
Plastic pipettes
Equipment Pretreatment Module™ Deparaffinization and Heat-Induced Epitope Retrieval by Thermo Scientific

Reagents:
Ethanol 70%
Ethanol 96%
Ethanol 100%
Xylene
Distilled Water

PROTOCOL MATERIALS

Protein Block Serum free **Dako Catalog #Ref. X0909** Step 17

Hydrogen Peroxide Solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #7722-84-1**

Step 13

Entellan ® **Merck MilliporeSigma (Sigma-Aldrich) Catalog #1.00869** Step 29

EnvisionTM Dako DAB **Dako Catalog #Ref. K3468** Step 22

Harris hematoxylin for histology **Bio-optica Catalog #05-06004E** Step 24

Antibody Diluent **Dako Catalog #Ref. 8006** Step 18

EnVision FLEX Target Retrieval Solution Low pH **Dako Catalog #K8005** Step 10

EnVision FLEX Target Retrieval Solution High pH **Dako Catalog #K8004** Step 10

EnvisionTM Flex Wash Buffer **Dako Catalog #Ref. K8007** In 5 steps

EnVisionTM Flex conjugated w/ HRP **Dako Catalog #Ref. K4061** Step 20

EMSURE Methanol **Supelco Catalog #1.06009.2500** In 2 steps

SAFETY WARNINGS

This protocol uses the following hazardous chemicals:

Xylene
Methanol
Hydrogen peroxide
DAB
Resinous Mounting Medium

Please comply with all safety measures and risk assessment protocols.

Latex gloves can be used for the entire protocol, except when manipulating the reagents previously stated, during which you should use nitrile gloves. Type IV biological disposable bin should be readily available.

BEFORE START INSTRUCTIONS

If FFPE - begin protocol after adhering sections to the slides for 1h at 60ºC

Preparation of Wash Buffer

1 Measure 20 mL of 20X EnvisionTM Flex Wash Buffer **Bio-optica Catalog #Ref. K8007**

2 Make up with distilled water to 1 L of total volume

Sample Preparation after sectioning - FFPE

3 10m

Deparaffinize slides with Xylene for 00:10:00

4 Change Xylene and deparaffinize for another 00:10:00 10m

5 Place slides in Ethanol 100% for 00:05:00 5m

6 Place slides in Ethanol 96% for 00:05:00 5m

7 Place slides in Ethanol 70% for 00:05:00 5m

8 To complete the hydration, place slides in distilled Water for 00:05:00 5m

Sample Preparation after sectioning - Frozen

9 **If frozen samples:** 10m
Remove from storage and let dry.
If embedded in OCT, put in distilled water for 00:05:00 at Room temperature .
If embedded in gelatin, put in PBS for 00:05:00 at 37 °C .

Antigen Retrieval

10 Perform antigen retrieval using PT Module by Thermo Scientific with a cycle of pre-heat to 65ºC and heat to 95ºC for 20 minutes.

Note

This equipment, when optimized, allows you to skip the step of deparaffinization in this section, by putting the slides directly through the antigen retrieval step.
This equipment allows two buffers with different pH, in which we use
EnVision FLEX Target Retrieval Solution High pH **Dako Catalog #K8004** and
EnVision FLEX Target Retrieval Solution Low pH **Dako Catalog #K8005** , depending on the optimization of the antibody.

Blocking Endogenous Peroxidase and Total Proteins 35m

11 Wash in 1X EnvisionTM Flex Wash Buffer **Bio-optica Catalog #Ref. K8007** 3 times for 5m
00:05:00 each

12 Put slides in 200 mL of 100% EMSURE Methanol **Bio-optica Catalog #1.06009.2500**

Note

Extemporaneous solution. 100% methanol can be used up to 3 times.

13 Put 6 mL of Hydrogen Peroxide Solution **Bio-optica Catalog #7722-84-1** at 4 °C in the 100% EMSURE Methanol **Bio-optica Catalog #1.06009.2500** with the slides

14 Cover the slide dish and incubate slides in the solution for 00:30:00 30m

Note

Hydrogen Peroxide must be added each time when immersing the slides, to obtain a concentration of 3%.

15 Wash in 1X EnvisionTM Flex Wash Buffer **Bio-optica Catalog #Ref. K8007** 3 times for 00:05:00 each

16 Draw a limit on the edge of the tissue with the hydrophobic PAP Pen

17 Put Protein Block Serum free **Bio-optica Catalog #Ref. X0909** on the tissue

Incubation and Washing 1h 54m 10s

18 Incubate with Primary antibody in the optimized concentration (if needed, dilute in Antibody Diluent **Bio-optica Catalog #Ref. 8006**) for 01:00:00 at Room temperature 1h

Note

Normally, around 100µL of diluted antibody per section, depending on the size of the tissue.

19 Wash in 1X EnvisionTM Flex Wash Buffer **Bio-optica Catalog #Ref. K8007** 3 times for 00:05:00 each

20 Incubate with secondary antibody 30m

EnVisionTM Flex conjugated w/ HRP **Bio-optica Catalog #Ref. K4061** for 00:30:00

Room temperature

Note

Be sure to match the secondary antibody to the host of the primary antibody (ex: if using mouse anti-CD3 primary, use anti-mouse secondary)

21 Wash in 1X EnvisionTM Flex Wash Buffer **Bio-optica Catalog #Ref. K8007** 3 times for 00:05:00 each

22 Incubate with EnvisionTM Dako DAB+ **Bio-optica Catalog #Ref. K3468** for 00:02:00 to 00:05:00 7m

Safety information

After incubation, pour DAB excess on a paper towel and dispose on type IV waste, with tips and eppendorfs used, avoiding spills at any cost.
Clean the incubation chamber and surfaces with Bleach.

23 Wash in distilled water for 00:05:00 5m

Counterstain and mount

17m 10s

24 Counterstain with Harris hematoxylin for histology **Bio-optica Catalog #05-06004E** for 00:00:10 — 10s

Note

filter Hematoxylin before use

25 Put slides in lukewarm running water for 00:05:00 — 5m

26 Dehydrate with 96% ethanol for 00:02:00 — 2m

27 Place in 100% ethanol for 00:02:00 — 2m

28 Clear in Xylene for 00:10:00 — 10m

29 Mount with appropriate mounting medium (i.e. Entellan ® **Bio-optica Catalog #1.00869**)

Note

Let the slides dry in a fume hood until complete polymerization of the mounting medium is achieved