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

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DAB ANTIBODY (IHC) STAINING PROTOCOL

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ABSTRACT

This is the basic protocol for antibody staining of formalin fixed paraffin embedded (FFPE) tissue.

ATTACHMENTS

452-959.docx

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64108

Keywords: DAB ANTIBODY (IHC) STAINING PROTOCOL, Formalin fixed paraffin embedded (FFPE) tissue, FFPE, ASAPCRN

Funders Acknowledgement:

ASAP

GUIDELINES

Principle:

For antibody staining to be successful, most FFPE tissue requires antigen retrieval of some kind. Formalin fixation cross-links proteins during the course of fixation. Antigen retrieval unlinks the proteins and opens up the antigen sites so that the antibody will be able to bind to them.

References:

Carson, Freida, *Histotechnology – A Self-Instructional Text*, 2nd Edition, ASCP Press, 1997

MATERIALS

Specimen Preparation:

10% Neutral buffered formalin fixed tissue, or 4% paraformaldehyde fixed tissue, paraffin embedded sections cut at ~ 5-6 microns and mounted on charged slides.

Materials and Equipment:

- Charged slides
- Coverslips
- Drying oven, 60 degrees
- Fume Hood
- Gloves
- Microtome
- Staining racks
- Timer

Reagents:

- Xylene
- 100% Alcohol
- 95% Alcohol
- 80% Alcohol
- Harris Hematoxylin
- Background Sniper (Biocare Medical)
- TRIS with tween 20X (Biolegend,[hydroxymethyl aminomethane])
- Secondary antibody; i.e. Donkey anti-Rabbit
- Tertiary link; i.e., Vector Laboratories Vectastain ABC-HRP kit
- 3% Hydrogen peroxide
- DAB (Diaminobenzidine) from Biolegend
- DAB substrate Buffer (Biolegend)
- Permount or other xylene compatible mounting media

Pretreatment reagents:

- Reveal Decloaker (Biocare Medical) preferred
- Citrate buffer solution if you don’t use the Reveal
- Specific case for Beta-amyloid antibody
- 70% Formic acid treatment

SAFETY WARNINGS

Precautions:

Personal Protection:
Gloves, lab coat, goggles, fume hood, and use of universal precaution practices.

Chemical Wastes:
Dispose of alcohols, dyes, and xylene in appropriate labeled waste containers as directed by the University of Minnesota Hazardous Chemical Waste Management Manual 5th Edition.

Hazards:

- Xylene = Flammable, Carcinogen, Skin irritant
- Eosin & Alcohols = Flammable, Skin irritant
- Hematoxylin = Skin irritant
- Avoid strong oxidizers with all listed chemicals

Deparaffinize tissue: Day 1

45m

1 **2 options:** 45m

1) Either place slides on a slide warmer with temperature set to approx. 57 °C. Leave the slides on the warming plate until the paraffin looks melted on all of the slides (about 10 - 00:15:00) **or** 2) Put slides in a 60 °C oven for about 00:30:00.

2 Place slides vertically in the gray plastic slide holders and run the slides through the following solutions in the staining set up located in the fume hood.

	A	B
	Xylene	5 minutes
	Xylene	5 minutes
	Xylene	10 minutes
	100% Alcohol	3 - 5 minutes
	100% Alcohol	3 - 5 minutes
	95% Alcohol x 2	3 - 5 minutes

	A	B
	80% Alcohol	3 - 5 minutes
	Filtered water	3 - 5 minutes

STEP CASE

Preferred Antigen retrieval steps:

34 steps

3 Fill the vegetable steamer with deionized water to the second line in the transparent corner section of the steamer.

3.1 Turn steamer on and push the up arrow button until the time is about 00:40:00. 40m

> **Note**
>
> This will allow the steamer to heat up and be ready for the prepared slides.

4 Add capillary gap slides (same number as the number of slides you are staining) in the slide holder to wet the slides.

4.1 Fill the plastic “boat” containers with approx. 20 mL of one of the antigen retrieval solutions (Reveal or citrate buffer).

4.2 Pick up a set of slides (rough side of the capillary gap slide facing the tissue section slide) and put the slides into one of the troughs in the boat container.

4.3 Push slides toward each other and allow the fluid to come up slowly between the two slides.

> **Note**
>
> Try not to get bubbles between the slides or this will obstruct the antigen retrieval process and give you uneven staining.

4.4 Steam for about 00:30:00 . 30m

5 Remove clear basket from the steamer and allow slides to cool for about 00:20:00 to Room temperature . 20m

6 Transfer the “boats” of slides to the inner steamer container and rinse with running water for about 00:20:00 . 20m

6.1 Unpeel the slides from each other and transfer the slides to the gray slide holder for a final rinse (~ 00:05:00). 5m

Staining steps: 2h 38m

7 10m

Dilute or use prediluted TRIS (1x concentration) for 00:10:00 , approx 250- 300 µL per slide.

8 1h 28m

100% Background Sniper for nearly 00:13:00 . Do not go longer than 00:15:00 . (alternat 10% normal goat serum made in 1X TRIS or PBS can be substituted for 01:00:00).

9

Make up antibody solution at desired concentration in a diluent of5% Sniper in 1X TRIS solution. Using approx. 100 µL per slide, make up enough antibody to cover all slides.

10 1h

Cover the slides with either a glass coverslip (24 x 60 mm) or parafilm and put in a 4 °C . refrigera Overnight .

DAY 2:

1h 20m

11 Take slides out of the refrigerator and warm up to Room temperature about 00:15:00 - 00:20:00. Remove the cover slips and rinse the slides with 1X TRIS solution, then incubate slides in 1X TRIS solution for about 00:05:00 x 2. 40m

12 **Secondary reagent/antibody for** 00:30:00 30m

Apply the secondary reagent appropriate to the antibody, i.e., if your antibody was raised in a rabbit, you will apply an anti- Rabbit link.

> **Note**
>
> You will need to make up enough reagent for all the slides with approx. 250-300 microliter per slide or enough of the reagent to totally cover the tissue.If your antibody was raised in some other animal, you will have to find a secondary to that animal, i.e., donkey anti-mouse, goat anti-chicken, etc. Usually a dilution of 1:500 in a diluent of 5% Sniper made in 1X TRIS is a good starting concentration to use. Jackson ImmunoResearch Labs supply a host of secondary antibodies (**make sure the secondary is biotinylated**).

13 Rinse slides with 1X TRIS, 00:10:00. 10m

14 3% Hydrogen Peroxide made in 1X TRIS for 00:10:00 x 2.Rinse between with 1X TRIS. 10m

15 Rinse slides with 1X TRIS, 5 minutes x 2.

15.1 Rinse slides with 1X TRIS, 00:05:00 x 2 (1/2). 5m

15.2 Rinse slides with 1X TRIS, 00:05:00 x 2 (2/2). 5m

16 TERTIARY Reagent for 00:30:00. 30m

16.1 The Tertiary Reagent is made up about 00:25:00 before you apply it. 25m

Note

We typically use the Vectastain Elite ABC kit, Peroxidase (standard) from VECTOR Laboratories. The staining concentration is 100 µL reagent A to 5 mL diluent of 5% Sniper made in 1X TRIS, then add 100 µL B to the solution, mix and let sit until applied to the tissue.

17 Rinse slides with 1X TRIS, 5 minutes x 2.

17.1 Rinse slides with 1X TRIS, 00:05:00 x 2 (1/2). 5m

17.2 Rinse slides with 1X TRIS, 00:05:00 x 2 (2/2). 5m

18 DAB (DIAMINOBENZINE) chromogen for approximately 00:03:00 to develop. 3m

Safety information

DAB is carcinogenic – wear gloves and make sure to dispose of waste solutions, pipets, and syringes in appropriate hazardous waste containers.

18.1 Make up enough DAB solution to cover all the slides, using 250 µL - 300 µL per slide as a guide.

Note

A dilution of 40 µL of concentrated DAB (Biolegend) per 1 mL DAB substrate Buffer (Biolegend) is usually good.

18.2 Filter the DAB solution with a 0.22 µL syringe filter into a new vial before use.

Note

Microscopic checks to check and stop the development of the chromogen is advised. If the chromogen develops very quickly, then you will want to dilute the antibody concentration.

18.3 After development put slides in slide holder in water container.

18.4 Fill clear basket with water then submerge slides in running water in sink.

18.5 Now counterstain.

Counterstaining with hematoxylin and coverslipping:

1h 20m

19

Filtered Harris Hematoxylin – several dips, return to water and check after a few minutes to see if the hematoxylin is strong enough, if not, return for more dips and repeat.

20

Tap water rinse for about 00:05:00 to both “blue” the hematoxylin and rinse the slides until the water runs clear. Put slides in container with filtered water for dehydration and clearing steps as follows: 5m

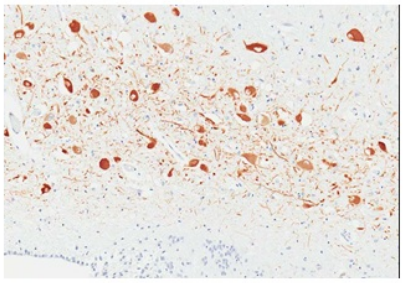
	A	B
	80% Alcohol	2 minutes
	95% Alcohol	2 minutes
	100% Alcohol	2 minutes
	100% Alcohol	2 minutes
	100% Alcohol	2 minutes
	Xylene	2 minutes
	Xylene	2 minutes
	Xylene	2 minutes

Note

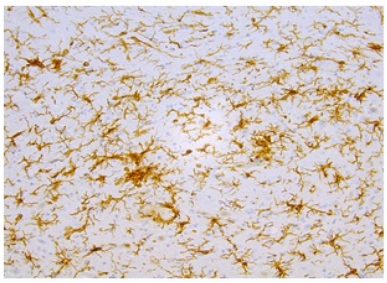
Leave slides in last container of xylene until they are mounted with Permount. Usually 2 drops of Permount is enough. Wipe off excess mounting media and allow to dry flat. Leave slides/slide holder to evaporate Xylene fumes in hood for a while.

Result examples:

Nuclei = Blue
Positive staining antigen = Brown
Background & cytoplasm= pale blue to colorless



Tau Stain



IBA1 stain