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We use this protocol and it's working

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Immunohistochemistry/Immunofluorescence

In 1 collection

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ABSTRACT

This protocol details about the immunohistochemisty/immunofluorescence staining techniques for tissue.

ATTACHMENTS

338-741.pdf

MATERIALS

Solutions and Reagents

0.5 M Tris (8 L)

	A	B	C
	Needed (mL)	Stock Solution	Final Concentration
	5 L	dH2O	
	485 g	Tris base	0.5 M
	240 mL	Concentrated HCl	
	pH to 7.6		
	To 8L	dH2O	

Reagents

	A	B	C	D	E
	Vendor	Catalog #	Qty	Unit Price	Description
	Vector Laboratories	H-3300	1	132.60	Antigen Unmasking Solution, Citric Acid Based
	Vector Laboratories	H-4000	1	120.00	ImmEdge Hydrophobic Barrier Pen

Keywords:
immunohistochemisty, immunofluorescence, formic acid retrieval, Sudan Black Treatment, ASAPCRN

	A	B	C	D	E
	Vector Laboratories	PK-6100	1	248.63	VECTASTAIN Elite ABC Kit (Standard)
	Vector Laboratories	SK-4105	1	138.13	ImmPACT DAB Peroxidase (HRP) Substrate
	Vector Laboratories	BA-2000	1	55	Biotinylated Horse Anti-Mouse IgG Antibody
	Vector Laboratories	BA-1100	1	140	Biotinylated Horse Anti-Rabbit IgG Antibody
	Sigma	199664-25G	1	66.60	Sudan Black B
	Thermo Fisher	6765001	1	46.41	Shandon Harris Hematoxylin (non acidic)
	Fisher Scientific	23-244-256	1	22.96	Cytoseal 60; 4 oz.
	Southern Biotech	0100-01	1	45.14	DAPI Fluoromount-G

Antigen Unmasking Solution Citrate-Based **Vector Laboratories Catalog #H-3300**

ImmEdge hydrophobic barrier pap pen **Vector Laboratories Catalog #H-4000**

VECTASTAIN Elite ABC HRP Kit (Peroxidase, Standard) **Vector Laboratories Catalog #PK-6100**

ImmPACT® DAB Substrate Peroxidase (HRP) **Vector Laboratories Catalog #SK-4105**

Horse Anti-Mouse IgG Antibody (H L) Biotinylated **Vector Laboratories Catalog #BA-2000**

Horse Anti-Rabbit IgG Antibody (H L) Biotinylated **Vector Laboratories Catalog #BA-1100**

Sudan black B **Sigma Aldrich Catalog #199664**

Shandon™ Harris Hematoxylin, Nonacidified **Thermo Fisher Catalog #6765001**

Fluoromount-G **Southern Biotech Catalog #0100-01**

Day 1

2h 11m

1 Label slides with antibody and treatment to be used.

2 De-paraffinize slides in fresh xylenes, then in a descending ethanol series.

2.1 De-paraffinize slides 00:05:00 in fresh xylenes. (1/2) 5m

2.2 De-paraffinize slides 00:05:00 in fresh xylenes. (2/2) 5m

2.3 De-paraffinize slides in ethanol 100% for 00:01:00. 1m

2.4 De-paraffinize slides in ethanol 100% for 00:01:00. 1m

2.5 De-paraffinize slides in ethanol 95% for 00:01:00. 1m

2.6 De-paraffinize slides in ethanol 80% for 00:01:00. 1m

2.7 De-paraffinize slides in ethanol 70% for 00:01:00. 1m

3 **Formic Acid Retrieval** (If necessary, do here).

3.1 Immerse slides in ddH_2O for 00:01:00, place in recycled FA for 00:05:00 and wash in running tap H_2O for 00:10:00. 16m

4 **Microwave antigen retrieval** (CA; If necessary do here).

4.1 Dilute antigen unmasking solution (Vector Labs, citric acid) 1:100 in dH_2O (2.5 mL / 250 mL dH2O/boat).

4.2 Place in Biogenex EZ-Retriever microwave for 00:15:00 at 95 °C. 15m

4.3 Cool for 00:20:00 at Room temperature. 20m

4.4 Wash slides for 00:10:00 in running tap H_2O. 10m

5 Immerse in freshly prepared Methanol/H_2O_2 (150 mL Methanol + 30 mL stock 30% H_2O_2) 00:30:00. 40m

> **Note**
>
> DO NOT GET ON SKIN OR LEAVE SPILL ON BENCH.

Then, wash in running tap H_2O for 00:10:00.

Note

*This step is not necessary for immunofluorescence.
*May use DI water/H_2O_2 (150 mL DI water + 50 mL stock 30% H_2O_2).

6 Wash in [M] 0.1 Molarity (M) Tris buffer, pH 7.6 00:05:00. Discard all Tris washes. 5m

7 Block in [M] 0.1 Molarity (M) Tris/2% FBS (Tris/FBS) 00:05:00+. Keep blocking solution for up to 2 weeks @ 4 °C. 5m

8 Dilute primary antibodies in Tris/FBS, and prepare humidified chamber(s) by soaking towel in the middle of the slide chamber(s).

9 Wipe excess fluid off back of slides and from around tissue and apply 200 µL of primary antibody to slides. Make sure antibodies cover all sections.

Note

Hydrophobic pen may be used at this point if desired, *but CANNOT be used for immunofluorescence*.

10 Incubate at 4 °C in humidified chamber Overnight. 5m

Day 2

2h 11m

11 Rinse off antibody from tissue using Tris.

> **Note**
>
> Carefully direct spray from wash bottle around tissue, NOT directly on it.

12 Wash in Tris 00:05:00. 5m

13 Block in Tris/FBS 00:05:00. 5m

14 Dilute Vector biotinylated IgG 1:1000 in Tris/FBS and apply 200 µL to wiped slides.

14.1 ***For immunofluorescence**, dilute fluorescent secondary antibodies 1:500 in Tris/FBS and apply 200 µL to wiped slides. Keep slides in the dark from here on. Incubate at 4 °C in humidified chamber overnight or at Room temperature for 03:00:00 or overnight at 4 °C. Proceed to Day 3. 3h

15 Incubate at Room temperature in humidified chamber 01:00:00. 1h

16 Rinse biotinylated IgG using Tris.

17 Wash in Tris 00:05:00. 5m

18 Block in Tris/FBS 00:05:00 . 5m

19 Mix AB solution (Vector peroxidase standard) in Tris/FBS to a dilution of 1:000 (ie add 1 µL of A and 1 µL of B to 1 mL of [M] 0.1 Molarity (M) Tris/2%FBS). Vortex and let sit 00:15:00 before use. Then, apply 200 µL of AB solution to wiped slides. 15m

20 Incubate at Room temperature in humidified chamber for 01:00:00 . 1h

21 Rinse off AB using Tris.

22 Immerse in Tris 00:05:00 . 5m

23 Make Vector DAB solution (1 drop of DAB per mL of Stable DAB Buffer) .

24 Apply 200 µL of DAB to each slide and incubate until a visible brown signal is seen and well developed.

> **Note**
>
> Development time may differ by antibody, but all sections treated with the same antibody should be developed for the same amount of time.

25 Rinse with Tris and place in dH_2O. Wash 00:05:00 in dH_2O. Filter Harris hematoxylin. 5m

26 Counterstain briefly with Harris hematoxylin (~ 00:00:15 , depending on age). 15s

27 Wash in running tap H_2O 00:05:00 . 5m

28 Dehydrate and clear in ascending ethanol and xylenes.

28.1 Dehydrate and clear in 70% ethanol for 00:01:00 and 70% xylenes for 00:05:00 . 6m

28.2 Dehydrate and clear in 80% ethanol for 00:01:00 and 80% xylenes for 00:05:00 . 6m

28.3 Dehydrate and clear in 95% ethanol for 00:01:00 and 95% xylenes for 00:05:00 . 6m

28.4 Dehydrate and clear in 100% ethanol for 00:01:00 and 100% xylenes for 00:05:00 . 6m

28.5 Dehydrate and clear in 100% ethanol for 00:01:00 and 100% xylenes for 00:05:00 . 6m

29 Coverslip with cytoseal.

30 Dry in tissue processor closet Overnight . 5m

Day 3 (Immunofluorescence) 16m 10s

31 Filter Sudan Black. This process can take a long time, so start early.

32 Rinse off AB using Tris.

33 Wash in running tap H_2O for 00:05:00 . 5m

34 Wash in Tris for 00:05:00 in green boats. 5m

35 **Sudan Black Treatment** (0.3% Sudan Black B in 70% Ethanol)

35.1 Use a control slide (usually 1 positive primary and 1 secondary only) to titrate for background reduction without changing signal intensity (usually 00:00:10 to 00:01:00). 1m 10s

35.2 Image before and after Sudan black treatment for various times.

35.3 Treat all slides identically.

36 Wash in [M] 0.1 Molarity (M) Tris 00:05:00 in green boats. 5m

37 Coverslip using non-photobleaching reagent (FluorMount with DAPI). Allow to dry completely before imaging on scanner.