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Protocol status: Working

We use this protocol and it's working



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Abstract

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

Attachments



[338-741.pdf](#)

527KB

Materials

Solutions and Reagents

0.5 M Tris (8 L)

	A	B	C
	Needed (mL)	Stock Solution	Final Concentration
	5 L	dH ₂ O	
	485 g	Tris base	0.5 M
	240 mL	Concentrated HCl	
	pH to 7.6		
	To 8L	dH ₂ O	

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
	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)

	A	B	C	D	E
	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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #199664**

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











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

Troubleshooting



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 ddH₂O for  00:01:00 , place in recycled FA for  00:05:00 and wash in running tap H₂O 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 dH₂O ( 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 H₂O.

10m



5 Immerse in freshly prepared Methanol/H₂O₂ ( 150 mL Methanol +  30 mL stock 30% H₂O₂)  00:30:00 .

40m



Note




DO NOT GET ON SKIN OR LEAVE SPILL ON BENCH.

Then, wash in running tap H₂O for  00:10:00 .

Note




*This step is not necessary for immunofluorescence.

*May use DI water/H₂O₂ ( 150 mL DI water +  50 mL stock 30% H₂O₂).

6 Wash in  0.1 Molarity (M) Tris buffer,  7.6  00:05:00 . Discard all Tris washes.


5m



7 Block in  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 μ L of A and 1 μ L 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 μ L 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 μ L 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 dH₂O. Wash  00:05:00 in dH₂O. Filter Harris hematoxylin.  
- 26 Counterstain briefly with Harris hematoxylin (~  00:00:15 , depending on age). 
- 27 Wash in running tap H₂O  00:05:00 .  
- 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 . 
- 28.2 Dehydrate and clear in 80% ethanol for  00:01:00 and 80% xylenes for  00:05:00 . 
- 28.3 Dehydrate and clear in 95% ethanol for  00:01:00 and 95% xylenes for  00:05:00 . 
- 28.4 Dehydrate and clear in 100% ethanol for  00:01:00 and 100% xylenes for  00:05:00 . 
- 28.5 Dehydrate and clear in 100% ethanol for  00:01:00 and 100% xylenes for  00:05:00 . 
- 29 Coverslip with cyto seal.
- 30 Dry in tissue processor closet  Overnight .  

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 H₂O 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  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.