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

3,3-Diaminobenzidine Tetrahydrochloride (DAB) Immunohistochemistry on Mouse Brain Tissue Sections

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ABSTRACT

This protocol details the staining for free floating mouse brain sections.

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MATERIALS

Ø Pyrex® Staining Dish Ted Pella Inc. Catalog #36754-60

⊗ 8-Section Staining Nets **Ted Pella Inc. Catalog #**36154-64

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

⊠ Pierce[™] DAB Substrate Kit **Thermo Scientific Catalog #**34002

Fisherbrand™ Superfrost™ Plus Microscope Slides **Fisher**Scientific Catalog #12-550-15

Recipes

10X TBS

А	В	
1M Tris	500 ml	
5M NaCl	300 ml	
H20	200 mL	

1X TBS + 0.1% triton \rightarrow 1 liter

A	В	
10X TBS	100 ml	
Triton X	1 ml	
Water	899 ml	

TBS \rightarrow 1 liter

A	В	
1M Tris	50 ml	
5M NaCl	30ml	
Water	919 ml	

TBST (0.1% Tween-20) \rightarrow 1 liter

	A	В	
	10X TBS	100 ml	
Г	Tween-20	1ml	
	Water	899 ml	

0.2M Phosphate Buffer

	A	В	
Na2HPO4		22.72 g	
NaH2PO4		5.52 g	
Г	H20	1000 mL	

0.1M Phosphate Buffer

A	В	
0.2M PB	500 mL	
H20	500 mL	

Cresyl Violet (500 mL)

A	В
H20	500 mL
Cresyl Violet Acetate	0.5g
100% Glacial Acetic Acid	1.25mL

Warm to 60°C and stir until completely dissolved filter with whatman paper (wrap in foil to protect from light)

Quenching solution \rightarrow 100ml

A	В
30% hydrogen peroxide (Sigma H1009)	1 ml
Methanol	99 ml

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ABC mix→60ml

A		В	
	TBS	60 ml	
	Reagent A	24 drops	
	Reagent B	24 drops	

DAB Solution→30ml

	A	В	
	PBS	30 ml	
	(DAB reagent 1)	12 drops buffer pH = 7.5	
	(DAB reagent 2) 24 drops DAB		
(DAB reagent 3) 12 drops hydrogen		12 drops hydrogen peroxide	

vortex between reagents

Blocking solution→100ml

A	В	
NGS	10 ml	
TBS + triton	90 ml + 0.1%	

Primary Antibodies:

A	В	С	D
Target	Species	Conc	Manufacturer
lba1	Rabbit	1:1000	WAKO 019-19741
GFAP	Mouse	1:1000	Sigma G3893
pSer129-Synuclein	Rabbit	1:1000	Abcam ab51253
тн	Rabbit	1:2000	Novus Biological N300109
pSer202, pThr205 Tau (AT8)	Mouse	1:1000	Thermofisher MN1020
GFP	Rabbit	1:1000	Thermofisher A-11122

Secondary Antibodies:

A	В	С	D	E
Target	Species	Conc	Manufacturer	
Mouse	Goat	1:500	BA-9200-1.5	Biotinylated
Rabbit	Goat	1:500	BA-1000-1.5	Biotinylated

Day 1

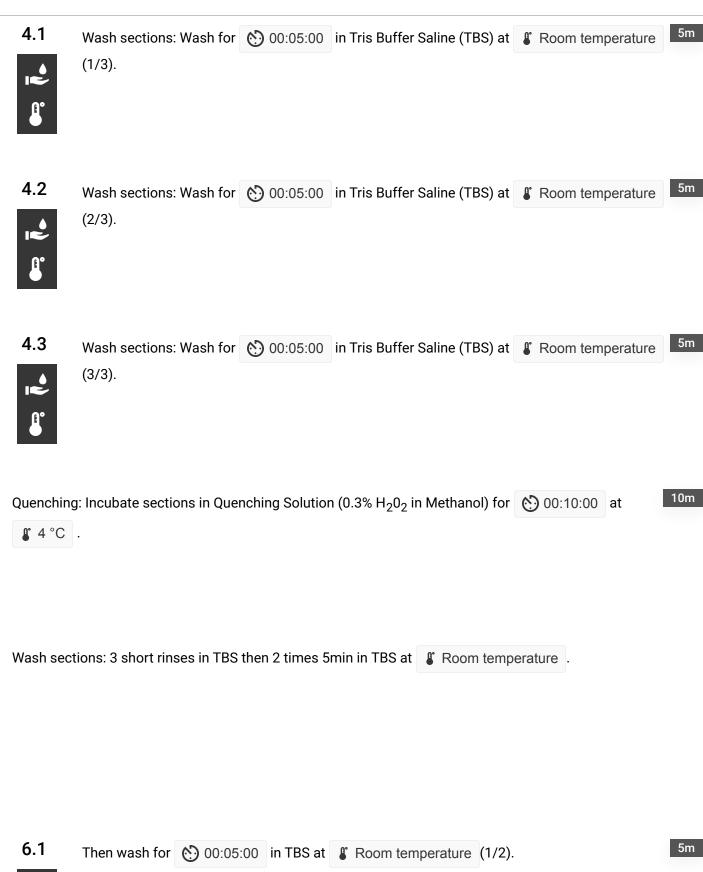
2d 1h 35m

- 1 Staining protocol for 35µm free floating mouse brain sections.
- 2 Staining is performed in glass staining dishes (Pyrex 36754-60) using 8-section staining nets (Ted Pella 36154-64).
 - 2.1 The sections can be transferred between wells using a paint brush.
 - 2.2 Volume of solution:



- 1. \triangle 20 mL \triangle 30 mL for antibodies or ABC mix.
- 2. \perp 50 mL for washing.
- 3 Gently rock the plates during the washing and incubation steps.
- 4 Wash sections: 3 times (5 min per wash) in Tris Buffer Saline (TBS) at Room temperature.





5

6



6.2 Then wash for 00:05:00 in TBS at Room temperature (2/2). 5m



7 Block sections in blocking solution (10% Normal Goat Serum (NGS) + 0.1% Triton-X in TBS) for 1h



♦ 01:00:00 at \$\mathbb{s}\$ Room temperature .

8 Primary Antibodies: Dilute antibodies in 5% NGS + 0.1% Triton-X in TBS.

2d



Day 4

8.1

8.2 Cover dishes with parafilm for this step.

1d 3h 47m



Wash 3 times (10 min each wash) in TBST (0.1% Tween 20 in TBS) at Room temperature.

Incubate in primary antibody solution for 48:00:00 at 4 °C.



9.1 Wash for 00:10:00 in TBST (0.1%Tween 20 in TBS) at Room temperature (1/3).



9.2 Wash for 00:10:00 in TBST (0.1%Tween 20 in TBS) at Room temperature (2/3).

9.3 Wash for 00:10:00 in TBST (0.1%Tween 20 in TBS) at 8 Room temperature (3/3).



10 Secondary Antibodies:



10.1 Dilute secondary antibodies in TBST.

10.2 Biotinylated goat anti-rabbit IgG (1:500 dilution) or Biotinylated goat anti-mouse IgG (1:500 dilution).

10.3 Incubate for (5) 02:00:00 at (8 Room temperature or (5) 24:00:00 at (8 4 °C).



1d 2h

10m





- 11 Wash sections 3 times (10min each wash) in TBST at Room temperature.
 - 11.1 Wash for 🕙 00:10:00 in TBST at 🖁 Room temperature (1/3).

10m

11.2 Wash for (5) 00:10:00 in TBST at (8) Room temperature (2/3).

10m

11.3 Wash for 00:10:00 in TBST at 8 Room temperature (3/3).

10m

- 12 Prepare ABC solution using VECTASTAIN® Elite® ABC-HRP Kit, Peroxidase (Standard) (PK-6100).
 - 12.1 ABC mix: 4 5 mL TBS + 2 drops of reagent A (vortex) + 2 drops reagent B (vortex) let stand



- Room temperature for 00:30:00 before using.
- 12.2 Vortex between reagents (after adding reagent A and after adding reagent B).



12.3 Make 4 30 mL ABC solution per dish.

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9

13 Incubate sections in ABC solution for 👏 01:00:00 at 🖁 Room temperature .

1h



14 Wash sections 3 times (10min each wash) in TBST at 3 Room temperature.



14.1 Wash for 00:10:00 in TBST at Room temperature (1/3).





14.2 Wash for 00:10:00 in TBST at Room temperature (2/3).





14.3 Wash for 00:10:00 in TBST at Room temperature (3/3). 10m



15 Wash sections 2 times (5 min each wash) in TBS at 8 Room temperature . 15.1

Wash sections for 00:05:00 in TBS at 8 Room temperature (1/2).

5m



15.2

Wash sections for 00:05:00 in TBS at 8 Room temperature (2/2).

5m



16

Prepare DAB solution using DAB Substrate Kit, Peroxidase (HRP), with Nickel, (3,3'-diaminobenzidine) (SK-4100).



- 1. TBS + 2 drops of Reagent 1 (Buffer pH=7.5) + 4 drops Reagent 2 (DAB) + 2 drops Reagent 3 (H2O2).
- 2. Vortex between reagents (after reagent 1, after reagent 2 and after reagent 3).
- 3. Make 🚨 30 mL DAB solution per dish.
- 17

Revelation with DAB chromogen (until cells are visible):

2m

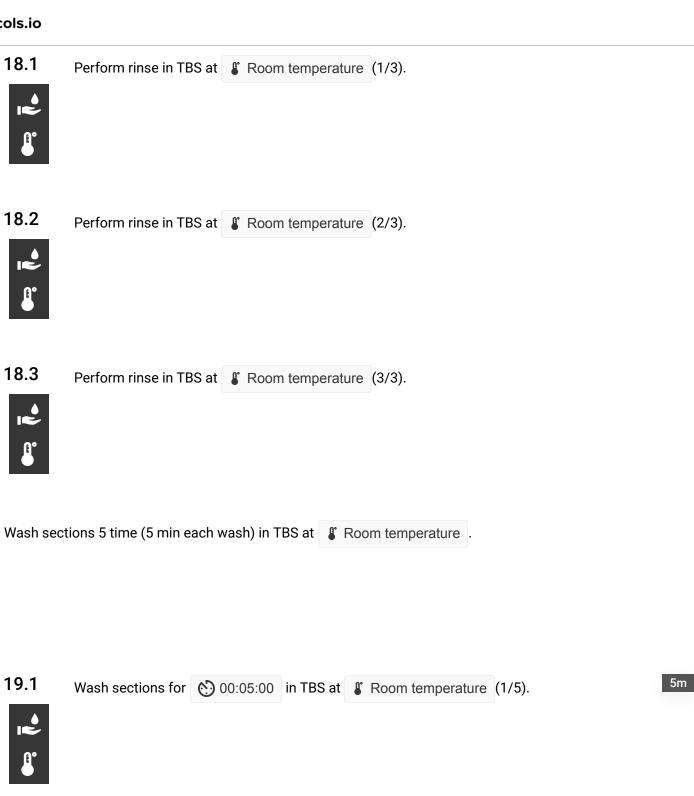


typically about 00:02:00 for TH.

- All dishes must be developed for the same amount of time do them together, ask a friend for help if you have more than 2 dishes of tissue.
- 18

Perform 3 short rinses in TBS at 8 Room temperature .







Wash sections for 00:05:00 in TBS at 8 Room temperature (2/5).

5m

19



Wash sections for 00:05:00 in TBS at 8 Room temperature (3/5).

5m

19.4

Wash sections for 00:05:00 in TBS at 8 Room temperature (4/5).

5m



19.5 Wash sections for 00:05:00 in TBS at 8 Room temperature (5/5).

5m



20 Mount sections:

- 20.1 Use a petri dish filled with PBS to mount the sections on superfrost plus slides (Fisher Scientific 12-550-15).
 - This is easiest to do using a medium sized paint brush.
- 20.2

After mounting let slides dry at Room temperature (000:02:00 - 000:10:00) then in miliQ H₂O.

20.3

Dry slides Overnight at 8 Room temperature before moving to the next step.

5m



Day 5 or 6

9h 18m

Rehydrate slides in H_2O for $\bigcirc 00:02:00$ - $\bigcirc 00:03:00$ at $\bigcirc Room$ temperature.



II.

22 Dehydrate slides in sequential EtOH washes (70%, 95%, 100% EtOH) for 00:05:00 each wash at



Room temperature

23

Clear tissue for 00:05:00 in fresh Xylene I then incubate in fresh Xylene for ≥ 00:05:00.





- Keep slides in Xylene until coversliping.
- Perform Xylene steps and coversliping in a chemical fume hood.

24 Use Entellan mounting medium (Electron Microscopy Sciences 14802) to coverslip slides.



- 24.1 Apply one line of Entellan across the middle of the slide.
- 24.2 Cover with glass coverslip and gently press down with forceps.





Let slides dry in the fume hood for 01:00:00 – then dry on the bench Overnight a. 1h 5m



Room temperature .

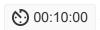
24.4 Gently remove excess Entellen with a razor blade.

Optional Cresyl Violet Protocol (NISSL stain)

10m

25 After rehydration and before ethanol steps (dehydration).

26 Incubate slides in fresh cresyl violet for 600:10:00 at 8 Room temperature.



10m

27 Proceed with dehydration steps normally.

28 Must stain with cresyl violet for TH stereology in substantia nigra.

Secondary Antibodies

29

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A	В	С	D	E
Target	Species	Conc	Manufacturer	
Mouse	Goat	1:500	BA-9200-1.5	Biotinylated
Rabbit	Goat	1:500	BA-1000-1.5	Biotinylated