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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

- 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
- Entellan **Electron Microscopy Sciences Catalog #**14802

Recipes

10X TBS

	A	B
	1M Tris	500 ml
	5M NaCl	300 ml
	H2O	200 mL

1X TBS + 0.1% triton → 1 liter

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

TBS → 1 liter

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

TBST (0.1% Tween-20) → 1 liter

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

0.2M Phosphate Buffer

	A	B
	Na2HPO4	22.72 g
	NaH2PO4	5.52 g
	H2O	1000 mL

0.1M Phosphate Buffer

	A	B
	0.2M PB	500 mL
	H2O	500 mL

Cresyl Violet (500 mL)

	A	B
	H2O	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→100ml

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

ABC mix→60ml

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

DAB Solution→30ml

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

vortex between reagents

Blocking solution→100ml

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

Primary Antibodies:

	A	B	C	D
	Target	**Species**	**Conc**	**Manufacturer**
	Iba1	Rabbit	1:1000	WAKO 019-19741
	GFAP	Mouse	1:1000	Sigma G3893
	pSer129-Synuclein	Rabbit	1:1000	Abcam ab51253
	TH	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	B	C	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. 20 mL - 30 mL for antibodies or ABC mix .
2. 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 .

4.1 Wash sections: Wash for 00:05:00 in Tris Buffer Saline (TBS) at Room temperature (1/3). 5m

4.2 Wash sections: Wash for 00:05:00 in Tris Buffer Saline (TBS) at Room temperature (2/3). 5m

4.3 Wash sections: Wash for 00:05:00 in Tris Buffer Saline (TBS) at Room temperature (3/3). 5m

5 Quenching: Incubate sections in Quenching Solution (0.3% H_2O_2 in Methanol) for 00:10:00 at 4 °C . 10m

6 Wash sections: 3 short rinses in TBS then 2 times 5min in TBS at Room temperature .

6.1 Then wash for 00:05:00 in TBS at Room temperature (1/2). 5m

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 01:00:00 at Room temperature . 1h

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

8.1 Incubate in primary antibody solution for 48:00:00 at 4 °C . 2d

8.2 Cover dishes with parafilm for this step.

Day 4 1d 3h 47m

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

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

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

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

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 02:00:00 at Room temperature or 24:00:00 at 4 °C . 1d 2h

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 00:10:00 in TBST at Room temperature (2/3). 10m

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

12 Prepare ABC solution using VECTASTAIN® Elite® ABC-HRP Kit, Peroxidase (Standard) (PK-6100).

12.1 ABC mix: 5 mL TBS + 2 drops of reagent A (vortex) + 2 drops reagent B (vortex) let stand at Room temperature for 00:30:00 before using. 30m

12.2 Vortex between reagents (after adding reagent A and after adding reagent B).

12.3 Make 30 mL ABC solution per dish.

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 Room temperature .

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

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

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 Room temperature .

15.1 Wash sections for 00:05:00 in TBS at Room temperature (1/2). 5m

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

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

1. 5 mL 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

> **Note**
>
> 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 Room temperature .

18.1 Perform rinse in TBS at Room temperature (1/3).

18.2 Perform rinse in TBS at Room temperature (2/3).

18.3 Perform rinse in TBS at Room temperature (3/3).

19 Wash sections 5 time (5 min each wash) in TBS at Room temperature .

19.1 Wash sections for 00:05:00 in TBS at Room temperature (1/5). 5m

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

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

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

19.5 Wash sections for 00:05:00 in TBS at 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 (00:02:00 - 00:10:00) then [illegible] in miliQ H_2O. 12m

20.3 Dry slides Overnight at Room temperature before moving to the next step. 5m

Day 5 or 6

9h 18m

21 Rehydrate slides in H_2O for 00:02:00 - 00:03:00 at Room temperature . 5m

22 Dehydrate slides in sequential EtOH washes (70%, 95%, 100% EtOH) for 00:05:00 each wash at Room temperature . 5m

23 Clear tissue for 00:05:00 in fresh Xylene I then incubate in fresh Xylene for ≥ 00:05:00 . 10m

a. Keep slides in Xylene until coversliping.
b. 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.

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

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 00:10:00 at Room temperature . 10m

27 Proceed with dehydration steps normally.

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

Secondary Antibodies

29

	A	B	C	D	E
	Target	**Species**	**Conc**	**Manufacturer**	
	Mouse	Goat	1:500	BA-9200-1.5	Biotinylated
	Rabbit	Goat	1:500	BA-1000-1.5	Biotinylated