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DAB Staining for Tyrosine Hydroxylase (TH) on Free-floating Fixed NHP Brain Tissue

Forked from Standard DAB Staining for Free-floating Fixed NHP Brain Tissue

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We use this protocol and it's working

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Abstract

This protocol details the procedure for immunohistochemical 3,3′-Diaminobenzidine (DAB) staining for Tyrosine Hydroxilase (TH) of free-floating fixed brain tissue sections using the avidin/biotin ABC complex and the Rabbit Polyclonal Anti-TH NB300-109 (Novus).

This protocol has been tested with free-floating non-human primate (NHP) brain tissue that has been fixed (10% formalin or 4% paraformaldehyde), cryoprotected (sucrose or glycerol gradients), and cryo-sectioned 50 µm.

Guidelines

This protocol has been optimized for using 6 well tissue culture plates [Falcon, 353046] to react individual sections. You will need **2+ mL** solutions for ***each*** well plate.
It is recommended to use new culture plates for antibody incubation and discard plates used for DAB.
Plates used for other steps can be washed and re-used.

Materials

Tissue:
Brain tissue sections (20 - 50 µm).

Materials/Equipment:
- Tissue culture plates or circular staining nets
- Orbital shaker
- Fume hood
- Nitrile Gloves
- Glass slides (charged or subbed)

Reagents:
- Phosphate-buffered saline (PBS)
- Phosphate-buffered saline (PBS) with 0.2% Triton-X
- Akoya Blocking Reagent Powder (SKU FP1012) to make TNB Blocking Buffer/
- Hydrogen Peroxide: H_2O_2 (3% or 30%)
- Distilled water: dH_2O
- Primary Antibody: Novus Biologicals Tyrosine Hydroxylase (TH) Polyclonal Rabbit.
- Secondary Antibody (to match the host of the primary antibody): biotinylated Goat-Anit Rabbit.
- Normal Serum Blocking Solution (e.g., Normal Goat Serum, S-1000-20)
- Vectastain Elite ABC Peroxidase Kit (Standard) (PK-6100) (Vector Laboratories)
- Vectastain ABC-HRP Kit, Peroxidase (Rabbit IgG) (PK-4001, Vector Laboratories)
- DAB Substrate Kit

Examples:
Peroxidase (HRP) with Nickel (3,3'-diaminobenzidine) (SK-4100) (Vector Laboratories)
ImmPACT DAB (SK-4105)

Safety warnings

Use appropriate care when using hydrogen peroxide (reactive, can cause skin/eye damage) and DAB (suspected carcinogen). Collect DAB solution for chemical waste disposal.

Part I (Day 1) 3h

1 Bring tissue to Room temperature in **phosphate-buffered saline (PBS)** on an orbital shaker for **30 minutes**. 00:30:00 . 30m

2 Prepare **Peroxide Solution (3 % H_2O_2)** in **dH_2O**. 5m
E.g., for 10 mL 3% H_2O_2 use:
- 1000 µL 30% H_2O_2
- 9000 µL dH_2O

3 Prepare **Blocking Serum Solution: Normal Goat Serum (NGS) in TNB Blocking Buffer.** 5m
E.g., in 10 mL buffer (TNB Blocking Buffer) add:
- 150 µL NGS (or 3 drops of normal serum if using an ABC kit, e.g. Vectastain ABC-HRP Kit Rabbit IgG PK-4001)

4 Prepare **Primary Antibody Solution: anti-TH NB300-109 at 1:1000 TNB Blocking Buffer.** 5m
E.g., for 10 mL **Primary Antibody Solution** use:
- 9990 µL TNB Blocking Buffer
- 10 µL anti-TH NB300-109

5 **Rinse** in **PBS with 0.2% Triton X (PBS-Tx)** on a shaker at Room temperature : 9m
3 × 3 minutes. 00:09:00

6 Quench endogenous peroxide in **Peroxide Solution (3 % H_2O_2)** on a shaker at 1h 20m
Room temperature :
2 × 10 - 15 minutes. 00:20:00 - 01:00:00

7 **Rinse** in **PBS with 0.2% Triton X (PBS-Tx)** on a shaker at Room temperature : 9m
3 × 3 minutes. 00:09:00

8 Incubate in **Blocking Serum Solution** on a shaker at RT: **1 hour**. 1h
01:00:00 .

DO NOT RINSE after blocking serum.

9 Incubate in **Primary Antibody Solution** on a shaker at 4 °C : **overnight x 3 (60 - 72 hours).** 72:00:00 . 3d

Part II (Day 2) 4h

10 Bring tissue (in the **Primary Antibody Solution**) to Room temperature on a shaker (**45 minutes**). 00:30:00 - 01:00:00 30m

11 Prepare **ABC Solution** in **PBS with 0.2% Triton X (PBS-Tx)** (**at least 30 minutes before use).** 00:30:00 . 5m

12 Prepare **Secondary Antibody Solution** (**1:200**) in **TNB Blocking Buffer**. 5m
In 10 mL TNB Blocking Buffer add:

- 150 µL Normal Goat Serum (NGS) (= 3 drops of normal serum if using an ABC kit, e.g. Vectastain ABC-HRP Kit Rabbit IgG PK-4001).
- 50 µL Secondary biotinylated goat-anti rabbit (= 1 drop of normal goat serum if using an ABC kit, e.g. Vectastain ABC-HRP Kit Rabbit IgG PK-4001).

13 **Rinse** in **PBS with 0.2% Triton X (PBS-Tx)** on a shaker at Room temperature : **3 × 3 minutes.** 00:09:00 . 9m

14 Incubate in **Secondary Antibody Solution** on a shaker at Room temperature : **30 minutes.** 00:30:00 . 30m

15 **Rinse** in **PBS with 0.2% Triton X (PBS-Tx)** on a shaker at Room temperature : **3 × 3 minutes.** 00:09:00 . 9m

16 Incubate in **ABC Solution** on a shaker at Room temperature : **60 minutes.** 01:00:00 . 1h

17 **Rinse** in **PBS with 0.2% Triton X (PBS-Tx)** on a shaker at Room temperature : 9m
3 × 3 minutes. 00:09:00 .

18 Prepare **Peroxide Substrate Solution** in dH_2O. 5m
To use the Vector Labs DAB Peroxidase Substrate Kit (SK-4100):
In 5 mL dH_2O:
- 2 drops Reagent 1
- 4 drops Reagent 2
- 2 drops Reagent 3
- [optional] 2 drops of Reagent 4 (Nickel) if a black reaction product is desired

Note: Mix well before use. Use immediately.

19 Incubate in **Peroxide Substrate Solution on** a shaker at Room temperature : 6m
3 - 10 minutes. 00:03:00 - 00:06:00 .

Note: Watch the tissue closely to avoid high background staining.

20 **Rinse** in buffer (e.g. PBS) on a shaker at Room temperature : 9m
3 × 3 minutes. 00:09:00 .

21 Mount tissue on glass slides (subbed or charged) in 1:8 PBS in dH_2O and let air dry.

22 Rinse slides with dH_2O and let air dry (preferably in a hood).

23 Coverslip clean and dry slides with Cytoseal 60 (Thermo Fisher #830-16).

Protocol references

https://vectorlabs.com/productattachments/protocol/VL_SK-4100_UserGuide_LBL02267.pdf
https://vectorlabs.com/products/vectastain-elite-abc-hrp-kit-standard
https://my.akoyabio.com/ccrz__ProductDetails?sku=FP1012&cclcl=en_US