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Standard DAB Staining for Free-floating Fixed NHP Brain Tissue V.2

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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 of free-floating fixed brain tissue sections using the avidin/biotin ABC complex.

This protocol has been tested with free-floating non-human primate (NHP) and rodent (mouse, rat) brain tissue that has been fixed (10% formalin or 4% paraformaldehyde), cryoprotected (sucrose or glycerol gradients), and cryo-sectioned 20 µm - 50 µm.

Guidelines

When using 6 well tissue culture plates [Falcon, 353046] to react individual sections, you will need **2+ mL** solutions for ***each*** well plate.

When using circular staining nets [e.g., Brain Research Laboratories #4115] to react multiple series of sections, you will need **50 mL** solutions for ***each***.

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)
- Hydrogen Peroxide: H_2O_2 (3% or 30%)
- Distilled water: dH_2O
- Primary Antibody
- Secondary Antibody (to match the host of the primary antibody)
- Normal Serum Blocking Solution (e.g., Normal Horse Serum, S-2000-20; Normal Goat Serum, S-1000-20)
- Vectastain Elite ABC Peroxidase Kit (Standard) (PK-6100) (Vector Laboratories)
- ABC-HRP Kit

  Examples:
  Vectastain ABC-HRP Kit, Peroxidase (Mouse IgG) (PK-4002, 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)

Troubleshooting

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 buffer (e g., Phosphate buffered saline, PBS) on an orbital shaker for 30 minutes. 00:30:00 . 30m

2 Prepare **Peroxide Solution (0.3 - 3 % H_2O_2)** in dH_2O. 5m
E.g., for 10 mL 0.3% H_2O_2 use:
- 100 µL 30% H_2O_2
- 9900 µL dH_2O

3 Prepare **Blocking Serum Solution** (e.g. Normal Horse, Normal Goat Serum) using a serum that matches the **host of the secondary antibody** (e.g. Normal Horse Serum for a Horse anti-Mouse secondary, Normal Goat Serum for a Goat anti-Rabbit secondary). 5m
E.g., in 10 mL buffer (PBS) add:
- 150 µL normal serum (or 3 drops of normal serum if using an ABC kit, e.g. Vectastain ABC-HRP Kit, Peroxidase Mouse IgG PK-4002, Rabbit IgG PK-4001)

4 Prepare **Primary Antibody Solution** at the appropriate dilution in buffer (e.g., 1:1000 in PBS). 5m

5 **Rinse** in buffer (e.g. PBS) on a shaker at Room temperature : **3 × 3-5 minutes.** 15m
00:03:00 - 00:05:00

6 Quench endogenous peroxide in **Peroxide Solution (0.3 - 3 % H_2O_2)** on a shaker at 1h
Room temperature : **30 - 60 minutes.** 00:30:00 - 01:00:00

7 **Rinse** in buffer (e.g. PBS) on a shaker at Room temperature : **3 × 3-5 minutes.** 15m
00:03:00 - 00:05:00

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

DO NOT RINSE after blocking serum.

9 Incubate in **Primary Antibody Solution** on a shaker at 4 °C Overnight , or longer (20 - 72 hours depending on the antibody). 20h

Part II (Day 2) 4h

10 Bring tissue (in the **Primary Antibody Solution**) to Room temperature on a shaker (**30 - 60 minutes**). 00:30:00 - 01:00:00 30m

11 Prepare **ABC Solution** in buffer (e.g. PBS) (**at least 30 minutes before use).** 00:30:00 . 5m

12 Prepare **Secondary Antibody Solution** (**1:200**) in buffer (e.g. PBS). 5m
In 10 mL buffer add:

- 150 µL (= 3 drops of normal serum from a Vector Labs kit) of normal serum (matched to the host of your secondary antibody)
- 50 µL (= 1 drop secondary antibody from a Vector Labs kit) of biotinylated secondary antibody (matched to the host of your primary Antibody)

13 **Rinse** in buffer (e.g. PBS) on a shaker at Room temperature : **3 × 3-5 minutes.** 15m
00:03:00 - 00:05:00 .

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

15 **Rinse** in buffer (e.g. PBS) on a shaker at Room temperature : **3 × 3-5 minutes.** 15m
00:03:00 - 00:05:00 .

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

17 **Rinse** in buffer (e.g. PBS) on a shaker at Room temperature : **3 × 3-5 minutes.** 15m
00:03:00 - 00:05:00 .

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
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 : **3 × 3-5 minutes.** 15m
00:03:00 - 00:05:00 .

21 Mount tissue on glass slides (subbed or charged) in 1:8 buffer 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