



VERSION 1

FEB 07, 2024

OPEN ACCESS



Protocol Citation: Andreea C. Bostan 2024. Standard DAB Staining for Free-floating Fixed NHP Brain Tissue . [protocols.io](https://protocols.io/view/standard-dab-staining-for-free-floating-fixed-nhp-c7eizjce) <https://protocols.io/view/standard-dab-staining-for-free-floating-fixed-nhp-c7eizjce>

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

Created: Jan 10, 2024

Last Modified: Feb 07, 2024

Standard DAB Staining for Free-floating Fixed NHP Brain Tissue V.1

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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 $\pm 20 \mu\text{m}$ -

$\pm 50 \mu\text{m}$.

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 larger wells to react a series of sections, you will need **50 mL** solutions for **each** well.

PROTOCOL integer ID: 93354

Keywords: ASAPCRN,
Immunostaining, DAB, NHP Brain
Tissue

Funders Acknowledgement:

Aligning Science Across
Parkinson's
Grant ID: ASAP-020519

MATERIALS

Tissue:

Brain tissue sections (20 - 50 μ m).

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)









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 00:30:00 30m.
- 2 Prepare **Peroxide Solution (0.3 - 3 % H₂O₂)** in dH₂O. 5m
 E.g., for 10 mL 0.3% H₂O₂ use:
 - 100 µL 30% H₂O₂
 - 9900 µL dH₂O
- 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 x 3-5 minutes.** 00:03:00 15m
 00:05:00





- 6 Quench endogenous peroxide in **Peroxide Solution (0.3 - 3 % H₂O₂)** on a shaker at  Room temperature : **30 - 60 minutes.**  00:30:00 -  01:00:00 1h
- 7 **Rinse** in buffer (e.g. PBS) on a shaker at  Room temperature : **3 x 3-5 minutes.**  00:03:00 -  00:05:00 15m
- 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**  00:30:00 **before use**). 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 x 3-5 minutes.** 00:03:00 - 00:05:00 . 15m
- 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 x 3-5 minutes.** 00:03:00 - 00:05:00 . 15m
- 16 Incubate in **ABC Solution** on a shaker at Room temperature : **60 minutes.** 01:00:00 . 1h
- 17 **Rinse** in buffer (e.g. PBS) on a shaker at Room temperature : **3 x 3-5 minutes.** 00:03:00 - 00:05:00 . 15m
- 18 Prepare **Peroxide Substrate Solution** in dH₂O. 5m
 To use the Vector Labs DAB Peroxidase Substrate Kit (SK-4100):
 In 5 mL dH₂O:
 - 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 : 00:03:00 - 00:06:00 . 6m
Note: Watch the tissue closely to avoid high background staining.

- 20 **Rinse** in buffer (e.g. PBS) on a shaker at  Room temperature : **3 x 3-5 minutes.**  00:03:00 -  00:05:00 . 
- 21 Mount tissue on glass slides (subbed or charged) in 1:8 buffer in dH₂O and let air dry.
- 22 Rinse slides with dH₂O and let air dry (preferably in a hood).
- 23 Coverslips clean and dry slides with Cytoseal 60 (Thermo Fisher #830-16).