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# DAB Immunohistochemistry (IHC) Staining for Stereological Analysis

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

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## Abstract

DAB (3,3'-diaminobenzidine) is oxidized in the presence of peroxidase and hydrogen peroxide resulting in the deposition of a brown, alcohol-insoluble precipitate at the site of enzymatic activity.DAB (3, 3'-diaminobenzidine) produces a dark brown reaction product and can be used for immunohistochemical and applications. This protocol details the DAB immunohistochemistry staining for stereological analysis of 40 µM slices cut with cryostat and stored in antifreeze.

## Guidelines

This protocol needs prior approval by the users' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee

## Materials

**Reaction mixture:**

| | A | B |
|---|---|---|
| | 0.2M acetate buffer | 5mL |
| | Nickel ammonium sulfate | 250mg |
| | β-D-Glucose | 20mg |
| | Ammonium chloride | 4mg |

**0.2 M Acetate Buffer pH 6.0:**
Prepare 1L of 0.2N acetic acid (11.5 ml glacial acetic acid and top up to 1L with H2Odd)
Mix 900 ml of 0.2M sodium acetate and 51.7 ml
Top up to 1L with H2Odd
Check pH

**Glucose oxidase (1mg/ml)**
2 mg of glucose oxidase
2 ml of acetate buffer 50 mM
Make aliquots of 80 ul in advance
Keep the powder and aliquots at -20 degrees

## Procedures

1w 6d 18h 29m 30s

1 Basic protocol for a peroxidase reaction using free floating sections. 10m

> **Note**
>
> The following steps should be done on a rotating table:

Wash in [M] 0.01 Molarity (M) PBS for 00:10:00 (use cell strainers for all washing steps).

2 Wash in [M] 0.01 Molarity (M) PBS **containing 0.9% $H_2O_2$** for 00:10:00 ( 90 µL in 10 mL PBS 0.01M) (Blocking of endogenous peroxidase). 10m

3 Wash in [M] 0.01 Molarity (M) PBS (3x 10 min).

3.1 Wash in [M] 0.01 Molarity (M) PBS for 00:10:00 (1/3). 10m

3.2 Wash in [M] 0.01 Molarity (M) PBS for 00:10:00 (2/3). 10m

3.3 Wash in [M] 0.01 Molarity (M) PBS for 00:10:00 (3/3). 10m

4 Incubate in primary antibodies for 48:00:00 @ 4 °C **under stiring**. 2d

- Diluted to its optimal titer (1:1000-1:10,000) in [M] 0.01 Molarity (M) containing 0.3% Triton X-100

> **Note**
>
> * Better to avoid sodium azide because of its blocking action on HRP

• TH Rabbit – 1:1000
• GFP Rabbit 1:5000
• 5-HT Rabbit 1:1000

5 Wash in [M] 0.01 Molarity (M) PBS (3x 10 min).

5.1 Wash in [M] 0.01 Molarity (M) PBS for 00:10:00 (1/3). 10m

5.2 Wash in [M] 0.01 Molarity (M) PBS for 00:10:00 (2/3). 10m

5.3 Wash in [M] 0.01 Molarity (M) PBS for 00:10:00 (3/3). 10m

6 Incubate in **biotinylated** secondary antibodies for 12:00:00 **at** 4 °C **under stiring**. 12h

• Diluted to 1:200 in 0.01 M PBS containing 0.3% Trition X-100 (this antibody is stored in 1:2 glycerol, consequently the concentration will be 1:100).

7 Wash in [M] 0.01 Molarity (M) PBS (3x 10 min).

7.1 Wash in [M] 0.01 Molarity (M) PBS for 00:10:00 (1/3). 10m

7.2 Wash in [M] 0.01 Molarity (M) PBS for 00:10:00 (2/3). 10m

7.3 Wash in [M] 0.01 Molarity (M) PBS for 00:10:00 (3/3). 10m

8 Incubate in streptavidin horseradish peroxidase for 03:00:00 @ Room temperature **under stiring.** 3h

• Diluted to 1:200 in [M] 0.01 Molarity (M) PBS containing 0.3% Triton X-100

9 Wash in [M] 0.01 Molarity (M) PBS (3x 10 min).

9.1 Wash in [M] 0.01 Molarity (M) PBS for 00:10:00 (1/3). 10m

9.2 Wash in [M] 0.01 Molarity (M) PBS for 00:10:00 (2/3). 10m

9.3 Wash in [M] 0.01 Molarity (M) PBS for 00:10:00 (3/3). 10m

10 Prepare a **reaction mixture** which contains:

| | A | B |
|---|---|---|
| | 0.2M acetate buffer | 5 mL |
| | Nickel ammonium sulfate | 250 mg |
| | β-D-Glucose | 20 mg |
| | Ammonium chloride | 4 mg |

Note

* Once the nickel ammonium sulfate is dissolved add 5 mL dd$H_2O$.

**For 0.2 M Acetate Buffer pH 6.0**

- Prepare 1 L of [M] 0.2 Molarity (M) sodium acetate ( 27.216 g for 1L)
- Prepare 1 L of [M] 0.2 Mass Percent acetic acid ( 11.5 mL glacial acetic acid and top up to 1L with $H_2O$dd)
- Mix 900 mL of [M] 0.2 Molarity (M) sodium acetate and 51.7 mL of [M] 0.2 Mass Percent acetic acid
- Top up to 1 L with $H_2O$ dd.
- Check the pH (Should be pH 6 ).

11 Prepare **DAB solution** (10mg/ml); 1 pill (10 mg) of DAB ( -20 °C ) in 1 ml aliquot of dd$H_2O$. 1m

Vortex for 00:01:00 until the pill is dissolved.

Note

**WARNING: THESE STEPS USE 3.3' DAB WHICH IS CARCINOGEN. THE RESULTING WASTE MUST BE THROWN IN A BLACK GARBAGE BAG AND THEN IN ETHIDIUM BROMIDE CONTAINER. THE TOOLS TO REUSE, SUCH AS BRUSH, MUST BE CLEANED WITH BLEACH AND $H_2O$.**

12 Prepare **Glucose oxidase** ( [M] 1 mg/mL )

**Glucose oxidase stock**:

- 2 mg of glucose oxidase
- 2 mL of acetate buffer [M] 50 millimolar (mM)
- Make aliquots of 80 µL in advance .
- Keep the powder and aliquots at -20 °C .

13 Add 250 µL of DAB (10mg/1mL); 1 mL **for 10 mL reaction mixture**) and 20 µL of glucose oxidase for every **2.5 mL** of mixture (1mg/mL); 80 µL **for 10 mL reaction mixture**) to 10 mL of reaction mixture.

14 Transfer the DAB reaction mixture in 10 ml syringe with 0.2 µm filter and place 1 mL of DAB reaction mixture per well (**Use a 12-wells plate**).

15 Rinse sections in [M] 0.1 Molarity (M) **acetate buffer** for 00:01:00 . 1m

**For 0.1 M Acetate Buffer pH 6.0**

- Mix 500 mL of [M] 0.2 Molarity (M) Acetate Buffer and 500 mL of $H_2O$dd.

16 Transfer sections to multi-wells containing DAB reaction mixture.

17 Wait 00:00:30 - 00:10:00 for sections to develop a dark blue/purple nuclear stain **under stirring** (for TH, 45s is sufficient) 10m 30s

18 Remove immediately the sections from DAB reaction mixture.

19 Wash in [M] 0.1 Molarity (M) **acetate buffer** for 00:10:00 (it will stop reaction). 10m

20 Mount sections on charged slides in 0.1M acetate buffer.

21 Allow slides to dry 48:00:00 - 96:00:00 . 6d

> **Note**
>
> (96h is better to avoid slices from coming off).

22 Defat slides in a series of ethanol baths 50-100% and then 00:02:00 in Xylene. 47m

> **Note**
>
> This step must be done under chemical hood.

- 00:02:00 $H_2O$
- 00:02:00 Cresyl Violet ( 00:30:00 at 37 °C before staining)
- 00:01:00 $H_2O$
- 00:01:00 $H_2O$
- 00:01:00 EtOH 50%
- 00:01:00 EtOH 70%
- 00:01:00 EtOH 90%
- 10 dips EtOH 90%

- 00:01:00 EtOH 100%
- 00:01:00 Isopropanol
- 00:02:00 Xylene
- 00:02:00 Xylene

23 Cover with Permount under chemical hood.

24 Let dry 48:00:00 - 72:00:00 under hood. 5d