Immunohistochemistry Protocol for Free-floating Fixed Tissue

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

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ABSTRACT

Immunohistochemistry protocol for staining free-floating fixed tissue in the Kordower Laboratory.

ATTACHMENTS

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GUIDELINES

HISTO- NOTES:

- Primate tissue staining dishes use 100 mL solution per dish
- Rodent tissue staining dishes 50 mL solution per dish
- If staining a large number of primate cases, incubate 1' & 2' Ab in individual cups to conserve volume of Ab used.
- Prepare bleach neutralizing solution prior to Step 12.
- Be conscious of tissue saturation while washing and incubating. i.e. Check that tissue is fully submerged in solution & not clumping. This will ensure proper penetration of antibodies & other reagents.
- Always include Positive & Negative Controls.
- Positive: Use relevant control tissue to confirm specific antibody detection. (i.e. pS129; control tissue should consist of nigral sections previously successfully stained for pS129).
- Negative: Ideally, use tissue that you know does not contain the targeted antigen. If not available, use a section of tissue not incubated in the 1' Ab (primary delete).
- When incubating 1' Ab overnight, leave on shaker in refrigerator.
- Can incubate in fridge on a shaker, covered in parafilm, over the weekend or up to 3 days.
- Select a secondary antibody directed against the species in which the primary antibody was raised (i.e. if a primary antibody raised in rabbit is used, an anti-rabbit secondary antibody raised in a species other than rabbit must be used).

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MATERIALS

- Dilution Media (DM) ([M] 0.2 Molarity (M) TBS plus [M] 0.05 % volume Triton X-100)
- [M] 0.2 Molarity (m) Tris-buffered saline (TBS)
- Sodium meta-periodate
- Normal Serum (species matching the host of the secondary antibody, e.g. horse, goat)
- Bovine Serum Albumin (BSA)
- Triton X-100
- Vectastain Elite ABC-HRP Kit (PK-6100)
- Imidazole
- Sodium Acetate
- 3,3-Diaminobenzidine Tetrahydrochloride (DAB)
- [M] 30 % (v/v) hydrogen peroxide
- [M] 0.2 Molarity (m) Phosphate-buffered saline (PBS)
- Household Bleach
- Primary antibody against the target antigen
- Secondary antibody directed against the species in which the primary antibody was raised (i.e. if a primary antibody raised in rabbit is used, an anti-rabbit secondary antibody raised in a species other than rabbit must be used).

DAY 1 (4 hrs)

1 Wash sections (6 x 00:10:00) in Dilution Media (DM) ([M] 0.2 Molarity (M) TBS plus [M] 0.05 % volume Triton X-100). 10m

1.1 Wash sections for 00:10:00 in DM (1/6). 10m

1.2 Wash sections for 00:10:00 in DM (2/6). 10m

1.3 Wash sections for 00:10:00 in DM (3/6). 10m

1.4 Wash sections for 00:10:00 in DM (4/6). 10m

1.5 Wash sections for 00:10:00 in DM (5/6). 10m

1.6 Wash sections for 00:10:00 in DM (6/6). 10m

2 Endogenous peroxidase inhibition (00:20:00). [M] 0.1 Molarity (M) Sodium meta-periodate in TBS. 20m

- 100 mL [M] 0.2 Molarity (m) Tris-buffered saline (TBS)
- 2.13 g Sodium meta-periodate

3 Wash (2 x 00:10:00) in DM. 10m

3.1 Wash for 00:10:00 in DM (1/2). 10m

3.2 Wash for 00:10:00 in DM (2/2). 10m

4 Serum blocking step (01:00:00 incubation): 1h

- 100 mL DM
- 3 mL Normal Serum (species matching the host of the secondary antibody, e.g. horse, goat)

- 2 g Bovine Serum Albumin (BSA)

5 Incubation in primary antibody (18:00:00 - 72:00:00). See antibody catalog for concentration of primary antibody. 3d 18h

- 100 mL DM
- 1 mL Normal Serum (species matching the host of the secondary antibody, e.g. horse, goat)
- 1 g BSA
- 0.5 mL Triton X-100

Note

Optionally, refrigerate 4 °C to keep antibody stable

DAY 2 (8 hrs) 2h 30m

6 Wash (6 x 00:10:00) in DM. 10m

6.1 Wash in DM for 00:10:00 (1/6). 10m

6.2 Wash in DM for 00:10:00 (2/6). 10m

6.3 Wash in DM for 00:10:00 (3/6). 10m

6.4 Wash in DM for 00:10:00 (4/6). 10m

6.5 Wash in DM for 00:10:00 (5/6). 10m

6.6 Wash in DM for 00:10:00 (6/6). 10m

7 Secondary antibody incubation (01:00:00) Concentration of secondary antibody is always 1:200 in solvent. 1h

- 100 mL DM
- 1 mL Normal Serum (species matching the host of the secondary antibody, e.g. horse, goat)
- 1 g BSA

8 Wash (6 x 00:10:00) in DM. 10m

Note

****(incubate ABC in solvent during these washes)**.**

8.1 Wash for 00:10:00 in DM (1/6). 10m

8.2 Wash for 00:10:00 in DM (2/6). 10m

8.3 Wash for 00:10:00 in DM (3/6). 10m

8.4 Wash for 00:10:00 in DM (4/6). 10m

8.5 Wash for 00:10:00 in DM (5/6). 10m

8.6 Wash for 00:10:00 in DM (6/6). 10m

9 Avidin-Biotin Complex (ABC) Step (01:00:00) - Vectastain Elite ABC-HRP Kit (PK-6100). 1h

- 100 mL DM
- 1 mL Normal Serum (species matching the host of the secondary antibody, e.g. horse, goat)
- 1 g BSA

9.1 Add ABC Reagent A and B to 1/10th of total desired volume of solvent.

9.2 Incubate for 00:30:00 at Room temperature. Then dilute 1:10 using the same solvent. 30m

Note

This is your working solution. See chart below for example volumes.

A	B	C	D
Working Solution	A (drops)	B (drops)	1/10th Working solution
25 mL	1	1	2.5 mL
50 mL	2	2	5 mL
100 mL	4	4	10 mL

10 Wash for 00:10:00 in DM. 10m

11 Wash for 00:10:00 with TBS. 10m

12 Wash (3 x 00:10:00) in [M] 0.2 Molarity (M) Imidazole/ [M] 1.0 Molarity (M) Sodium Acetate buffer pH 7.2 to pH 7.4 . 10m

- 1000 mL dH_2O
- 0.68 g Imidazole
- 6.8 g Sodium Acetate.
- Retain 100 mL of non-pH'd buffer for DAB preparation.

12.1 Wash for 00:10:00 in Imidazole/Sodium Acetate buffer (1/3). 10m

12.2 Wash for 00:10:00 in Imidazole/Sodium Acetate buffer (2/3). 10m

12.3 Wash for 00:10:00 in Imidazole/Sodium Acetate buffer (3/3). 10m

13 DAB Step (Neutralize DAB with bleach when done)

13.1 Make DAB solution

- 100 mL non-pH'd imidazole acetate buffer from above

- 50 mg 3,3-Diaminobenzidine Tetrahydrochloride (DAB)
- 2 g Nickel(II) sulfate hexahydrate **(Only used with certain primary antibodies, chromagen enhancer that changes brown DAB precipitate to blue-purple)**

13.2 Make [M] 1 % (v/v) Hydrogen Peroxide (H_2O_2)

- 3 mL of dH_2O
- 100 µL of [M] 30 % (v/v) hydrogen peroxide (H_2O_2)

13.3 Start reaction -- add 500 µL of [M] 1 % (v/v) hydrogen peroxide (H_2O_2) to the above DAB mixture just prior to use.
OR add 16.7 µL of [M] 30 % (v/v) hydrogen peroxide (H_2O_2), per 100 mL.

13.4 Place tissue in DAB solution. 11m

- Develop tissue for approximately 00:04:00 to 00:07:00.
- Timing is critical, ensure all tissue spends the same amount of time in DAB solution.

13.5 To monitor signal, move all tissue to imidazole buffer, remove one section and mount on an UNSUBBED slide and view under microscope. Place all tissue back in DAB solution to increase signal intensity, if needed.

14 Wash developed tissue in imidazole acetate buffer (3 x 00:10:00). 10m

Note

****Neutralize DAB with BLEACH!!!****

14.1 Wash developed tissue in imidazole acetate buffer for 00:10:00 (1/3). 10m

14.2 Wash developed tissue in imidazole acetate buffer for 00:10:00 (2/3). 10m

14.3 Wash developed tissue in imidazole acetate buffer for 00:10:00 (3/3). 10m

15 Store tissue in [M] 0.2 Molarity (m) Phosphate-buffered saline (PBS) in refrigerator 4 °C until mounted on slides.