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

(f) Immunohistochemistry Protocol for Free-floating Fixed Tissue with Tyramine Signal Amplification (TSA)

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ABSTRACT

Immunohistochemistry protocol for staining free-floating fixed tissue with Tyramine signal amplification (TSA) in the Kordower Laboratory. TSA allows a much lower concentration of primary antibody to be used (typically 1:10K or 1:15K) at the expense of an extra day of staining.

ATTACHMENTS

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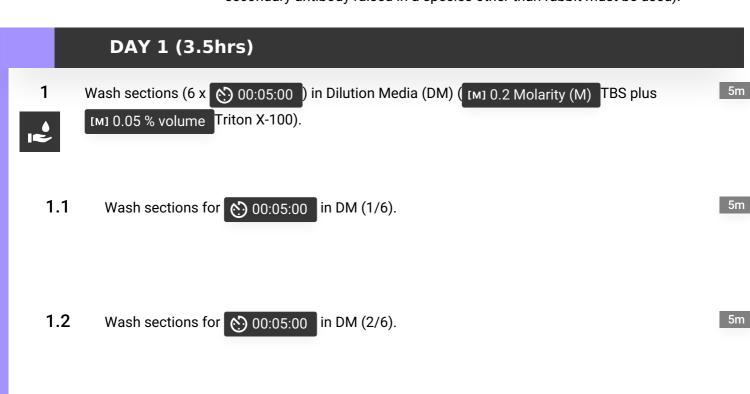
GUIDELINES

HISTO-NOTES:

- Primate tissue staining dishes use 🚨 100 mL solution per dish
- Rodent tissue staining dishes Ⅰ 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 antirabbit secondary antibody raised in a species other than rabbit must be used).

MATERIALS

- Dilution Media (DM) (IMI 0.2 Molarity (M) TBS plus IMI 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
- Sodium tetraborate decahydrate
- Boric Acid
- Biotin tyramide
- 3,3-Diaminobenzidine Tetrahydrochloride (DAB)
- Nickel(II) sulfate hexahydrate
- [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).



1.3 Wash sections for 00:05:00 in DM (3/6).
1.4 Wash sections for 00:05:00 in DM (4/6).

5m

5m

1.5 Wash sections for 00:05:00 in DM (5/6).

5m

1.6 Wash sections for 00:05:00 in DM (6/6).

5m

20m

- 2 Endogenous peroxidase inhibition (00:20:00). [M] 0.1 Molarity (M) Sodium metaperiodate in TBS.
 - Д 100 mL [м] 0.2 Molarity (m) Tris-buffered saline (TBS)
 - Z 2.13 g sodium meta-periodate
- 3 Wash (2 x (5) 00:00:00) in DM.

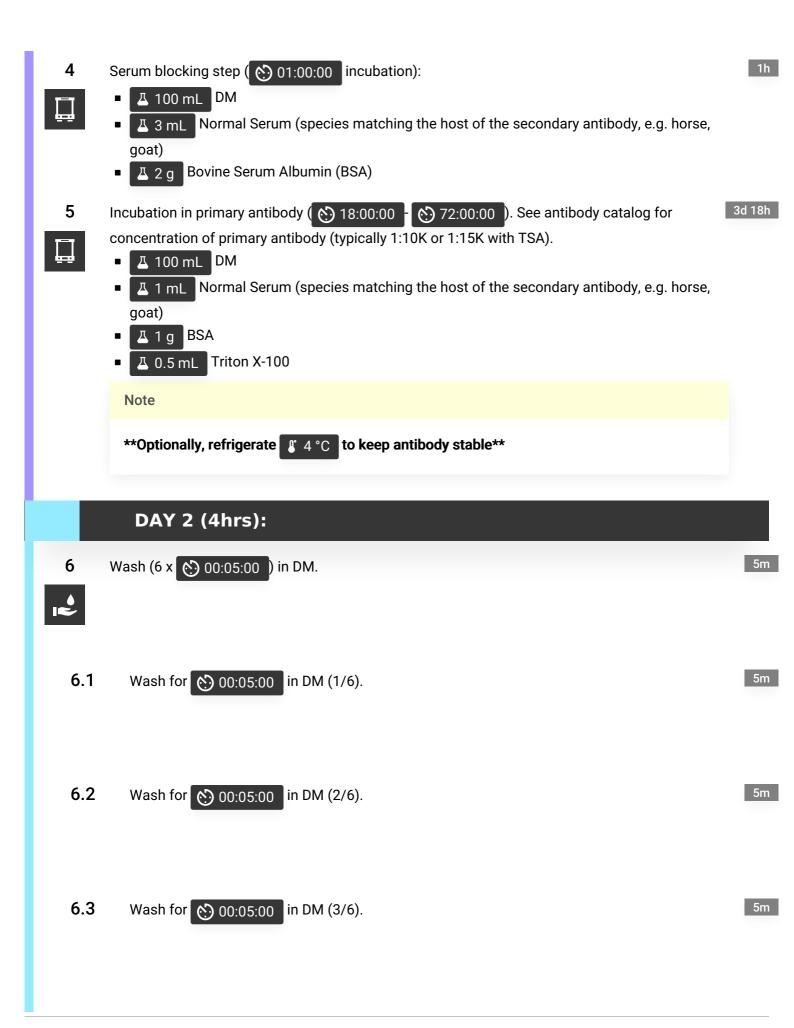


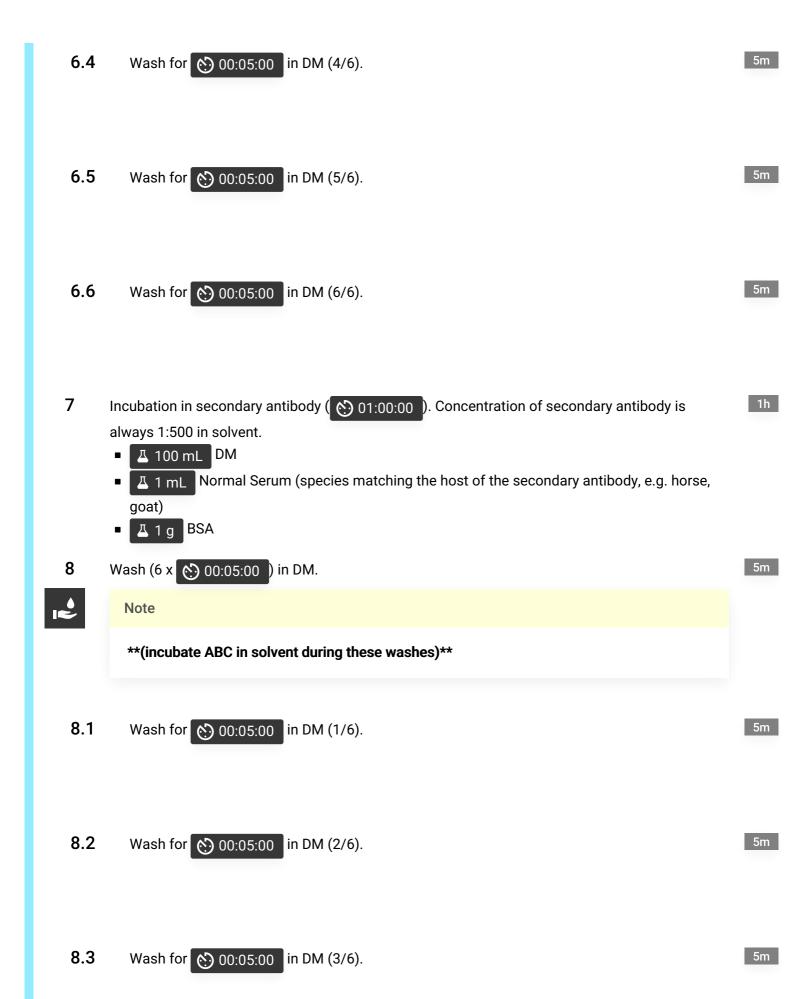
3.1 Wash for 00:10:00 in DM (1/2).

10m

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

10m







5m

8.5 Wash for 00:05:00 in DM (5/6).

5m

8.6 Wash for 00:05:00 in DM (6/6).

5m

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

1h

Note

**Re-use for step 15. Can be stored overnight in refrigerator 4 °C **

- 🗸 100 mL DM
- I mL Normal Serum (species matching the host of the secondary antibody, e.g. horse, goat)
- A 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 8 Room temperature

30m



Note

Then dilute 1:10 using the same solvent. This is your working solution. See chart below for example volumes.

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

10 10m Wash for (5) 00:10:00 in DM. 11 10m Wash 00:10:00 in TBS. 12 Wash (2 x 👏 00:10:00) in гмз 0.05 Molarity (M) Borate buffer 🕞 8.5 . 10m \perp 1000 mL dH₂O 🗸 4.77 g Sodium tetraborate decahydrate ☐ 2.32 g Boric Acid 12.1 Wash for 00:10:00 in Borate buffer (1/2). 10m 12.2 10m Wash for 00:10:00 in Borate buffer (2/2). 30m 13 Incubate sections with biotin tyramide solution (© 00:30:00). Note DO NOT USE IF >6 MONTHS OLD. △ 100 mL Borate buffer

- Δ 2 μL of [M] 50 mg/mL biotin tyramide stock
- \bot 10 μ L [M] 30 % (v/v) Hydrogen Peroxide (H₂O₂)
- Wash (3 x 00:10:00) in TBS (Antigen is now labeled with biotin).

10m



14.1 Wash for (5) 00:10:00 in TBS (1/3).

10m

14.2 Wash for 00:10:00 in TBS (2/3).

10m

14.3 Wash for 00:10:00 in TBS (3/3).

10m

DAY 3 (4 hrs):

40m

Repeat Actin Biotin Complex Step 9 then wash for 00:10:00 with TBS.

10m



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 dH₂O
- Д 0.68 g Imidazole
- <u>A 6.8 g</u> Sodium Acetate
- Retain 🗷 100 mL of non-pH'd buffer for DAB preparation
- 16.1 Wash for 00:10:00 in Imidazole/Sodium Acetate buffer (1/3).

10m

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

10m

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

10m

- 17 DAB step (Neutralize DAB with bleach when done)
- 17.1 Make DAB solution
 - Д 100 mL non-pH'd imidazole acetate buffer from above

 - A 2 g Nickel(II) sulfate hexahydrate **(Only used with certain primary antibodies, chromagen enhancer that changes brown DAB precipitate to blue-purple)**
- 17.2 Make [M] 1 % (V/V) Hydrogen Peroxide (H_2O_2)
 - of dH₂O
 - \bot 100 μ L of [M] 30 % (V/V) hydrogen peroxide (H₂O₂)
- 17.3 Start reaction -- add \bot 500 µL of [M] 1 % (V/V) hydrogen peroxide (H₂O₂) to the above DAB mixture just prior to use. OR add \perp 16.7 µL of [M] 30 % (v/v) hydrogen peroxide (H₂O₂), per \perp 100 mL
- 17.4 Place tissue in DAB solution.

- Develop tissue for approximately () 00:05:00
- Timing is critical, ensure all tissue spends the same amount of time in DAB solution.
- 17.5 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



