IMMUNOHISTOCHEMISTRY PROTOCOL: *DLX*-2 (*TES*-1) 08.93

D.D. Eisenstat, MD; A. Bulfone, MD; M. H. Porteus, MD, PhD; J.L.R. Rubenstein, MD, PhD_

General:

1. Frozen embryo (-80 C) sectioned in cryostat (-20 C), 10 microns thickness

Use Fisher Superfrost Plus slides at room temperature
Once section is dry, place in slide box on dry ice
When sectioning complete, slide boxes placed under vacuum, slightly ajar, at 4 C for at least 2 hours, then 3-4 desiccant capsules (Humicap 386) added, box placed in freezer bag with desiccant crystals (Drierite #23001 anhydrous calcium sulfate) at -80 C.

2. Stain reference slides (every 10 sections) with cresyl violet 0.5%
(Chroma brand, filtered before use.)

(a) Slides to room temperature, allow condensation to thaw
(b) Fix in 4% paraformaldehyde (freshly prepared) in 1X PBS pH 7.5 for 10 min at RT
(c) Wash 3 times with water
(d) Stain in cresyl violet 1-5 min at RT
(e) Wash 3 times in water
(f) Dehydrate in graded ethanols, 2 min each:
50%,75%, 85%, 95%, 100%, 100%, xylene, xylene
(g) Mount with Permount (Fisher); place on coverslip first (to be done in hood)

[**20X PBS in 0.5 liter**: 10 g Na_2HPO_4 (140mM), 3.6 g NaH_2Po_4 (60 mM), 76 g NaCl (2.6 M); pH to exactly 7.5; autoclave]

[**4% paraformaldehyde in 0.25 liter:** 10 g paraformaldehyde (Polysciences #00380), 12.5 mL 20x PBS pH 7.5, dH_20 to 250 mL; dissolve until clear at 65 C, filter (0.2μM Nalgene) in hood, bring to room temperature.]

<u>IMMUNOHISTOCHEMISTRY PROTOCOL: *DLX*-2 (*TES*-1)</u> 08.93

<u>Protocol A: VECTOR *Elite* ABC Kit</u>

(modified by D. Eisenstat, MD)

1) <u>Primary Antibody</u> to Dlx-2 is **M650**, dilution 1:50 (rabbit anti-mouse)
   [15 μL blocking serum, 3 μL antibody, 7.5 μL 20X PBS pH 7.5, 7.5 μL 1% thimerosal (Sigma #T5125), 117 μL dH_20: total volume 150 μL]

2) <u>Secondary Antibody</u> (**Vector BA-1000/ PK6101 kit**) biotinylated goat anti-rabbit, dilution 1:200
   [150 μL stock blocking serum, 10 mL 1X PBS , 50 μL biotinylated antibody stock]

3) <u>Blocking serum</u> (**Vector S-1000**): goat

4) <u>Control antibody</u> (**Vector I-1000**): rabbit IgG, dilution 1:1250 or 1:2500

5) <u>Tertiary substance</u>: (**Vector PK 6100/ 6101**) Elite ABC
   [2 drops (100 μL) solution 'A' to 5 mL 1X PBS, mix, add 2 drops 'B', mix well]

6) <u>Chromogenic substrate</u>: DAB (**Vector SK-4100**)
   [2 drops buffer stock solution added to 5 mL water, mix; add 4 drops DAB stock solution, mix; add 2 drops hydrogen peroxide solution, mix.]

<u>Day 1</u>

1. Thaw frozen sections at RT
2. Fix in 4% paraformaldehyde at RT 15-20 min
3. Wash 3 times in 1X PBS pH 7.5, 5 min/wash at RT
4. Add water to humidifier chamber
5. Dry excess PBS off slides; do not let slides dry out
6. Circle tissue section with Lipshaw ISOLATOR pen
7. Add 150 μL goat serum, undiluted.
8. Incubate overnight at 4 C.

<u>Day 2</u>

9. Prepare primary antiserum or control antibody in 10% blocking serum, 0.05% thimerosal ,1X PBS pH 7.5
10. Blot off excess serum and add 150μL primary antibody
11. Incubate 1-2 hours at RT
12. Wash as in step 3.

13. Prepare secondary antibody . Add 150 µL per slide.
14. Incubate 30-60 minutes at RT
15. Blot excess. Wash as per step 3.

IMMUNOHISTOCHEMISTRY PROTOCOL: *DLX*-2 (*TES*-1) 08.93

Protocol A: VECTOR *Elite* ABC Kit

Day 2 (continued)

16.* Prepare 250 mL of 3%# hydrogen peroxide (Sigma) in 100% methanol (Fisher). Quench endogenous peroxidases by washing for 30 min at RT. (* optional step; use if high background level of endogenous peroxidases) (# try 0.3% H_2O_2 first)
17. Blot excess. Wash as in step 3.
18. Prepare tertiary molecule: let stand at RT 30 min prior to use
19. Add 150 µL to slide. Incubate 30 min at RT.
20. Blot excess. Wash as in 3.
21. Prepare DAB substrate. Waste and excess DAB treated with 2% bleach prior to discarding.
22. Add 150 µL . Allow staining for 1- 30 minutes; usually 2-10 min. Observe under microscope.
23. Stop reaction in water for 5 min.
24. Dehydration in graded ethanols then xylene. (refer to cresyl violet protocol).
25. Mount with permount.

IMMUNOHISTOCHEMISTRY PROTOCOL: *DLX*-2 (*TES*-1) 08.93

Protocol B: Biotin / Streptavidin / Peroxidase

1) Primary Antibody to Dlx-2 is **M650** dilution 1:50 (rabbit anti-mouse)
   [15 μL blocking serum, 3 μL antibody, 7.5 μL 20X PBS pH 7.5, 7.5 μL 1% thimerosal (Sigma #T5125), 117 μL dH_20: total volume 150 μL]
2) Secondary Antibody **(Boehringer Mannheim (BMB) #1214659)** Sheep anti-rabbit IgG biotinylated F(ab)' fragment, dilution 1:200
   [150 μL blocking serum, 7.5 μL antibody, 75 μL 20X PBS pH 7.5, 1267.5μL dH_20: total volume 1500 μL]

3) Blocking serum (**Sigma S-7773**): sheep

4) Control antibody (**Vector I-1000**): rabbit IgG, dilution 1:1250 or 1:2500

5) Tertiary substance: (**BMB #1089-153)** Streptavidin-peroxidase, dilution 1:1000
   [1.5 μL streptavidin-peroxidase, 75 μL 20X PBS pH 7.5 , 1423.5 μL dH_20]
6) Chromogenic substrate: DAB (**Vector SK-4100**)
   [2 drops buffer stock solution added to 5 mL water, mix; add 4 drops DAB stock solution, mix; add 2 drops hydrogen peroxide solution, mix.]

Day 1

1. Thaw frozen sections at RT
2. Fix in 4% paraformaldehyde at RT 15-20 min
3. Wash 3 times in 1X PBS pH 7.5, 5 min/wash at RT
4. Add water to humidifier chamber
5. Dry excess PBS off slides; do not let slides dry out
6. Circle tissue section with Lipshaw ISOLATOR pen
7. Add 150 μL sheep serum, undiluted.
8. Incubate overnight at 4 C.

Day 2

9. Prepare primary antiserum or control antibody in 10% blocking serum, 0.05% thimerosal ,1X PBS pH 7.5
10. Blot off excess serum and add 150μL primary antibody
11. Incubate 1-2 hours at RT
12. Wash as in step 3.
13. Prepare secondary antibody Add 150 μL per slide.
14. Incubate 30-60 minutes at RT

15. Blot excess. Wash as per step 3.
16.* Prepare 250 mL of 3% # hydrogen peroxide (Sigma) in 100% methanol (Fisher). Quench endogenous peroxidases by washing for 30 min at RT. (* optional step; use if high background level of endogenous peroxidases) (# try 0.3% H_2O_2 first)

IMMUNOHISTOCHEMISTRY PROTOCOL: ***DLX*-2 (*TES*-1)** 08.93

Protocol B: Biotin / Streptavidin / Peroxidase

Day 2 (continued)

17. Blot excess. Wash as in step 3.
18. Prepare tertiary molecule:
19. Add 150 μL to slide. Incubate 30-60 min at RT.
20. Blot excess. Wash as in 3.
21. Prepare DAB substrate: Waste and excess DAB treated with 2% bleach prior to discarding.
22. Add 150 μL . Allow staining for 1- 30 minutes; usually 2-10 min. Observe under microscope.
23. Stop reaction in water for 5 min.
24. Dehydration in graded ethanols then xylene. (refer to cresyl violet protocol).
25. Mount with permount.