**GAD (Enzo) immunostaining with Fluoro Nissl counter**

**(Floating) XG**

Animal

0. If needed, 150 ug colchicine should be injected into lateral ventricle or targeted tissue two days before perfusion to enhance GAD signals in the soma.

Perfusion and Sectioning

1. Transcardially perfuse with cool saline followed by 4% paraformaldehyde and 0.2% picric acid with 0.1 M phosphate buffer (pH 7.2–7.3). Postfix overnight with the same fixative.

2. Section the brain via vibrating microtome into 40 to 50-µm-thick slices and harvest them in 24-well plates containing PBS.

Antibody application (Room temperature, Light shielding)

3. PBS wash.

4 10% NGS in PBS-X, 30 min on shaker

5. 1st antibody in PBS-XG, ON on shaker

6. PBS-X wash (quick × 1, 10 min × 2)

7. 2nd antibody in PBS-XG, 2 hr on shaker

8. PBS-X wash (quick × 1, 10 min × 2)

9. PBS wash

Mounting

10. Mount on gelatin-coated glass slides and Air-dry (30 min)

11. Circle with Liquid-blocker to make hydrophobic barrier

Counterstaining with fluorescent Nissl

12. PBS, 10 min.

13. Diluted NeuroTrace (Blue), 40-60 min.

14. PBS wash (quick × 1, 10 min × 2)

Coverslipping

15. Remove the hydrophobic barrier with KIMTECH wipes and cotton buds.

16. Coverslip with 50% (v/v) glycerol/2.5% (w/v) DABCO in PBS.

Observation

17. Observe with epifluorescence or confocal microscopy.

**Solutions**

**PBS-X** (total 550 ml)

20% Triton X-100 8.25 ml final 0.3%

in PBS 541.75 ml

**PBS-XG** (150 ml)

normal goat serum 1.5 ml (final 1%)

sodium azide 30 mg (final 0.02%)

in PBS-X 148.5 ml

**10%NGS in PBS-X** (5 ml)

normal goat serum 0.45 ml (final 10%)

in PBS-XG 4.55ml

**1st antibody**

Ms anti-GAD (Enzo Life Sciences Cat# BML-GC3108-0100, RRID:AB\_2632394) 1:4k dil.

in PBS-XG

**2nd antibody** (1:1000 dilution, 2 µg/ml final)

Goat anti-mouse IgG Alexa Fluor 488 (A-11029)

in PBS-XG

**Diluted NeuroTrace Solution** ( NeuroTrace Blue, N-21479)

Confocal microscopy (1:150 dilution with PBS)

Epifluorescence microscopy (1:300 dilution with PBS)

**Reagents**

BML-GC3108-0100 (Enzo Life Sciences): 100 µl

A-11029 (Invitrogen): 0.5 ml, 71,300 HUF

N-21479 (Invitrogen): 1 ml, \34,000

Normal Goat Serum S-1000 (Vector labratories): 20 ml

**References**