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

**(Floating) PBS-XD**

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% NDS in PBS-X, 30 min on shaker

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

6. PBS-X wash (quick × 1, 10 min × 2)

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

8. PBS-X wash (quick × 1, 10 min × 2)

9. PBS wash

Counterstaining with fluorescent Nissl

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

11. PBS wash (quick × 1, 10 min × 2)

Mounting

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

Coverslipping

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

Observation

14. 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-XD** (150 ml)

normal donkey serum 1.5 ml (final 1%)

sodium azide 30 mg (final 0.02%)

in PBS-X 148.5 ml

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

normal donkey serum 0.45 ml (final 10%)

in PBS-XD 4.55ml

**1st antibody** (1:10000 – 1:1000 dilution)

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

in PBS-XD

**2nd antibody** (1:500 dilution, 3–4 µg/ml final)

Donkey anti-mouse IgG Alexa 555 (A-31570) (1:1000 dilution, 2 µg/ml final)

Or

Donkey anti-mouse IgG Alexa 594 (A-21203) (1:1000 dilution, 2 µg/ml final)

Or

Donkey anti-mouse IgG Alexa 647 (A-31571): (1:1000 dilution, 2 µg/ml final)

in PBS-XD

**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-21203 (Thermo Fisher Scientific): 0.5 ml, 74,200 HUF

A-31570 (Thermo Fisher Scientific) 0.5 ml, 71,900 HUF

A-31571 (Thermo Fisher Scientific) 0.5 ml, 79,400 HUF

N-21479 (Thermo Fisher Scientific): 1 ml, 320 USD

Normal donkey Serum IHR-8135 (ImmunoBioScience): 20 ml, $60,00

**References**