**GAD67 (Frontier) immunostaining with Fluoro Nissl counter**

**(Floating) XG**

Animals

1. 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, Agarose-embedding, Sectioning

2. Transcardially perfuse with 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.

3. Remove the dura. If possible, the arachnoid membrane, the pia also.

4. Embed the brain into the 4% electrophoresis quality agarose.

5. Section the brain with vibrating blade microtome into 50-µm-thick slices and harvest them in 12 well plates containing PBS

Antibody application (Room temperature, Light shielding)

6. 10% NGS in PBS-X, 30 min on shaker

7. 1st antibody in PBS-XG, overnight on shaker.

8. PBS-X wash (quick × 1, 10 min × 2)

9. 2nd antibody in PBS-XG, 2 hr on shaker

10. PBS-X wash (quick × 1, 10 min × 2)

11. PBS wash

Counterstaining with fluorescent Nissl

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

13. PBS wash (quick × 1, 10 min × 2)

Mounting

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

Coverslipping

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

Observation

16. 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** (Yamada et al., 2001) (1 µg/ml final)

Rb anti-GAD67/65 (Frontier Institute Cat# GAD-Rb, RRID:AB\_2571698) 1:200 dil.

in PBS-XG

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

Goat anti-rabbit IgG Alexa 488 (A-11034)

Or

Goat anti-rabbit IgG Alexa 633 (A-21071)

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**

GAD-Rb-Af260 (frontier science): 20 µg, \37,800, 50 µg, \70,200

A-11034 (Thermo Fisher Scientific): 0.5 ml, 71,900 HUF

A-21071 (Thermo Fisher Scientific): 0.5 ml, 71,900 HUF

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

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

**References**

Yamada K, Fukaya M, Shimizu H, Sakimura K, Watanabe M (2001) NMDA receptor subunits GluRepsilon1, GluRepsilon3 and GluRzeta1 are enriched at the mossy fibre-granule cell synapse in the adult mouse cerebellum. Eur J Neurosci 13:2025-2036.