7TM Antibodies
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7TM Phospho-Immunohistochemistry Protocol

This protocol is designed and validated for the staining of perfusion-fixed, free-floating cryosections from mouse brain but can be adapted to other tissues and species.

1. Buffers and Reagents

Use double distilled water for buffer preparation or water with the same grade of purity.

- Protein Phosphatase-Inhibitors (+PPIs) (1 tablet PhosSTOP per 10 ml). We recommend Sigma Aldrich 04906845001.
- Tyrode's solution (NaCl 8.00 g/L, KCl 0.20 g/L, $CaCl_2$ 0.13 g/L, $MgCl_2$ x 6 H_2O 0.10 g/L, NaH2PO_4 x H_2O 0.05 g/L, $NaHCO_3$ 1.00 g/L, water-free Glucose 1 g/L)
- Zamboni's fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate solution (Heat 80 g paraformaldehyde (PFA in 350 mL saturated picric acid in a 500 mL bottle to 60 °C and add 30-40 mL 2.52% NaOH in water until solution is cleared. Filter into 2 L bottle and fill up with 0.1 M phosphate solution (6.62 g NaH_2PO_4 and 44.8 g Na_2HPO_4 in 2 L water).
- Biotinylated Tyramine (BT). (Preparation of BT as described in PMID 7897179: Dissolve 100 mg NHS-LC-biotin (Pierce) in 40 mL 50 mM borate buffer pH 8.0. Then add 30 mg tyramine-HCl (Sigma), agitate solution overnight at room temperature. Clear solution by filtration and store aliquots at 4 °C.)
- PBS: Phosphate-Buffered Saline (137 mM NaCl, 2.7 mM KCl, 2 mM KH_2PO_4, 10 mM Na_2HPO_4)
- PBST: PBS containing 0.3% Tween 20
- NDS (normal donkey serum, e.g., Sigma Aldrich S30-100ml)
- Methanol
- H_2O_2 30%
- Triton X-100
- ABC-Kit (e.g., Vectastain Elite ABC Kit, e.g., VEC-PK-6100)
- Fluorescently-labeled streptavidin (e.g., AlexaFluor 555)
- DAPI
- mounting medium (e.g., Eukitt)
- SuperFrost Plus glass slides (e.g., ThermoFisher 15438060)
- Tween 20

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2. Animal perfusion and tissue preparation

1. Animals are deeply anaesthetized. (e.g., Isoflurane)
2. Animals are then subjected to a transcardial perfusion with calcium-free Tyrode's solution containing protein phosphatase-inhibitors (+PPIs) (1 tablet PhosSTOP per 10 ml) followed by Zamboni's fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate solution +PPIs.
3. Brain, spinal cord and other tissues of interest are rapidly dissected and postfixed in the same fixative for 4 h at room temperature.
4. The tissue is then cryoprotected by immersion in 10% sucrose +PPIs followed by 30% sucrose +PPIs for 48 h at 4 °C.
5. For free-floating sections, tissue is cut into 40 µm sections using a freezing microtome.

3. Staining and Mounting

1. Wash free-floating sections in PBS + PPIs.
2. For quenching of endogenous peroxidase, incubate sections with PBS/methanol (1:1) containing 0.3% H_2O_2 for 30 minutes at room temperature.
3. Wash sections twice with PBS containing 0.3% Tween 20 (PBST) for 15 min at room temperature.
4. For blocking of nonspecific binding, incubate sections with PBS containing 10% NDS and 0.3% Triton X-100 for 2 hours at room temperature.
5. Incubate sections with 500 µL Phospho-IHC-Grade 7TM Antibodies in PBS containing 2% NDS and 0.3% Triton X-100 at 4 °C overnight under gentle agitation.
6. Wash sections twice with PBST for 15 min at room temperature.
7. Incubate sections with 500 µL biotinylated anti-rabbit-IgG in PBS containing 10% NDS and 0.3% Triton X-100 for 2 hours at room temperature.
8. Wash sections twice with PBST for 15 min at room temperature.
9. Incubate sections with 500 µL of AB solution (containing 5 µL component A and 5 µL componenet B per mL PBS) for 1 hour at room temperature. Prepare AB solution 30 min before use according to the instructions of the manufacturer.
10. Wash sections twice with PBST for 15 min at room temperature.
11. Incubate sections with 500 µL BT solution (3 µL BT per mL PBS) containing 0.3% H_2O_2 for 20 minutes at room temperature.
12. Wash sections twice with PBST for 15 min at room temperature.

13. Incubate sections with 500 µL fluorescently labeled streptavidin (e.g., Alexa Fluor 555) in PBS containing 10% NDS and 0.3% Triton X-100 at 4 °C overnight.
14. Wash sections twice with PBST for 15 min at room temperature.
15. If nuclear counterstain is desired, incubate sections with 500 µL DAPI solution (1 µL/mL distilled water) for 5 minutes at room temperature.
16. Wash sections twice with PBST for 15 min at room temperature.
17. Mount sections onto SuperFrost Plus glass slides and coverslip using Eukitt mounting media.