Protocol for ENT4 Antibody (Cat. No. 463 005) Immunohistochemistry Formaldehyde (IHC) Tissue Preparation

Tissue preparation and fixation are important for the success of immunohistochemical experiments. First, it is crucial, that the animal is perfused with saline or phosphate buffered saline (PBS) to remove residual IgG containing blood from blood vessels. Otherwise, IgGs may be bound by cross-reactive secondary reagents and cause undesired background staining. Second, the tissue is fixed to maintain tissue integrity. A commonly used fixative is formaldehyde (FA), which is known for good tissue preservation. Scientists use self-made FA fixation solutions produced by dissolving paraformaldehyd (PFA) in PBS, or they apply ready-to-use FA fixation solutions containing different amounts of methanol for stabilization. In our standard tissue preparation protocol, we apply a ready-to-use FA solution with a low amount of methanol. The optimal fixation time varies between minutes to hours, depending on tissue and antibody. Too short or too long fixation times may lead to bad tissue integrity or masking of antigens. In our standard protocol, we apply 24 h 4% FA fixation to obtain a good tissue integrity. However, some antibodies need shorter/milder FA fixation or even methanol or acetone fixation, because the epitope is prone to be masked by FA crosslinking (please check the remarks sections for deviating fixation protocols).

Vibratome sections

Materials and reagents

- Cold 0.9% saline containing 17 U/ml Heparin
- **Fixation buffer:** 4% FA in PBS pH 7.4 (room temperature)
- **TBS:** Tris buffered saline (50 mM Tris, 150 mM NaCl), pH 7.2
- **PBS:** Phosphate buffered saline (200 mM NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4), pH 7.4
- **Cryoprotectant buffer:** 25% glycerol, 25% ethylene glycol, 50% PBS pH 7.4

Procedure

1. Transcardially perfuse with 30-50 ml cold 0.9% saline containing 17 U/ml Heparin with a rate of 5 ml/min until the tissue is cleared from blood.

2. Perfuse with **fixation buffer** 30-50 ml with a rate of 5 ml/min.

3. After tissue dissection, postfix tissue in **fixation buffer** for 24 h at 4°C.

4. Rinse tissue in **TBS** and incubate tissue in TBS for 24 h at 4°C to stop FA fixation process.

5. Cut tissue 25-50 µm with a vibratome in ice-cold **PBS** and store at -20°C in **cryoprotectant buffer** until staining (Protocol for Immunohistochemistry Formaldehyde (IHC) Fluorescence Staining - Free Floating).

Cryostat sections

Materials and reagents

- Cold 0.9% saline containing 17 U/ml Heparin
- **Fixation buffer:** 4% FA in PBS pH 7.4 (room temperature)
- **TBS:** Tris buffered saline (50 mM Tris, 150 mM NaCl), pH 7.2
- **PBS:** Phosphate buffered saline (200 mM NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4), pH 7.4
- **Sucrose solution:** 30% sucrose in PBS
- **Cryoprotectant buffer:** 25% glycerol, 25% ethylene glycol, 50% PBS pH 7.4
- Tissue-Tek®
- Slides SuperFrost® plus

Procedure

1. Transcardially perfuse with 30-50 ml cold 0.9% saline containing 17 U/ml Heparin with a rate of 5 ml/min until the tissue is cleared from blood.

2. Perfuse with **fixation buffer** 30-50 ml with a rate of 5 ml/min.

3. After tissue dissection, postfix tissue in **fixation buffer** for 24 h at 4°C.

4. Rinse tissue in **TBS** and incubate tissue in TBS for 24 h at 4°C to stop FA fixation process.

5. Incubate tissue in **sucrose solution** until it sinks to the bottom (1-3 days).

6. Freeze tissue on dry ice and store at -80°C until cutting.

7. Cut tissue with a cryostat:
   - Sections on slides: Cut tissue 10-20 µm and mount on slides. Dry sections for 30 min at 57°C. Store slides at -20°C until staining procedure (Protocol for Immunohistochemistry Formaldehyde (IHC) Fluorescence Staining - Slide Mounted).
   - Sections free floating: Cut tissue 25-50 µm with a cryostat and store in **cryoprotectant buffer** at -20°C until staining procedure (Protocol for Immunohistochemistry Formaldehyde (IHC) Fluorescence Staining - Free Floating).

Note: This protocol has been validated in the SYSY Antibodies laboratories to ensure consistent and reliable staining results. However, for achieving the best specific signal with minimal background, the optimal antibody concentration, incubation temperature, and incubation duration should be optimized for each experiment.