Protocol for iNOS Antibody (Cat. No. 484 017) Immunohistochemistry Paraffin embedded (IHC-P) Tissue Preparation

Tissue (or ce**ll) p**reparation and fixation are important for the success of immunohistochemical experiments. Many tissue processing parameters have been identified as affecting IHC-P staining results. These include the delay between tissue removal and fixation (cold ischaemia), the duration of fixation (see: Influence of Formalin Fixation Duration on Staining Intensity in FFPE Tissues), and the choice of fixative. FFPE tissues are most often fixed in either 10% neutral buffered formalin (FA) solution or fresh 4% formaldehyde solution (PFA) made from paraformaldehyde powder. In our standard tissue preparation protocol, we use a ready-to-use formalin solution containing a low concentration of methanol. When working with tissue from mice or rats, it is crucial that the animal is perfused with saline or phosphate buffered saline (PBS) to remove residual IgG containing blood from blood vessels. This reduces the undesired background when secondary reagents that cross-react with endogenous IgGs are used, e.g. mouse-on-mouse. In our standard protocol, we fix standard samples in 4% FA for 24 hours to obtain good tissue integrity.

Tissue preparation and fixation are also important for human samples. SYSY Antibodies purchased these samples from an external provider.

Mouse and rat FFPE

Materials and reagents

- Cold 0.9% saline containing 17 U/ml Heparin
- **Fixation buffer:** 4% FA in PBS pH 7.2 (room temperature)
- **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
- **Dehydrate solution:** Xylene, 100% ethanol, 90% ethanol, 70% ethanol, Xylene
- Paraffin

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. Continue the perfusion using the **fixation buffer** at a rate of 5 ml/min.

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

3. Transfer tissue to **70% ethanol** and incubate until **paraffin** embedding at 4°C.

4. Dehydration, clearing and paraffin infiltration steps are performed in an automated tissue processing system:
   - 70% ethanol 1h
   - 90% ethanol 1h
   - 90% ethanol 1h
   - 100% ethanol 1h
   - 100% ethanol 1h
   - 100% ethanol 1h
   - Xylene 1h
   - Xylene 1h

- Paraffin 45 min
- Paraffin 45 min
- Paraffin 45 min
- Paraffin 45 min

5. Remove the paraffin infiltrated tissue specimens form the automated tissue processing system and orient them in a metal base mold. Pour in the melted paraffin and carefully transfer the mold onto a cool plate

6. After the paraffin has solidified, the mold can be removed and the FFPE block is ready for sectioning on the microtome

Cell pellet FFPE

Materials and reagents

- Neubauer counting chamber
- **1% low-melt-Agarose in** 1x PBS boiled up and kept warm at 47-50°C
- **10% neutral buffered formalin** (FA) solution
- Round bottom 96-well plate or 2ml Eppendorf tube
- **1x 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
- **Dehydrate solution:** Xylene, 100% ethanol, 90% ethanol, and 70% ethanol, Xylene
- Paraffin

Procedure:

1. Harvest cells and centrifuge them to create a pellet.

2. Wash the cells twice in 1x PBS by centrifugate your cells for 10 min at 300 x g and removal of the supernatant.

3. Prepare a single-cell suspension in 1xPBS, count the cells, then calculate the required number. Use 1-2x 10^6 cells for one FFPE block.

4. To fix the cells, add an equal volume of **8% FA** solution to the cell suspension (e.g. 4 ml cells suspension in PBS + 4 ml 8% FA solution) and mix well (final concentration is 4%).

5. After **20 min**, centrifuge the fixed cells for **10 min** at 300g.

6. Reuspend the fixed cells in 70 - 90 µl of **1x PBS** per FFPE block, then transfer the cell suspension volume per FFPE block to a **96-well round-bottom plate** well or a **2 ml Eppendorf tube**.

7. Add an equal amount of heated **1% low-melt-agarose** to the fixed cells. Mix the two solutions, taking care not to create bubbles.

8. Cool the plate or tube with the cell/agarose mixture in the fridge for **10 min**.

9. Use a small spoon or spatula to remove the cell/agarose pellet and transfer it to a new 15 ml tube containing fresh **4% FA solution**. Incubate for **8 hours** or **overnight** at 4°C.

10. After the final fixation, transfer the cell/agarose pellets to **70% ethanol** and keep them there until paraffin embedding. The

subsequent dehydration, clearing and paraffin infiltration steps are performed using an automated tissue processing system following step 5 of the procedure for mouse and rat tissue.

FFPE sections with a microtome

1. Cool the FFPE block.
2. Cut tissue into 2.5 to 5 µm sections and place the sections onto a warm water bath.
3. Pick up the sections onto Superforst plus slides and dry overnight at 37°C.

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.