Protocol for iNOS Antibody (Cat. No. HS-484 008) Immunohistochemistry Paraffin embedded (IHC-P) Chromogenic Staining (DAB)

Tissue Fixation and Section Thickness

- 3.7% formaldehyde (24 h), 3.5 µM paraffin sections on superfrost slides

Materials and reagents

- **Food Steamer** (e.g. Braun, Multigourmet)
- Staining Containers with slide holders (e.g. Tissue-Tek)
- **Protein Block, Serum-Free** (Agilent X0909)
- **Antibody diluent** (Agilent S2022)
- **Primary antibody:** iNOS antibody (cat. no. HS-484 008)
- **Secondary antibody:** Biotinylated secondary rabbit antibody Jackson (111-065-144)
- **ABC HRP Kit, Standard** (Vectorlabs PK-4000)
- **ImmPACT DAB** (Vectorlabs SK-4105)
- **Hydrogen peroxide 30%** (Merck 1.07298.0250)
- **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
- **TBST:** Tris buffered saline (50 mM Tris, 150 mM NaCl) pH 7.6, 0.05% Tween 20
- **Antigen Retrieval buffer**
  - **Citrate Buffer** (10 mM Citrate, 0.05% Tween 20, pH 6.0)
- **Deparaffinize and re/de-hydrate solution:** Xylene, 100% ethanol, 90% ethanol, 80% ethanol and 70% ethanol, 2-propanol
- **Optional:** Hematoxylin Solution (Mayer's, Modified) or other nuclear counterstain
- **Optional:** Avidin/Biotin Blocking Kit (Vectorlabs SP-2001)
- **Non-aqueous mounting medium**
- **Coverslip**

Procedure

1. **Deparaffinize and hydrate tissue sections**
   - Xylol 2 x 5 min
   - 100% EtOH 2 x 2 min
   - 90% EtOH 1 x 2 min
   - 80% EtOH 1 x 2 min
   - 70% EtOH 2 x 2 min
   - Deionized Water 1 x 20 sec
   - PBS 1 x 2 min
     - Keep the slides in PBS until ready to perform the Antigen Retrieval.
     - Do not allow the slides to dry out.

2. Performe **Antigen Retrieval (AR)** using a **food steamer.**

a. Heat the steamer with a suitable staining container filled with Antigen Retrieval buffer to **~97°C.**
   - **Citrate Buffer** (10 mM Citrate, 0.05% Tween 20, pH 6.0)

b. Transfer the sections into the staining box, wait until the temperature reaches **97°C.**

c. Incubate the sections in the steamer for **30 min.**

d. Remove the staining container from the steamer and allow the slides to cool down for **20 min** (target end temperature **~60°C**).

3. Wash slides in PBS, 3 x 1 min.

4. Blocking endogenous peroxidase activity: Incubate the sections with **3% hydrogen peroxide** in PBS (freshly prepared!) for **5 min.**

5. Wash slides in PBS, 2 x 1 min.

6. Wash slides in TBST, 1 x 2 min.
   - *Optional: Perform Avidin-Biotin-Block according to manufacturer's instructions.*
   - *Note: Certain tissues (e.g. liver, kidney) contain high levels of endogenous biotin. The Avidin-Biotin blocking step is recommended when using the ABC system for these tissues. If the background problem persists, consider trying a polymer-based detection system instead of biotinylated secondary antibody / ABC system.*

7. Blocking non-specific binding in **Protein Block, Serum-Free** for **10 min.**

8. Drain slides (do not rinse).

9. Apply **primary antibody** diluted in **Antibody Diluent** and incubate in a humidified chamber for **1 h** at **room temperature (RT).**
   - **Suggested dilution: 1:1000** in **Antibody Diluent**

10. Wash slides in TBST, 3 x 2 min.

11. Apply **secondary antibody** diluted in **Antibody Diluent** for **30 min** at **RT.**
    - **Suggested concentration:** 5 µg/ml
    - Perform step 12 in the interim

12. Prepare the **ABC-reagent:** 5 ml PBS + 1 drop A + 1 drop B, incubate for **30 min.**

13. Wash slides in TBST, 3 x 2 min.

14. Apply the **ABC reagent** for **30 min** at **RT.**

15. Apply the **DAB substrate, 1-10 min.**
    - Observe the staining with a microscope!
    - Development times may differ depending upon the level of antigen.

16. Stop the DAB reaction with deionized water.
    - *Optional: Counterstain, follow the manufacturer's instructions for counterstaining and bluing.*

17. Wash slides in deionized water for 1 min.

18. **Dehydrate tissue sections:**
    - 70% EtOH 2 x 10 sec
    - 80% EtOH 1 x 10 sec
    - 90% EtOH 1 x 10 sec
    - 2-Propanol 2 x 1 min
    - Xylol 3 x 2 min

19. Mount slides in a suitable **organic mounting medium** and add a **coverslip.**

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.