

# Reference Protocol for Anti-iNOS (HS-484 008) Immunohistochemistry using DAB as Chromogen

## Tissue Fixation

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

## Materials and Reagents

- Food Steamer Braun, Multigourmet
- Staining Containers with slide holders (e.g. Tissue-Tek)
- Protein Block, Serum-Free Agilent X0909
- Antibody diluent Agilent S2022
- Biotinylated anti-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 (pH 7.4)
- TBST (TBS, 0.05% Tween 20, pH 7.6)
- Antigen Retrieval buffer:  
**Citrate Buffer (10 mM Citrate, 0.05% Tween 20, pH 6.0)**
- 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

## Method

### 1. Deparaffinize and hydrate tissue sections

- |                    |            |
|--------------------|------------|
| a. Xylol           | 2 x 5 min  |
| b. 100% EtOH       | 2 x 2 min  |
| c. 90% EtOH        | 1 x 2 min  |
| d. 80% EtOH        | 1 x 2 min  |
| e. 70% EtOH        | 2 x 2 min  |
| f. Deionized Water | 1 x 20 sec |
| g. 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. **Antigen Retrieval (AR) using a food steamer**
  - a. Heat the steamer with a suitable staining container filled with Antigen Retrieval buffer to ~97°C
  - 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**
  - a. 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
7. **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.*
8. Block in Protein Block, Serum-Free for **10 min**
9. **Drain slides (do not rinse)**
10. **Apply primary antibody diluted in Antibody Diluent** and incubate in a humidified chamber for **1 h at room temperature**  

**\*Suggested dilution: 1:1000 in Antibody Diluent\***
11. Wash slides in TBST, 3 x 2 min
12. **Apply secondary antibody diluted in Antibody Diluent for 30 min at room temperature.**  

**\*Suggested concentration: 5 µg/ml\***  
**\*Perform step 13 in the interim\***
13. **Prepare the ABC-reagent:** 5 ml PBS + 1 drop A + 1 drop B, incubate for 30 min
14. Wash slides in TBST, 3 x 2 min
15. **Apply the ABC reagent for 30 min at room temperature**
16. Wash slides in TBST, 3 x 2 min
17. **Apply the DAB substrate, 1-10 min**  

**\*Observe the staining with a microscope!**  
**Development times may differ depending upon the level of antigen\***
18. Stop the DAB reaction with deionized water
19. **Optional: Counterstain**
  - a. Follow the manufacturer's instructions for counterstaining and bluing
20. Wash slides in deionized water for 1 min
21. **Dehydrate tissue sections:**
  - a. **70% EtOH** 2 x 10 sec
  - b. **80% EtOH** 1 x 10 sec
  - c. **90% EtOH** 1 x 10 sec
  - d. **2-Propanol** 2 x 1 min
  - e. **Xylo** 3 x 2 min
22. **Mount slides in a suitable organic mounting medium and add coverslip**

**Note:** The SYSY standard protocol generates good results in the SYSY labs and may be used as a reference. However, to achieve the highest

*specific signal and lowest non-specific background signal, the best antigen retrieval condition, antibody concentration, incubation temperature, and incubation time must be determined individually. Please also refer to our general protocols.*