IHC-P: Staining Protocol - Fluorescent Detection

In fluorescent immunostainings, fluorophore-conjugated secondary antibodies are used to localize antigen-antibody complexes. Fluorescent detection allows easier multiplexing, especially for co-localized targets, and has a higher dynamic range when high and low abundant targets have to be visualized on one slide. However, many FFPE tissues, especially spleen and kidney, show high tissue autofluorescence making the interpretation of assay results particularly difficult in green- and red-channel fluorophores. Autofluorescence Quenching Kits can reduce tissue autofluorescence and improve signal-to-noise ratio.

Important: Some proteins have special requirements for good detection. Please refer to the **remarks** sections for IHC on the respective data sheet.

Tissue preparation

For the preparation of paraffin embedded tissues for immunohistochemistry, please refer to our **tissue preparation protocols.**

Materials and reagents

- Water bath (alternatively: microwave oven, pressure cooker, vegetable steamer)*
- Staining containers with slide holders
- **Blocking buffer:** 10% normal serum, 0.3% Triton X-100 in Tris buffered saline (TBS) (normal serum should be from the host-species the secondary reagents originate from)
- **Antibody incubation buffer:** 5% normal serum, 0.3% Triton X-100 in TBS (normal serum should be from the host-species the secondary reagents originate from)
- Fluorochromated secondary antibody
- **Washing buffer:** 50 mM Tris, pH 7.3, 150 mM NaCl
- **Antigen retrieval buffer:** 10 mM citrate, 0.05% Tween 20, pH 6.0 or 10 mM Tris pH 9.0, 1 mM EDTA, 0.05% Tween 20. Please check IHC-P remarks on the respective data sheet.
- Xylene, 100% ethanol, 90% ethanol, 80% ethanol and 70% ethanol
- **Optional:** DAPI or other nuclear counterstain
- **Mounting medium**

Deparaffinization and rehydration

Deparaffinize and hydrate tissue sections

1. Xylene 2x 5 min
2. 100% EtOH 2x 2 min
3. 90% EtOH 1x 2 min
4. 80% EtOH 1x 2 min
5. 70% EtOH 2x 2 min
6. Deionized water 1x 20 sec
7. TBS

Keep the slides in TBS until ready to perform the antigen retrieval. Do not allow the slides to dry out.

Antigen retrieval (using a water bath)*

1. Heat the water bath with a suitable staining container filled with **antigen retrieval buffer** to **95°C.**
2. Transfer the sections into the staining box, wait until the temperature reaches 95°C.
3. Incubate the sections in the water bath for **40 min.**
4. Remove the staining container and allow the slides to cool down to room temperature.
5. **Optional:** Some antibodies require an additional **antigen retrieval** step with formic acid. Please check IHC-P remarks on the respective data- or factsheet. If step is performed wash slides 3 times with TBS before formic acid treatment. Incubate slides for **3 min** in **88% formic acid**.

Blocking

1. Wash slides in TBS, 3x 1 min.
2. Block in **Blocking Buffer** for 30 min at RT.

Antibody incubation

1. Drain slides (do not rinse).
2. Apply primary antibody diluted in **antibody incubation buffer** and incubate in a humidified chamber for overnight **at 4°C.**
3. Wash slides in TBS, 3x 2 min.
4. Apply secondary antibody diluted in **antibody incubation buffer** for 1 h at room temperature. *Avoid bright light when working with the secondary antibody to minimize photo bleaching of the fluorescent dye.* **Important Note:** This step can be omitted when fluorophore conjugated primary antibodies are used. In **multiplex** staining, make sure to use secondary antibodies cross-adsorbed against the host species of the other primary antibody used in your experiment. Ideally, all secondary antibodies should come from the same host species. If not, make sure that they have been cross-adsorbed against IgGs of the host-species of the other secondary antibody as well. This avoids cross-reaction between the secondary antibodies.
5. Wash slides in TBS, 3x 2 min.

Counterstain (optional)

1. Follow the manufacturer's instructions for counterstaining.
2. Wash slides in deionized water for 1 min.

Mounting

1. Mount slides in a suitable mounting medium and add coverslip.

*****For an alternative Antigen Retrieval protocol using a vegetable steamer check protocol-ihc-paraffin-chromogenic.*

Note: The SYSY standard protocol generates good staining results in the SYSY labs and may be used as suggestion. 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.