

# Reference Protocol for Anti-Ki67 (HS-398 003) Immunohistochemistry using DAB as Chromogen

## Tissue Fixation

- 3.7% formaldehyde (24 h), 3.5  $\mu$ M 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: 1:100 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) Xylol	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.*