SYSY | HistoSure

Reference Protocol for Anti-Ki67 (HS-398 003) 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: 1:100 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.