Immunohistochemistry Protocol for Paraffin-embedded Tissues

Solutions and reagents

1. Xylene
2. Ethanol, anhydrous denatured, histological grade (100%, 95%, 85%, 75%)
3. Washing buffer:

1XPBST,	**1 L**
10X PBS	100 mL
dH_2O	900 mL
Tween-20	1 mL

10X PBS,	**1 L**
$NaH_2PO_4 \cdot 2H_2O$	2.84 g
$Na_2HPO_4 \cdot 12H_2O$	27.2 g
NaCl	90 g
dH_2O	1000 mL

The pH should be about 7.2. Adjust if necessary with 1 M NaOH or 1 M HCl

4. Antigen Retrieval Solution:

① 10mM Sodium Citrate Buffer, pH 6.0

$C_6H_8O_7 \cdot H_2O$	0.21g
$C_6H_8Na_3O_7 \cdot 2H_2O$	2.9g
dH_2O	1000 mL

Adjust pH to 6.0

②10mM Tris Buffer with 1mM EDTA,pH 8.0 or 8.5 or 9.0

Tris	1.21 g
EDTA	0.37 g
dH_2O	1000 mL

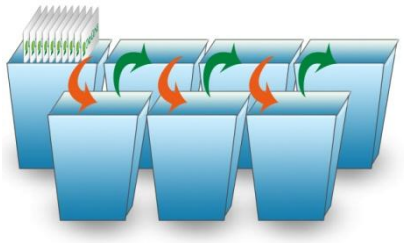
Adjust pH* to 8.0 or 8.5 or 9.0

**Note: Please refer to the antibody for individual antigen retrieval buffer and working conditions.*

5. 3% Hydrogen Peroxide
6. Hematoxylin QS
7. Permanent Mounting Medium

Protocol

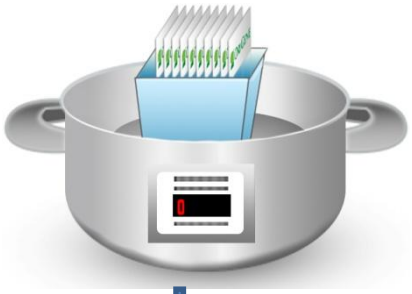
A. Deparaffinization and Rehydration



Heat slides in an oven at 60 °C for 5 min.
Wash slides 3 times for 10 min each in xylene.
Wash slides 3 times for 3 min each in 100% ethanol.
Wash slides in 95% ethanol, 1 min.
Wash slides in 85% ethanol, 1 min.
Wash slides in 75% ethanol, 1 min.

Rinse slides for 5 min in distilled water.

B. Antigen Retrieval*



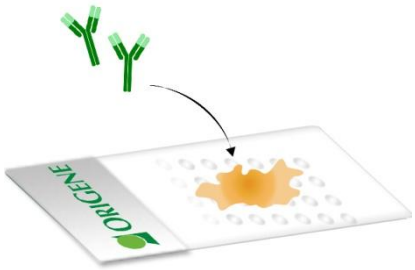
Put the slides in Antigen Retrieval Solution and keep 120℃ for 2.5 min in pressure cooker.
Then cool down at room temperature.
**Note: Please refer to the antibody for individual antigen retrieval buffer and working conditions.*

Wash slides three times with distilled water (2 min each).

C. Staining

Inactivate endogenous peroxidase by covering tissue with 3% hydrogen peroxide for 15 min.

Wash slides twice with 1X PBST (2 min each).



Dilute primary antibody in the IHC Antibody Diluent per recommendation on the data sheet.
Apply primary antibody to each section and incubate 90 min at room temperature*. Make sure the primary antibody solution covers the tissue evenly.
**Note: Please refer to the antibody for individual buffer and working conditions.*

Wash slides three times with 1X PBST (2 min each).

Apply to each section secondary antibody and incubate for 15 min at room temperature*.
**Note: Please refer to the antibody for individual buffer and working conditions.*

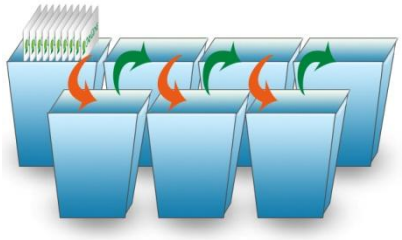
Wash slides three times with 1X PBST (2 min each).

Add freshly prepared DAB substrate to the sections.
Incubate tissue sections with the substrate at room temperature until suitable staining develops (generally about 5 min).

Rinse sections with water.

Counterstain with Hematoxylin QS for 3 min.

Rinse sections with water.

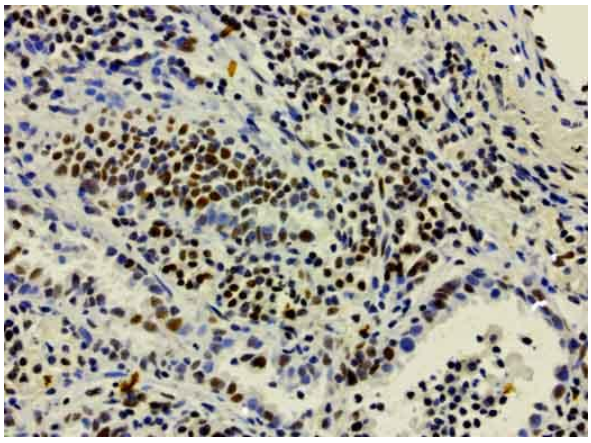


Wash slides in 75% ethanol, 1 min.
Wash slides in 85% ethanol, 1 min.
Wash slides in 95% ethanol, 1 min.
Wash slides in 2 changes of 100% ethanol rinses, 1 min each.
Wash slides in 3 changes of xylene, 1 min each.

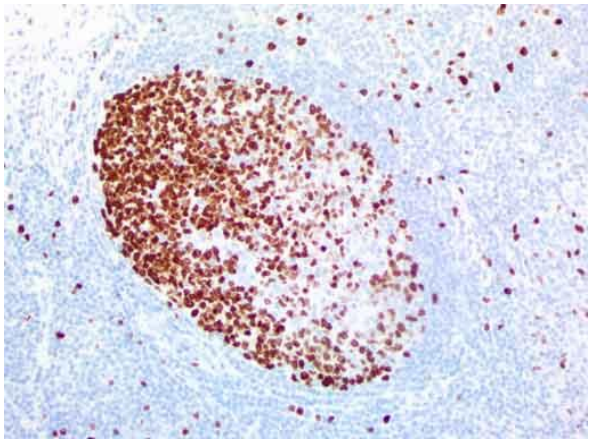
Mount coverslips on slides using Permanent Mounting Medium.

Allow slides to dry overnight at room temperature and then analyze the results with microscope.

Example



IHC staining (FFPE) of OriGene's ERCC1 UltraMAB (clone 4F9) of adenocarcinoma of lung (bronchioloalveolar carcinoma). (UM500008).



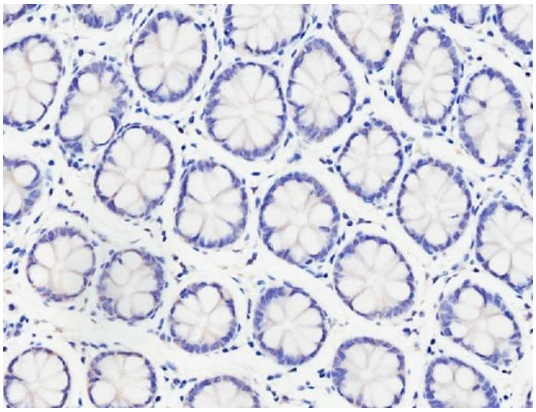
IHC staining of OriGene's MKI67 UltraMAB (clone UMAB107) of normal human tonsil tissue. (UM800033).

Useful links:

View our list of over 140,000 human tissues
View our list of the Ultra Specific Antibodies for Anatomic Pathology
View our list of detection kits developed by GBI Labs, Inc

Troubleshooting

No staining



1. Forgot to add primary antibody or secondary antibody to the antibody diluent
>Repeat protocol. Pay attention to application of antibody to diluent and tissue.
2. Antibody concentration too dilute
>Apply more antibodies, and/or incubate longer (e.g. incubate overnight at 4°C). Re-titer antibody.
3. The primary antibody and the secondary antibody are not compatible
>Use a secondary antibody that was raised against the species in which the primary was raised (e.g. primary antibody is raised in mouse, use anti-mouse secondary antibody).
4. Antibody dissociates from antigen during protocol.
> Use a lower concentration of detergent in washing buffers. Replace with higher affinity antibody or Re-optimize wash steps.
5. The antibody may not be suitable for IHC procedures
> Check the antibody datasheet from the supplier to see if it has been validated for IHC application, and what type of IHC (formalin fixation paraffin embedded, fresh frozen, etc.).
6. The amplification kit may have lost its activity due to improper storage, improper dilution or extensive freezing/thawing
> Run positive controls to ensure that the primary/secondary antibody is working properly.
7. The protein is not present in the tissue of interest
> Check the literature to find the positive tissue or run a positive control recommended by the supplier of the antibody.
8. The protein of interest is not abundantly present in the tissue
>Use an amplification step to maximize the signal. For example, use a biotin conjugated secondary antibody and a conjugated streptavidin to amplify the signal, or use detection kit with high sensitivity, such as GBI Labs Polink-2 HRP polymer detection kit.
9. Improper protocol used for HIER
>Follow standard protocol listed above or refer to recommend protocol in antibody insert.

10. Incorrect preparation of substrate-chromogen mixture

>Repeat substrate-chromogen treatment with correctly prepared reagent.

>Staining intensity is decreased when excess DAB/DAB+ is present in the working reagent.

11. Deparaffinization may be insufficient

>Deparaffinize sections longer and use fresh xylene.

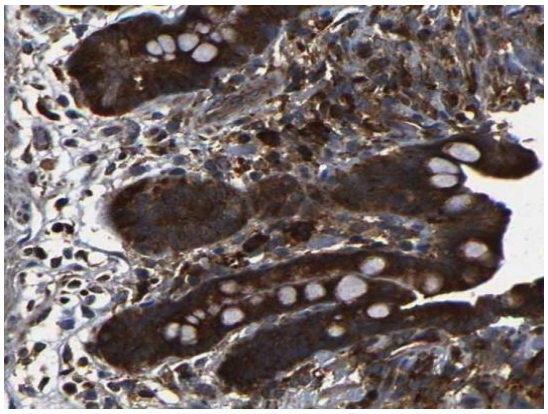
12. The protein is located in the nucleus and the antibody (nuclear protein) cannot penetrate the nucleus

> Add a strong permeabilizing agent like Triton X to the antibody dilution buffer. Recommend using HIER buffer with detergent. GBI Labs B22C-xx Accel pH 8.7 or B21-xxTEE pH 9.0

13. The PBS buffer is contaminated with bacteria that damage the phosphate groups on the protein of interest

>Add 0.01% sodium azide in the PBS antibody storage buffer or use fresh prepared PBS.

High background



1. The primary antibody concentration too high

>Titrate the antibody to the optimal concentration, or dilute the antibody further and incubate at 4°C.

2. The secondary antibody cross-reacts with the screening tissue

> Do not use the secondary antibody raised from the same species as tissue screened. Run a control with secondary antibody without primary antibody. If you see staining with your secondary only, change to a secondary antibody that has been pre-adsorbed against the immunoglobulin of the species of your samples.

3. Blocking might be absent or insufficient

>Block with serum from the host of the secondary antibody. Increase the blocking incubation time and may consider changing to another blocking agent.

4. Tissue not washed enough

>increase the detergent in the washing buffer, or increase the washing time for each wash step

5. Tissue not fixed properly

>Adjust the fixation protocol.

6. Amplification of detection too strong

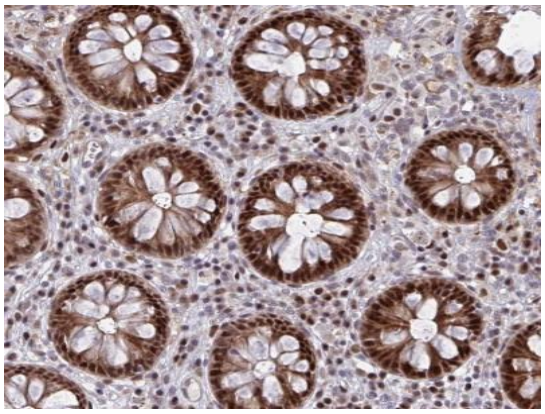
>Reduce amplification incubation time and dilute the secondary antibody.

7. Too much substrate was applied

>Substrate needs to be diluted further, or reduce the substrate incubation time.

8. Permeabilization has damaged the membrane and removed the membrane protein (membrane protein)
>Use a less stringent detergent (e.g.) Tween 20 instead of Triton X). Or simply remove permeabilizing agent from your buffers.

Non-specific staining



1. Primary or secondary antibody concentration too high
>Try decreasing the antibody concentration and/or the incubation period.
2. Forgot/Insufficient to block endogenous peroxidase or phosphatase
> Protocol should always have the step to block endogenous peroxidases and/or phosphatase with 3% H_2O_2 for peroxidase or 2mM Levanisol for phosphatase.
3. Antibody and tissue from same host species
>Use a primary antibody raised against a different species from your tissue. Use kits such as GBI Labs Klear Mouse D52-xx for mouse antibodies on mouse tissues.
4. Serum block step insufficient
>Change blocking formula. Goat, horse or donkey serum is interchangeable and will block different background.
5. The sections have partially dried out
>Keep sections at high humidity container and do not let them dry out throughout the procedure.
6. Burn/Hot spots appear on tissue
>Centrifuge the antibodies at12000g for 5 minutes to get rid of the pellet. Keep and use the supernatant.