# 6-Color Manual IHC Kit (pH9.0)

**[Product No.]**

OM642682

**[Principle of action]** The 6 color IHC detection kit, get rid of the traditional immunofluorescence experimental conditions of antibody species sources of restrictions and constraints, without ventilation kitchen and traditional dewaxing repair tank, the traditional three times xylene dewaxing, three to five times gradient ethanol hydration and antigen repair integration into a solution, DAIP and sealing integration into a solution, effectively shorten the Three fluorescent dyes immunohistochemical experiments used in the reagent one-stop ready, with high sensitivity of HRP polymer secondary antibody (Anti-Rabbit/Mouse) and fluorescence color solution supporting the use, can be a color seven antibodies (without considering the source of antibody species), making the kit has the characteristics of convenient operation, rapid, high sensitivity.

**[Packaging Specifications]** 1mL 2mL 5mL

**[Storage conditions and validity]**

1, storage requirements: see the table.

2, validity: one year

**[Self-provided materials]**

1, suitable heating device, dye vat, pipette, cover glass and other commonly used consumables

2, anhydrous alcohol

3, neutral gum

4, PBST, pure water and other commonly used reagents

**[Components in kit]**

| Reagent | Name | Size | | | Storage |
|---|---|---|---|---|---|
| | | **1mL** | **2mL** | **5mL** | |
| Reagent 9A | Dewaxing and Repair 20X | 10 mL | 10 mL | 200mL | 2~8°C |
| Reagent B | Blocking solution | 1mL | 2mL | 5mL | 2~8°C |
| Reagent C | Polymer HRP Mouose/Rabbit | 6mL | 6mL*2 | 30mL | 2~8°C |
| Reagent D-488 | 488 labeled Tyramide | 1mL | 2mL | 5mL | 2~8°C |
| Reagent D-525 | 525 labeled Tyramide | 1mL | 2mL | 5mL | 2~8°C |
| Reagent D-594 | 594 labeled Tyramide | 1mL | 2mL | 5mL | 2~8°C |
| Reagent D-680 | 680 labeled Tyramide | 1mL | 2mL | 5mL | 2~8°C |

| | | | | | |
|---|---|---|---|---|---|
| Reagent D-750 | 750 labeled Tyramide | 1mL | 2mL | 5mL | 2~8°C |
| Reagent G | DAPI/mounting solution | 1mL | 2mL | 5mL | -20°C |
| Reagent F | Stripping solution 20X | 8mL | 8mL*2 | 40mL | 2~8°C |

**[Intended Use]**

Immunohistochemical fluorescence staining, with Omnimabs conventional rabbit/mouse antibodies or ready-to-use antibody.

**[Sample requirements]**

Paraffin or frozen sections, cell slides. Recommended with Omnimabs conventional concentrated or ready-to-use primary antibody.

Paraffin sections: primary antibody application indicated IHC-p

Frozen sections: primary antibody application indicated IHC-f

Cell slides: primary antibody application indicated IF or ICC.

Some frozen or tissue sections are easy to fall off after repeated heating, the tissue can be puted into the 24/12/6 well plate.

**[Read before use]**

1, Use deionized water to dilute reagent 9A, volume ratio 1:19.

2, If you need to send or vertical preservation slices, please drop nail polish or neutral gum around the cover glass to dry.

3, Paraffin slices need to be put into the oven at 65 °C for one hour before use. Freezing slices, cell climbing slices have no this step.

4, TSA staining is more sensitive than conventional fluorescence staining and TMB staining. If the fluorescence intensity is too high, you can try to double dilute of primary antibody, or double dilute of Tyramide reagent.

5, The expression abundance of indicators can be determined by conventional staining or pre-experiment. Usually, the highest expression index is stained first, and the lowest expression index is stained last.

6, 525, 555, 594 markers are different degrees red under the microscope. Please use special imaging instruments and analytical software to distinguish the spectra.

7, The dewaxing antigen repair solution in this kit is suitable for most primary antibodies. If the staining effect is not good, you can use the general public formula to dewax the slices and repair antigen.

8, Because of the small adhesion ability of frozen slices and cell climbing slices, the pre-experiment of **step 9** can be carried out separately before use this kit to ensure that the phenomenon of dropping slices does not occur.

**[Recommended fluorescence channel for imaging]**

| Reagent | Nmae | Exi | Emi | Filter | Color |
|---|---|---|---|---|---|
| Reagent D-350 | 350 labeled Tyramide | 350 | 450 | DAPI | |
| Reagent D-435 | 435 labeled Tyramide | 435 | 470 | Aqua,Opal470 | |
| Reagent D-488 | 488 labeled Tyramide | 496 | 519 | FITC，Opal520 | |

| | | | | | |
|---|---|---|---|---|---|
| Reagent D-525 | 525 labeled Tyramide | 530 | 560 | Cy3,SpGold,Opal540 | |
| Reagent D-555 | 555 labeled Tyramide | 555 | 567 | TRITC, Cy3,SpOr,Opal570 | |
| Reagent D-594 | 594 labeled Tyramide | 590 | 617 | SpRed,TxRed,Opal620 | |
| Reagent D-647 | 647 labeled Tyramide | 650 | 665 | Cy5,Opal650 | |
| Reagent D-680 | 680 labeled Tyramide | 680 | 700 | Cy5.5,Opal690 | |
| Reagent D-750 | 750 labeled Tyramide | 750 | 796 | Cy7,Opal780 | |
| Reagent G | DAPI/mounting solution | 345 | 455 | DAPI | |

* Due to the overlap of excitation and emission spectra, Opal540/570 can be observed mixed under fluorescence microscope objective.Cy5/Cy5.5 can be observed mixed

** Using the full-spectrum imaging system can split separate spectra to achieve more than 6 colors imaging

**[Procedure for paraffin sections]**

1. Put reagent 9A (dewaxing repair 2-in-1) working solution into the repair box and heat it to boiling.
2. Put the section into the boiling reagent 9A (dewaxing repair 2-in-1) working solution (to ensure the pH value of the repair solution, metal section holder cannot be used), and the liquid completely immerses the tissue on the section. Heat it continuously for 30 min.

**Note:** Antigen repair is depended on a variety of factors such as the primary antibody supplier, tissue type, etc., and the commonly used antigen repair reagents or other validated repair conditions in the laboratory can be used.This step can be omitted for tissues with frozen slides or cell-crawling slides which are easy to fall off.

3. Remove the repair box from the heating source and cool it to room temperature.
4. Take out the section into a beaker filled with distilled water, and then soak it with distilled water 5-6 times.
5. Drain the slide for a few seconds, wipe the water around the tissue with filter paper, drop reagent B (peroxidase blocking buffer) 50-100ul (it can cover the tissue), incubate at room temperature for 15 min, and wash it with PBST for 2 min × 3 times.
6. Drain the slide for a few seconds, wipe off the moisture around the tissue with a filter paper, circle the tissue with an immunohistochemical pen (end to end should be closed, and cannot be drawn on the tissue), dilute the primary antibody, and drop the corresponding primary antibody onto the tissue until completely covered. Allow to incubate at room temperature or 37 °C for 1 to 2 hours, or incubate at 37 °C for 30 minutes after overnight at 4 °C in a wet box, and rinse with PBST for 2 minutes × 3 times.
7. Drain the slide for a few seconds, wipe off the moisture around the tissue with a filter paper, drop reagent C (HRP polyclonal anti-rabbit/mouse secondary antibody) working solution 50-100ul (to cover the tissue), incubate at room temperature for 30 minutes, and rinse with PBST for 2 minutes × 3 times.
8. Add fluorescent reagent D-594 working solution 50-100ul (to cover the tissue), after 10 minutes, rinse with PBST for 2 minutes × 3 times.
9. Add Reagent F (antibody stripping buffer, preheated required): Add an appropriate amount of antibody stripping buffer preheated to 37℃ to cover the tissue, incubate at 37℃ for 5-20 minutes. After spin-drying, no washing is needed; add

an appropriate amount of antibody stripping buffer again to cover the tissue and incubate at 37℃ for another 5-20 minutes, then discard the stripping buffer (a total incubation time of 20 minutes for the two stripping steps is usually sufficient). Wash the tissue three times with PBST for 5 minutes each time.

10. Repeat steps 6-9, and change the fluorescent dye to reagent D-488, and incubate the second primary antibody.
11. Repeat steps 6-9, and change the fluorescent dye to reagent D-680, and incubate the 3th primary antibody.
12. Repeat steps 6-9, and change the fluorescent dye to reagent D-525, and incubate the 4th primary antibody.
13. Repeat steps 6-8, and change the fluorescent dye to reagent D-750, and incubate the 5th primary antibody.
14. Drop a drop of reagent G (about 30-50 ul) on the tissue section, cover the cover glass, let the section contact the sealing solution, try to avoid bubbles, scan or take a picture.

**Note:** The above fluorescence sequence is applicable to situations where the expression levels of various indicators are not significantly different. The fluorescence dye sequence can be adjusted based on the expression of corresponding indicators and the results of preliminary experiments. For example, high-expression indicators can be labeled preferentially, followed by low-expression indicators. Some tissues may be affected by multiple rounds of heating, which may affect the binding of primary antibodies to proteins. It is recommended to adjust the staining sequence through multiple rounds of heating with a single color.

**[Procedure for cell slices/frozen sections]**

1. **Optional step**: fixation: after restoring the section to room temperature, use 4% paraformaldehyde (not provided in the kit) for 10-15min, wash with 1xPBS three times, 5 minutes each time. This step is usually added in the cell crawling slices. The frozen slices are not fixed before OCT embedding. It is recommended to add this step.
2. **Optional step**: antigen repair: reagent 9A (dewaxing repair two-in-one) working solution is put into the repair box, heated to boiling. The slides were placed in boiling reagent 9A (dewaxing repair 2-in-1) working solution (to ensure the pH value of the repair solution, metal slide holders were not used), and the liquid completely immersed the tissue on the slides. The repair box was removed from the heating source and cooled to room temperature.

**Note:** antigen repair is depended on a variety of factors such as the primary antibody supplier, tissue type, etc., and the commonly used antigen repair reagents or other validated repair conditions in the laboratory can be used.This step can be omitted for tissues with frozen slides that are easy to fall off, or for cell-crawling slides or water bath repair (see Step 9).

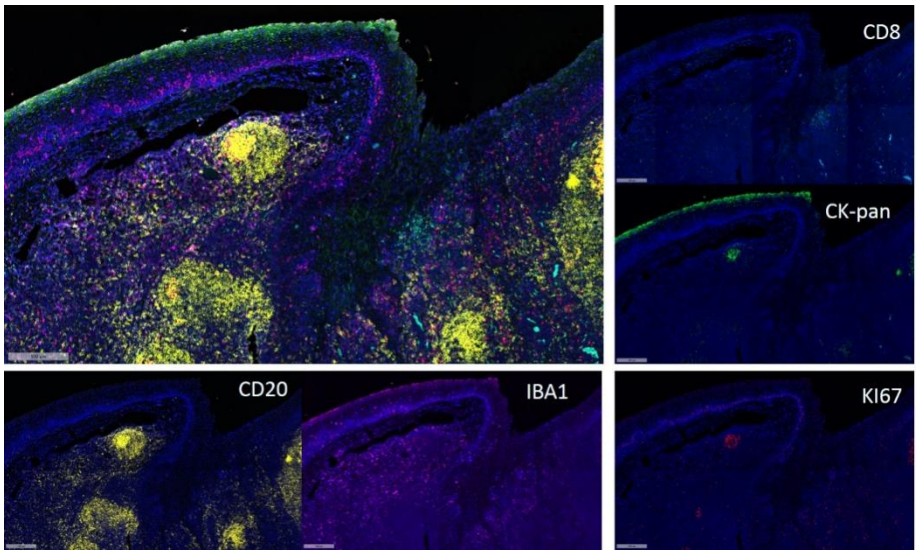
3. **Optional steps**: permeate with 0.5% Triton X-100 (not provided in the kit) at room temperature for 20 min (this step is omitted for antigens expressed on the cell membrane).
4. Wash with 1xPBS three times, 2 minutes each time.
5. Drain the slide for a few seconds, wipe off the moisture around the tissue with filter paper, drop reagent B (peroxidase blocking buffer) 50-100ul (enough to cover the tissue), incubate at room temperature for 15 min, and wash with PBST for 2 min × 3 times.
6. Drain the slide for a few seconds, wipe off the moisture around the tissue with filter paper, circle the tissue with an immunohistochemical pen (the ends should be closed, and cannot be drawn onto the tissue), dilute the primary antibody, and drop the corresponding primary antibody onto the tissue until completely covered. After incubation at room temperature or 37 °C for 1 to 2 hours, or after incubation at 4°C overnight and rewarming at 37 °C for 30 minutes, PBST washed 2 min × 3 times.
7. Drain the slide for several seconds, wipe off the moisture around the tissue with filter paper, drop reagent C (HRP polyclonal anti-rabbit/mouse secondary antibody) working solution 50-100ul (can cover the tissue), incubate at room temperature for 30 minutes, PBST washed 2 min × 3 times.
8. Add fluorescent reagent D-594 working solution 50-100ul (can cover the tissue), 10 minutes later, PBST washed 2 min × 3 times.

9. Add Reagent F (antibody stripping buffer, preheated required): Add an appropriate amount of antibody stripping buffer preheated to 37℃ to cover the tissue, incubate at 37℃ for 5-20 minutes. After spin-drying, no washing is needed; add an appropriate amount of antibody stripping buffer again to cover the tissue and incubate at 37℃ for another 5-20 minutes, then discard the stripping buffer (a total incubation time of 20 minutes for the two stripping steps is usually sufficient). Wash the tissue three times with PBST for 5 minutes each time.
10. Repeat steps 6-9, and change the fluorescent dye to reagent D-488, and incubate the second primary antibody.
11. Repeat steps 6-9, and change the fluorescent dye to reagent D-680, and incubate the 3th primary antibody.
12. Repeat steps 6-9, and change the fluorescent dye to reagent D-525, and incubate the 4th primary antibody.
13. Repeat steps 6-8, and change the fluorescent dye to reagent D-750, and incubate the 5th primary antibody.
14. Drop a drop of reagent G (about 30-50 ul) on the tissue section, cover the cover glass, let the section contact the sealing solution, try to avoid bubbles, scan or take a picture.
**Note:** The above fluorescence sequence is applicable to situations where the expression levels of various indicators are not significantly different. The fluorescence dye sequence can be adjusted based on the expression of corresponding indicators and the results of preliminary experiments. For example, high-expression indicators can be labeled preferentially, followed by low-expression indicators. Some tissues may be affected by multiple rounds of heating, which may affect the binding of primary antibodies to proteins. It is recommended to adjust the staining sequence through multiple rounds of heating with a single color.

**[Product Performance Indicators]**
1. Compliance: The positive control result is positive, and the location of positive staining should be accurate, without background staining; the blank control and negative control staining results are negative.
2、Intra-batch repeatability: There is no significant difference in the intensity and location of staining in tissue sections from the same tissue source.
3. Inter-batch repeatability: There is no significant difference in the intensity and localization of staining between different batches of reagents on tissue sections of the same tissue origin.

**[Precautions]**
1. This product is only for scientific research and is not for other purposes.
2. Requires professional use.
3. Apply appropriate protective measures to avoid contact between reagents and the skin and eyes.
4. Waste liquid treatment: Conduct harmless treatment and comply with relevant environmental protection requirements.
5. The operation process needs to keep the wet tissue of the slide, such as dry pieces, will lead to non-specific staining results.
6. In the process of continuous heating repair, using small fire to maintain boiling, do not heat the dewaxing repair reagent splash out of the beaker.

**[Frequently Asked Questions]**
1, deep staining: a high concentration of anti-high, too long time.
2, light or no staining: a low concentration of anti-low, too short time.
3, no specific staining: slice dewaxing is not complete. Can be appropriately prolonged baking time.




Multiplex Fluorescence immunohistochemical analysis of Human tonsil tissue (formalin-fixed paraffin-embedded section). CK-pan mouse mAb(green), Ki-67 rabbit mAb(red), Iba 1 mouse mAb(purple),CD8 a mouse mAb(cyan), CD20 mouse mAb(yellow), The section was incubated in 5 rounds of staining; sequentially for Anti-antibodies; each using a separate fluorescent tyramide signal amplification system. EDTA based antigen retrieval (pH 9.0, 20 minutes) was used in between rounds of tyramide signal amplification to remove the antibody from the previous round, to avoid any cross-reactivity. DAPI (dark blue) was used as a nuclear counter stain. Microscopy and pseudocoloring of individual dyes was performed using a Slideviewer Imaging System (Excilone).

**[Related product]**

| Catalog | Product Name | Size |
|---|---|---|
| OM642680 | 4-Color Manual IHC Kit | 1mL 2mL 5mL |
| OM642679 | 3-Color Manual IHC Kit | 1mL 2mL 5mL |
| OM642681 | 5-Color Manual IHC Kit | 1mL 2mL 5mL |
| OM642682 | 6-Color Manual IHC Kit | 1mL 2mL 5mL |
| OM642683 | 7-Color Manual IHC Kit | 1mL 2mL 5mL |

**[Individual components]**

| Catalog | Reagent | Size |
|---|---|---|
| OM642685 | Polymer HRP Goat Anti Mouse/Rabbit IgG(H+L) | 3mL 10mL 100mL |
| OM642693 | 350 labeled Tyramide | 2mL 5mL 10mL |
| OM642694 | 435 labeled Tyramide | 2mL 5mL 10mL |
| OM642695 | 488 labeled Tyramide | 2mL 5mL 10mL |
| OM642696 | 525 labeled Tyramide | 2mL 5mL 10mL |
| OM642697 | 555 labeled Tyramide | 2mL 5mL 10mL |
| OM642698 | 594 labeled Tyramide | 2mL 5mL 10mL |
| OM642699 | 647 labeled Tyramide | 2mL 5mL 10mL |

| | | |
|---|---|---|
| OM642700 | 680 labeled Tyramide | 2mL 5mL 10mL |
| OM642701 | 750 labeled Tyramide | 2mL 5mL 10mL |
| OM642702 | Antigen Repair 20X pH9.0 | 250mL |
| OM642703 | Antigen Repair 20X pH6.0 | 250mL |
| OM642704 | DAPI/mounting solution | 5mL |
| OM642705 | Stripping solution 20X | 200mL |