4-Color Multiple Fluorescent Staining Kit (mIHC)

1. Product Introduction

This 4-Color Multiple Fluorescent Staining Kit (mIHC) is suitable for multiplex immunofluorescence staining of tissue samples, including paraffin sections, frozen sections, cell slides, cell smears, and organoids. It is particularly suitable for multiplex fluorescent immunolabeling using primary antibodies from the same source, but can also be used with antibodies from different sources. The primary principle of TSA is based on tyramide signal amplification (TSA), hereinafter referred to as TSA technology. TSA utilizes the peroxidase reaction of tyramide (wherein a fluorescently labeled tyramide forms a covalent binding site under the catalysis of HRP and H_2O_2). This generates a large number of enzymatic reactions, which then bind to surrounding protein residues (including tryptophan, histidine, and tyrosine residues), forming a large amount of fluorescein deposited at the antigen-antibody binding site, thereby amplifying the signal. This kit uses multiple fluorescent dyes to label tyramide, resulting in strong and stable fluorescence, making it suitable for repeated immunolabeling and multiplex fluorescent staining.

2. Product Information

Product form	liquid
Product Specifications	20T , 50T , 100T
Storage conditions	2-8 ℃
Conditions of Transport	Low temperature
Validity period	12 months

Components	Sizes			Dilutions
	20T	50T	1 00T	
TSA- CY3	10 μL	25 μL	50 μL	1:200
TSA-FITC	10 μL	25 μL	50 μL	1:200
TSA-CY5	10 μL	25 μL	50 μL	1:200
TSA diluent	6mL	15mL	30mL	Ready-to-use
3% H_2O_2	2mL	5mL	10mL	Ready-to-use

Blocking solution	6mL	15mL	30mL	Ready-to-use
Primary antibody diluent	6mL	15 mL	30mL	Ready-to-use
Antibody elution reagent	4mL	10mL	20mL	Ready-to-use
HRP- goat anti-rabbit/mouse IgG	6mL	15mL	30mL	Ready-to-use
DAPI staining	2mL	5mL	10mL	Ready-to-use
Anti-fluorescence fading mounting medium	2mL	5mL	10mL	Ready-to-use

3. Experimental Procedure

A. Paraffin sections

1. Dewaxing: Soak the sections in xylene 1 (15 min), xylene 2 (15 min), anhydrous ethanol 1 (5 min), anhydrous ethanol 2 (5 min), 95% ethanol (5 min), 85% ethanol (5 min), and 75% ethanol (5 min) in sequence, and finally rinse the sections with water.
2. Antigen retrieval: Antigen retrieval typically uses 1× citric acid (pH 6.0) as the retrieval solution. High-temperature, high-pressure retrieval is performed. Place the sections in a pressure cooker, add an appropriate amount of retrieval solution, close the pressure cooker, and wait for 2 minutes after the steam has risen. Cool to room temperature, and retrieval is complete. [For weakly expressed markers, EDTA (pH 9.0) can be used as the retrieval solution. For easily detached tissues such as bone and brain, microwave retrieval is recommended. The retrieval temperature is controlled at approximately 80°C, and 2× citric acid (pH 6.0) can be used as the retrieval solution.]
3. Endogenous enzyme blocking: Prepare a 3% H_2O_2 solution with pure water, place the sections in the solution, and incubate at room temperature for 20 minutes. If the tissue is prone to falling off, the H_2O_2 concentration and incubation time can be appropriately reduced.
4. Blocking: Add blocking solution to the tissue and incubate at 37°C for 30 minutes.
5. Primary antibody incubation: Dilute the primary antibody to an appropriate concentration using antibody diluent, add the antibody to the tissue, and incubate at 4°C overnight or at 37°C for 1 hour.

6. Secondary antibody incubation: Add HRP enzyme-labeled secondary antibody to the tissue and incubate at 37°C for 1 hour.
7. Incubate with TSA reagent: add TSA-CY3 to the tissue, incubate at 37° C for 30 minutes, and then wash three times with PBST, each time for 5 minutes.
8. Antibody elution: Add antibody elution solution to the tissue and incubate at room temperature for 15 minutes (37C is better).
9. Blocking: Add blocking solution to the tissue and incubate at 37°C for 30 minutes.
10. Primary antibody incubation: Dilute the primary antibody to an appropriate concentration in antibody diluent and incubate at 4°C overnight or at 37°C for 1 h.
11. Secondary antibody incubation: Add HRP enzyme-labeled secondary antibody to the tissue and incubate at 37°C for 1 hour.
12. Incubate TSA reagent: add TSA-FITC to the tissue, incubate at 37° C for 30 min, and then wash with PBS three times.5 minutes per session.
13. Antibody elution: Add antibody elution solution to the tissue and incubate at room temperature for 15 minutes (37C is better).
14. Blocking: Add blocking solution to the tissue and incubate at 37°C for 30 minutes.
15. Primary antibody incubation: Dilute the primary antibody to an appropriate concentration in antibody diluent and incubate at 4°C overnight or at 37°C for 1 h.
16. Secondary antibody incubation: add HRP enzyme secondary antibody to the tissue and incubate at 37°C for 1h.
17. Incubate with TSA reagent: add TSA-CY5 to the tissue, incubate at 37° C for 30 minutes, and then wash with PBS three times, 5 minutes each time.
18. DAPI nuclear staining: Add DAPI working solution to the tissue, incubate at room temperature for 5 minutes, and then wash three times with PBS for 5 minutes each. Drain the solution and add anti-fading mounting medium to the tissue, then cover with a coverslip. Store the prepared fluorescent sections at 4° C in the dark.

B. Frozen sections

1. Rewarming: Place the frozen sections at room temperature and return them to room temperature for later use.
2. Antigen retrieval:

   (1) Frozen sections made from fixed samples:

   Place the slice in a repair box filled with repair solution, heat it to 60-70℃ in a microwave oven, maintain this temperature for about 5-10 minutes, and cool it to room temperature to complete antigen repair.

   (2) Frozen slices made from fresh tissue:

   Place the slices in fixative and fix at room temperature for 10-15 minutes. Wash the slices 2-3 times with PBS. Antigen retrieval is not required for subsequent experiments.
3. Endogenous enzyme blocking: Prepare 3% H_2O_2 solution with pure water, place the slices in the solution, and incubate at room temperature for 20 minutes.For tissues that are prone to falling off, the H_2O_2 concentration and incubation time can be appropriately reduced.
4. Blocking: Add blocking solution to the tissue and incubate at 37°C for 30 minutes.
5. Primary antibody incubation: Dilute the primary antibody to an appropriate concentration using antibody diluent and incubate at 4°C overnight or at 37° C for 1 hour.
6. Secondary antibody incubation: Add HRP enzyme-labeled secondary antibody to the tissue and incubate at 37°C for 1 hour.
7. Incubate with TSA reagent: add TSA-CY3 to the tissue, incubate at 37° C for 30 minutes, and then wash with PBST three times, 5 minutes each time.
8. Antibody elution: Add antibody elution solution to the tissue and incubate at room temperature for 20 minutes (37C is better).
9. Blocking: Add blocking solution to the tissue and incubate at 37°C for 30 minutes.
10. Primary antibody incubation: Dilute the primary antibody to an appropriate concentration using antibody diluent and incubate at 4°C overnight or at 37° C for 1 hour.
11. Secondary antibody incubation: Add HRP enzyme-labeled secondary antibody to the tissue and incubate at 37°C for 1 hour.

12. Incubate with TSA reagent: add TSA-FITC to the tissue, incubate at 37° C for 30 minutes, and then wash with PBS three times, 5 minutes each time.
13. Antibody elution: Add antibody elution solution to the tissue and incubate at room temperature for 20 minutes (37° C is better).
14. Blocking: Add blocking solution to the tissue and incubate at 37°C for 30 minutes.
15. Primary antibody incubation: Dilute the primary antibody to an appropriate concentration using antibody diluent and incubate at 4°C overnight or at 37° C for 1 hour.
16. Secondary antibody incubation: Add HRP enzyme-labeled secondary antibody to the tissue and incubate at 37°C for 1 hour.
17. Incubate with TSA reagent: add TSA-CY5 to the tissue, incubate at 37° C for 30 minutes, and then wash with PBS three times, 5 minutes each time.
18. DAPI nuclear staining: Add DAPI working solution to the tissue, incubate at room temperature for 5 minutes, and then wash three times with PBS for 5 minutes each. Drain the solution and add anti-fading mounting medium to the tissue, then cover with a coverslip. Store the prepared fluorescent sections at 4° C in the dark.

C. Cell slides

1. Antigen retrieval (optional, if cells are fixed for too long)

Heat the repair solution to 60-70° C, then drop the heated repair solution onto the slide and then cool it to room temperature.

2. Cell permeabilization: Cells should be permeabilized with 0.5% Triton-100 at room temperature for 20 minutes.
3. Blocking: Add blocking solution to the slide and incubate at 37°C for 30 minutes.
4. Primary antibody incubation: Dilute the primary antibody to an appropriate concentration using antibody diluent, add it dropwise to the slide, and incubate at 4°C overnight or at 37° C for 1 hour.
5. Secondary antibody incubation: Add HRP enzyme-labeled secondary antibody to the tissue and incubate at 37°C for 1 hour.
6. Incubate with TSA reagent: add TSA-CY3 to the slide, incubate at 37° C for 30 minutes, and

then wash three times with PBST, each time for 5 minutes.

7. Antibody elution: Add antibody elution solution to the slide and incubate at room temperature for 15 minutes (37° C is better).
8. Blocking: Add blocking solution to the slide and incubate at 37°C for 30 minutes.
9. Primary antibody incubation: Dilute the primary antibody to an appropriate concentration using antibody diluent, add it dropwise to the slide, and incubate at 4°C overnight or at 37° C for 1 hour.
10. Secondary antibody incubation: Add HRP enzyme-labeled secondary antibody to the tissue and incubate at 37°C for 1 hour.
11. Incubate TSA reagent: add TSA-FITC to the slide, incubate at 37℃ for 30min, and then wash with PBS 3 times.5 minutes per session.
12. Antibody elution: Add antibody elution solution to the slide and incubate at room temperature for 15 minutes (37° C is better).
13. Blocking: Add blocking solution to the slide and incubate at 37°C for 30 minutes.
14. Primary antibody incubation: Dilute the primary antibody to an appropriate concentration using antibody diluent, add it dropwise to the slide, and incubate at 4°C overnight or at 37° C for 1 hour .
15. Secondary antibody incubation: Add HRP enzyme-labeled secondary antibody to the tissue and incubate at 37°C for 1 hour.
16. Incubate with TSA reagent: add TSA-CY5 to the slide, incubate at 37° C for 30 minutes, and then wash with PBS three times, 5 minutes each time.
17. DAPI nuclear staining: Add DAPI working solution to the slide, incubate at room temperature for 5 minutes, and then wash three times with PBS, 5 minutes each time. Add anti-fluorescence mounting medium to the slide, place the slide upside down on the slide, and allow to dry. Store the prepared fluorescent slide at 4° C in the dark.

4. Fluorescein Information

Fluorescein	Ex (nm)	Em (nm)	Color	Filters
TSA-FITC	490	520	green	FITC channel
TSA-CY3	550	570	orange-red	CY3 channel
TSA-CY5	640	670	red	CY5 channel

5. Precautions

1. Selection of primary antibodies

1.1 Monoclonal antibodies are preferred as primary antibodies, followed by polyclonal antibodies;

1.2 If the sample is mouse, try to avoid choosing mouse-derived primary antibodies. If you choose a mouse primary antibody, the secondary antibody will not only bind to the primary antibody but also to endogenous IgG in the tissue, resulting in nonspecific staining.

2. If the tissue is prone to falling off, it can be repaired in a 60° C water bath.

3. Compared to fluorescent secondary antibodies, TSA kits offer higher sensitivity and stronger signals. Therefore, the primary antibody concentration should be lowered. Generally, the dilution ratio recommended in the antibody data sheet should be increased to minimize background fluorescence caused by nonspecific binding. A gradient of primary antibody concentrations is recommended for optimal results.

4. If the background fluorescence is strong, it is recommended to add a tissue autofluorescence quenching step.

5. To ensure the effectiveness of antibody elution and fluorescent multiple labeling, it is recommended to perform TSA single labeling tests on each antibody before formal multiple labeling. After confirming that each antibody single labeling can produce a relatively ideal positive result, the antigen retrieval conditions, antibody sequence and other experimental conditions of the multiple labeling can be determined based on the single labeling test results.

6. If some antibodies have high titers and strong affinities and are difficult to elute completely, the elution times can be increased.

7. The antibody elution solution has strong fluidity. If the slide is not placed horizontally, the reagent will easily flow out of the circle, affecting the elution effect. It is necessary to pay attention to placing the slide flat during the operation.

8. Wear gloves, masks, and lab coats during operation to avoid contact between the reagents and skin and eyes. If the reagents accidentally come into contact with sensitive areas, rinse immediately with plenty of water.