3-Color Multiple Fluorescent Staining Kit (mIHC)

1. Product Introduction

This 3-Color Multiple Fluorescent Staining Kit (mIHC) is suitable for double immunofluorescence staining of tissue samples, including paraffin sections, frozen sections, cell slides, cell smears, and organoids. It is particularly suitable for double 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 technology utilizes the peroxidase reaction of tyramide (wherein a fluorescently labeled tyramide forms a covalent binding site under HRP-catalyzed H_2O_2). This generates a large number of enzymatic reactions, which then react with surrounding protein residues (including tryptophan, histidine, and tyrosine residues), forming a large amount of fluorescent pigment deposited at the antigen-antibody binding site, thereby amplifying the signal.

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	100T	
TSA- CY3	10μL	25μL	50μL	1:200
TSA-FITC	10μL	25μL	50μL	1:200
TSA diluent	4mL	10mL	20mL	Ready-to-use
3% H_2O_2	2mL	5mL	10mL	Ready-to-use
Blocking solution	4mL	10mL	20mL	Ready-to-use
Primary antibody diluent	4mL	10mL	20mL	Ready-to-use
Antibody elution reagent	2mL	5mL	10mL	Ready-to-use

HRP- goat anti-rabbit/mouse IgG	4mL	10mL	20mL	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 TSA reagent: add TSA-CY3 to the tissue, incubate at 37℃ for 30min, then wash with

PBST 3 times. 5 minutes per session.

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 each time.
13. 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 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 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. DAPI nuclear staining: Add DAPI working solution to the tissue, incubate at room temperature for 5 minutes, and then wash with PBS 3 times, 5 minutes each time. Drain the liquid, add anti-fluorescence quenching mounting medium to the tissue, and cover with a coverslip.

    Store the slides at 4° C away from light.

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℃ 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℃ 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 (37C is better).
8. Blocking: Add blocking solution to the slide and incubate at 37℃ 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℃ 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℃ 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. DAPI nuclear staining: add DAPI working solution to the slide, incubate at room temperature for 5 minutes, and then wash with PBS 3 times, 5 minutes each time. Add anti-fluorescence quenching mounting medium to the slide, turn the slide upside down on the slide, dry it properly, and The prepared fluorescent films were stored at 4° C away from light.

4. Fluorescein Information

Fluorescein	Ex (nm)	Em (nm)	Color	Filters
TSA-FITC	490	520	green	FITC channel
TSA-CY 3	550	570	orange-red	CY3 channel

5. Precautions

1. Selection of primary antibodies

1.1Primary antibodies should preferably be monoclonal 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 factor should be increased appropriately based on the recommended dilution ratio in the antibody datasheet to reduce 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.