TSA Fluorescence Triple Staining Plus Kit（Rabbit secondary antibody）

Code：RK50026P

Size：10 T / 50 T

◆ Product Overview

The main principle of this product is based on the tyramide signal amplification (TSA, Tyramide signal amplification) technology, hereinafter referred to as TSA technology.The main principle of TSA technology is to utilize the peroxidase reaction of tyramide (that is, the fluorescent-labeled tyramide salt forms covalent bond sites under the catalysis of HRP and H_2O_2), generating a large number of enzymatic reactions. The product can combine with the surrounding protein residues (including tryptophan, histidine, tyrosine residues, etc.) and form a large amount of fluorophore deposition at the antigen-antibody binding site, achieving signal amplification.Meanwhile, through antibody elution, multiple fluorescence staining labels are achieved using different fluorescent tyramines.

The fluorescence spectrum data is as follows:

Fluorochrom	Ex/Em（nm）	Detecting laser device（nm）	Equivalent detection channel
TYR-520	490/520	488	Alexa Fluor 488，DyLight 488，FITC，FAM，Cy2
TYR-570	550/570	552/561	Alexa Fluor 555，Cy3，Tetramethylrhodamine
TYR-620	590/620	594	Alexa FLuor 594，mCherry，Texas Red Dye，DyLight 594
TYR-690	630/690	638/640	Alexa Fluor 647，APC，Cy5
TYR-480	450/480	436	CFP ，Cerulean，BV480

◆ Product Formation

Numb	Name	Size（10 T）	Size（50 T）	Storage
RK50026P-1	TYR-520 plus fluorochrome (200×)	5 μL (Concentrated type)	25 μL (Concentrated type)	2-8℃，Protect from Light
RK50026P-2	TYR-570 plus fluorochrome (200×)	5 μL (Concentrated type)	25 μL (Concentrated type)	2-8℃，Protect from Light
RK50026P-3	TYR-690 plus fluorochrome (200×)	5 μL (Concentrated type)	25 μL (Concentrated type)	2-8℃，Protect from Light
RK50026P-4	TSA buffer	2.4 mL	12 mL	2-8℃
RK50026P-5	Polymer-HRP goat anti-rabbit secondary antibody（Ready-to-use）	2.4 mL	12 mL	2-8℃
RK50026P-6	DAPI staining solution (Ready-to-use)	1 mL	5 mL	2-8℃，Protect from Light
RK50026P-7	Antibody diluent	3 mL	15 mL	2-8℃
RK50026P-8	Peroxidase inhibitor solution	3 mL	15 mL	2-8℃，Protect from Light
RK50026P-9	Antifade mounting medium	1 mL	5 mL	2-8℃

Sample requirements: Paraffin tissue sections, frozen tissue sections, cell slides (the frozen sections and cell

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slides need to be used in conjunction with the RM02984P multiplex fluorescence staining specific antibody elution solution).

◆ Storage

Store at 2-8℃, with a validity period of 12 months. Avoid direct sunlight.

◆ Operating Steps

1、Sample preparation:

1）Paraffin section：Place the slices in xylene 15 minutes each time, then in xylene 15 minutes, followed by absolute ethanol 5 minutes each time, then absolute ethanol 5 minutes, 95% ethanol 5 minutes, 85% ethanol 5 minutes, 75% ethanol 5 minutes, and finally distilled water 5 minutes.

2）Frozen section：The frozen sections were fixed for 10-30 minutes, then washed with TBST for 5 minutes, repeated 3 times. Triton X-100 lysis buffer (0.3%) was added and the cells were lysed for 20 minutes (this step can be omitted if the expression of outer membrane proteins of the cell membrane is not to be detected). The cells were washed with TBST for 5 minutes, repeated 3 times.

3）Cell slides or cell preparations：The cell samples were fixed for 15 minutes, then washed with TBST for 5 minutes three times. Triton X-100 lysis buffer (0.3%) was added and the cells were lysed for 10 minutes (this step can be omitted if the expression of outer membrane proteins of the cell membrane is not to be detected). The cells were washed with TBST for 5 minutes three times.

2、Antigen retrieval: The tissue sections were placed in a fixative box filled with pH 9.0 EDTA alkaline antigen fixative solution or pH 6.0 citrate fixative buffer solution in the microwave oven for medium heat for 8 minutes, then turned off for 8 minutes, and then switched to medium-low heat for 7 minutes. During this process, it is necessary to prevent excessive evaporation of the buffer solution and avoid drying the slides (other heat-fixing methods such as high-temperature high-pressure under atmospheric conditions for 1-2 minutes or 100℃ water bath for 20 minutes can also be used). After natural cooling, the slides were placed in TBST and shaken in the decolorization shaker for 3 times, each time for 5 minutes. **(It is recommended to use EDTA (pH 9.0) for the first antigen retrieval. In the subsequent step 8, for the antibody elution of paraffin sections, the citrate retrieval solution should be uniformly used)**

Note: The fixative and fixation conditions are determined based on the type of tissue and the type of antigen. For frozen sections/cell slides, this step can be omitted. For samples that are prone to degrading (such as bone tissue), mild fixation methods such as enzymatic fixation or water bath fixation are recommended.

3、Block endogenous peroxidase： Dip the slides into the peroxidase blocking solution, incubate at room temperature in the dark for 15 minutes. Place the slides in TBST and shake them in the decolorization shaker for 3 times, each time for 5 minutes.

4、Non-specific target blockade： After slightly drying the slices, use an immunohistochemical pen to draw circles around the tissue (to prevent the leakage of blocking solution and antibodies). Drop 3% BSA solution

(or other blocking solutions, or the antibody dilution solution in the kit) evenly over the tissue within the circles. Let it incubate at room temperature for 30 minutes.

5、Incubation of specific primary antibody: Gently remove the sealing solution, and add the primary antibody X diluted with the antibody dilution solution to the slide. Place the slide flat in a light-proof humid box at 4°C for overnight incubation or incubate at 37°C for 1-2 hours (add a small amount of water in the humid box to prevent the evaporation of the antibody).

6、Incubate the Polymer-HRP secondary antibody: The slides were placed in TBST and shaken in a decolorization shaker for 3 times, each time for 5 minutes. After slightly drying the slides, a HRP secondary antibody corresponding to the species of the primary antibody was dropped onto the tissue in the circle. The tissue was incubated at room temperature in the dark for 50 minutes, and then washed 3 times with TBST, each time for 5 minutes.

7、Fluorescent dye staining: The concentrated fluorescent dye and TSA buffer were mixed evenly at a dilution ratio of 1:200. 50 μL of the fluorescent dye working solution was dropped onto the section. It was advisable to cover the tissue evenly. The reaction was carried out at room temperature for 2-15 minutes (the optimal incubation time is 5-10 minutes). The section was washed with TBST for 3 times, each time for 5 minutes. **(The dilution ratio of the concentrated fluorescent dye to TSA buffer can be flexibly adjusted and optimized according to specific circumstances. The dilution ratio range is 1:50 to 1:400. Generally, a dilution ratio of 1:200 can meet the needs of the vast majority of experiments)**

Note: The preliminary experiment can be stained for 1 minute first. After washing off the fluorescent dye, observe the staining effect under the microscope. If the staining is weakly positive, continue to add more fluorescent dye to enhance the staining intensity until it is appropriate, then proceed to the next step.

8、Antibody elution:

1) Paraffin section: Place in the antigen retrieval solution and incubate at 95°C in a water bath for 25-40 minutes (adjust the time flexibly according to the affinity of different antibodies); discard the elution solution, and wash three times with TBST, each time for 5 minutes. **(Lactic acid repair solution)**

2) Frozen section/Cell sample/Bone tissue: Add an appropriate amount of the multiplex fluorescent staining-specific antibody elution solution (item number RM02984P) preheated to 37°C and fully dissolved to cover the sample. Place it at 37°C for 5-20 minutes, then discard the elution solution; add an appropriate amount of the antibody elution solution again to cover the sample and place it at 37°C for 5-20 minutes, then discard the elution solution; wash with TBST 3 times, each time for 5 minutes. (When the temperature is low, the antibody elution solution is prone to precipitate, so it needs to be preheated to 37°C until fully dissolved before use)

9、Repeat steps 3 to 8 (Switch to using another type of TYR fluorescent dye) -Second round marking.

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10、Repeat steps 3 to 7（Switch to using another type of TYR fluorescent dye）-Third round of marking.

11、Re-dyeing the cell nucleus with DAPI: After the slices are dried, add 50-100 μL of the ready-to-use DAPI staining solution into the wells, ensuring a uniform coverage of the tissue. Incubate at room temperature in the dark for 5-20 minutes.

12、Seal sheet: The slices were placed in TBST and shaken in a decolorization shaker for 3 times, each time for 5 minutes. After slightly drying the slices, they were sealed with anti-fluorescence quenching mounting agent, avoiding the formation of bubbles as much as possible.

13、Microscopic examination and photography: The slices were observed and images were collected under a fluorescence microscope / confocal / multi-channel fluorescence scanner / multispectral imaging system.

◆ **Notice**

1. This product is for scientific research purposes only and is not intended for clinical diagnosis.

2. Before starting the experiment, it is necessary to carefully read the instructions.

3. Optimization of the dilution ratio of the TYR-xxx Plus fluorescent dye: The TYR-xxx Plus fluorescent dye included in this kit has a relatively high sensitivity, which is 10 to 50 times that of the conventional ordinary TSA kit. Therefore, it is necessary to pay attention to not having too strong a fluorescence signal during the detection. If the signal is too strong, the concentration of the fluorescent dye/reaction time/antibody concentration needs to be reduced; if the signal is too weak, the concentration of the fluorescent dye/reaction time/antibody concentration needs to be increased. The higher the concentration of the fluorescent dye, the more exponentially the fluorescence signal will increase during the detection. Generally, a dilution ratio of 1:200 can meet the needs of most experiments, a dilution ratio of 1:50 can obtain the strongest detection signal, and a dilution ratio of 1:500 is equivalent to the detection signal of the conventional ordinary TSA.

4. The expiration date of the diluted TYR-xxx Plus fluorescent dye solution: The TYR-xxx Plus fluorescent dye is recommended to be prepared immediately before use. If the dye solution is not used up at one time, the remaining diluted dye solution can be stored under light protection at -20℃ for two weeks.

5. Selection of the primary antibody: ①Specificity: Choose highly specific primary antibodies, and prefer monoclonal antibodies over polyclonal antibodies (as they are less likely to cause non-specific binding); ② Sample type: paraffin section（The primary antibody needs to be verified by IHC-P）、 frozen section（The primary antibody needs to be verified by IHC-Fr）、Cell sample（The primary antibody needs to be verified through ICC/IF methods）。③Species: The species of the primary antibody should be different from that of the actual sample being tested (for example, if testing a mouse sample, the primary antibody should use a rabbit monoclonal antibody rather than a mouse monoclonal antibody) to avoid non-specific binding caused by the secondary antibody; ④Dilution rate: Due to the higher sensitivity and stronger signal amplification of the TSA kit, after the primary antibody meets the application requirements of IHC, the dilution ratio of the primary antibody should be increased by 5 times on the basis of the recommended dilution ratio for IHC.

6. The general principle for selecting the sequence of indicators/antibodies when performing staining with dyes: ①Fluorescent dye spectral separation: It is preferable to select fluorescent dyes with non-overlapping emission spectra (such as 520, 570, 620 and 690, etc.) to avoid the situation where overlapping detection channels of fluorescent dyes lead to color mixing in the detection results. Usually, short-wavelength dyes (such as 520) are used for labeling first, followed by long-wavelength dyes (such as 690) for labeling, in order to reduce the interference of photobleaching on the intensity of the fluorescent signal; ②Expression abundance of the target antigen: Firstly, mark the antigens with low abundance or weak expression, and then mark the antigens with high abundance or high expression. Avoid strong signals masking weak signals. If the antigenic epitopes of different antigens being detected are adjacent, then prioritize marking the antibodies with greater steric hindrance; ③The affinity of the primary antibody: The primary labeling uses antibodies with relatively weaker affinity (easily elutable), to avoid interference in the subsequent labeling and staining due to incomplete elution. The final labeling uses antibodies with relatively higher affinity (more difficult to elute).

7. The issue of thickness in the preparation of slice samples: ①Paraffin section: The slice thickness is generally around 3-5 μm. Under normal circumstances, after antigen retrieval, the Polymer-HRP polymeric secondary antibody can effectively penetrate the tissue; ②Frozen section: The slice thickness is generally around 10 μm. However, for thick slices (20-40 μm, such as in mouse brain and other areas), it may hinder the effective penetration of the Polymer-HRP polyplexed secondary antibody into the tissue. Therefore, for thick slices, the permeabilization and membrane disruption time can be appropriately extended, and the incubation time of the Polymer-HRP polyplexed secondary antibody can also be prolonged (if necessary, the secondary antibody can be incubated at 4°C in the dark overnight to ensure effective tissue penetration of the Polymer-HRP polyplexed secondary antibody). Particularly, the larger the thickness of the frozen section, the higher the risk of disintegration. Therefore, for frozen sections, it is recommended to perform no more than three-color detection.

8. Possible causes for color bleeding: Color crossover usually refers to the overlapping of the emission spectra of different fluorescent dyes, which leads to signal interference during detection. Color crossover is related to many factors, such as: ①imaging device: The wavelength range of the filter is relatively wide, which may cause the signals of adjacent fluorescence channels to overlap. An excessively long exposure time may result in overexposure of the fluorescence signal; ②Insufficient antibody elution: High-affinity antibodies that remain present, such as those for cytokeratin (CK) and FoxP3, due to their tight binding, require an extended elution time; ③Unbalanced signal strength: Excessive concentration of fluorescent dyes or prolonged reaction time, too high concentration of primary antibody or improper repair strength; ④Antibody sequence and staining procedure: The antibody sequence design is unreasonable. Antibodies that are difficult to remove (such as FoxP3) should be placed in the last round of staining to avoid residual effects that may affect the subsequent results. For the first round, it is recommended to select targets suitable for EDTA fixation. For subsequent

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rounds, use citrate fixation instead. Human operational errors include not thoroughly removing the antibodies from the previous round, confusing the antibody sequence, or forgetting the fixation steps; ⑤The quality of the sample: Organ necrosis or contamination. The necrotic areas or uncleaned tissues are prone to adsorb fluorescent dyes, resulting in non-specific signals. Insufficient dewaxing of paraffin sections or poor fixation of frozen sections may lead to an increase in the staining background; ⑥Selection of fluorescent dyes and antibodies: Similar emission spectra of fluorescent dyes can lead to color interference. Therefore, a combination of dyes with a greater spectral interval should be selected. Polyclonal antibodies are prone to non-specific binding. It is recommended to use monoclonal antibodies that have been verified by knockout experiments, and choose antibodies labeled for IHC/mIHC.

9. Experimental group setup: It is recommended to set up a blank control group (i.e., a group without incubating with the primary antibody) to rule out non-specific staining issues.

10. The usage amount corresponding to the 10 T/50 T specifications is 50 μL per 1 T, Please note: If there are differences in experimental conditions, resulting in excessive usage, it will not be possible to meet the requirement of 50 uses. For example, for cell slides in 6-well plates and 12-well plates, if the usage is too large, it will not be able to meet the 50 usage requirements.

11. If the background fluorescence is strong, it is recommended to add a step for quenching the tissue's intrinsic fluorescence.

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