# 冰冻切片免疫荧光单标 TSA 实验报告

## 一、实验原理

酪酰胺信号放大(TSA)技术是一类利用辣根过氧化酶（HRP）对靶蛋白进行标记的酶学检测方法，类似常规免疫组化的 DAB 显色方法 。TSA 技术同样采用 HRP 标记的二抗，同样有对应的“显色”步骤 HRP 催化加入反应体系的酪胺荧光素底物，产生活化荧光底物，活化底物可与抗原上的酪氨酸等残基共价结合，使样品上稳定的共价结合酪胺荧光素。之后用热修复法洗去非共价结合的一抗-二抗-HRP 复合物，重复下一种一抗-hrp 二抗来进行第二轮孵育，如此往复就可实现多重标记。

## 二、实验器材及试剂

### 1、实验器材

| 名称 | 厂家 | 型号 |
|---|---|---|
| 冰冻切片机 | 达科为深圳医疗设备有限公司 | 6250 |
| 烤箱 | 天津市莱玻特瑞仪器设备有限公司 | GFL125 |
| 微波炉 | 美的 | M1-L213B |
| 盖玻片 | 江苏汇达医疗器械有限公司 | 710510 |
| 载玻片 | 海门市神鹰实验仪器厂 | 188109 |
| 脱色摇床 | 武汉赛维尔生物科技有限公司 | SYC-Z100 |
| 涡旋仪 | 武汉赛维尔生物科技有限公司 | MX-F |
| 掌上离心机 | 武汉赛维尔生物科技有限公司 | DS-S 100 |
| 显微镜 | NIKON | ECLIPSE E100 |
| 组化笔 | Gene tech | GT1001 |

| 移液枪 | Dragon | KE003068 |
|---|---|---|
| 3D 扫描仪 | 3D HISTECH | Pannoramic SCAN |

2、主要实验试剂

| 试剂 | 厂家 | 货号 |
|---|---|---|
| OCT 包埋剂 | 杭州浩克生物技术有限公司 | HK2061 |
| 甲醇 | 国药集团化学试剂有限公司 | 67561 |
| 抗原修复液 EDTA(9.0) | 杭州浩克生物技术有限公司 | HKI0004 |
| BSA 牛血清白蛋白 | 杭州浩克生物技术有限公司 | HKW2084 |
| PBS 缓冲液 | 杭州浩克生物技术有限公司 | HK0002 |
| 一抗 | | |
| 二抗：HRP 超敏山羊抗兔鼠通用二抗 | 杭州浩克生物技术有限公司 | HKI0029 |
| FlareXXXX | 杭州浩克生物技术有限公司 | |
| DAPI | 杭州浩克生物技术有限公司 | HKI0005 |
| 抗荧光淬灭封片剂 | 杭州浩克生物技术有限公司 | HKI0007-1 |

## 三、实验步骤

1. **冰冻切片固定：**冰冻切片从冰箱拿出来复温，晾干水分，纯甲醇固定 10min，后于 37℃或室温干燥，在 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min。
2. **抗原修复：**组织切片置于盛满 EDTA 抗原修复缓冲液（PH9.0）的修复盒中于微波炉内进行抗原修复。低火 10min，此过程中应防止缓冲液过度蒸发，切勿干片。自然冷却后将玻片置于 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min。（修复液和修复强度根据组织来确定）
3. **阻断内源性过氧化物酶：**切片加上 3%的双氧水，室温孵育 25min，将玻片置 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min。

4. **画圈：**用组化专用的组化笔沿着组织外围轮廓画一个与组织间隔 3-4 毫米的小圈，然后加入足量的 PBS 保证后续依次加入的封闭血清，一抗，二抗，以及显色剂能完全覆盖组织，而不沿着玻片流走。
5. **血清封闭：**切片稍甩干后用组化笔在组织周围画圈（防止抗体流走），在圈内滴加用 3%BSA 室温封闭 30min 以上。
6. **加一抗：**轻轻甩掉封闭液，在切片上滴加 PBS 按一定比例配好的一抗，切片平放于湿盒内 4°C 孵育过夜。（湿盒内加少量水防止抗体蒸发）
7. **加二抗：**玻片置于 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min。切片稍甩干后在圈内滴加与一抗相应种属的二抗覆盖组织，避光室温孵育 50min。
8. **信号放大：**将玻片置于 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min 。滴加相对应颜色 Flare 信号放大试剂，3-5min.将玻片置于 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min。
9. **DAPI 复染细胞核：**玻片置于 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min。切片稍甩干后在圈内滴加 DAPI 染液，避光室温孵育 8min。
10. **封片：**玻片置于 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min。切片稍甩干后用抗荧光淬灭封片剂封片。
11. **镜检拍照：**切片于尼康倒置荧光显微镜下观察并采集图像。

## 四、结果判读

DAPI 染出来的细胞核在紫外的激发下为蓝色，阳性表达为相应荧光试剂标记的颜色（具体荧光通道参考下表）。

| Flare 染料 | 激发波长 | 发射波长 |
| --- | --- | --- |
| DAPI 蓝色 | 350 | 420 |
| Flare480 青绿 | 450 | 480 |
| Flare520 绿 | 490 | 520 |
| Flare570 红 | 550 | 570 |

| Flare620 橙 | 590 | 620 |
| --- | --- | --- |
| Flare690 粉 | 630 | 690 |
| Flare780 红外 | 750 | 780 |

## 五、注意事项

1. 实验过程中切片勿干片；
2. 实验操作中小心枪头刮伤组织；
3. 冰冻切片注意防脱。

# Frozen section fluorescent single label staining experiment report (TSA)

## 1. Experimental principle

Tyramide signal amplification (TSA) technique is a kind of enzymatic detection method using horseradish peroxidase (HRP) to label target protein, which is similar to the conventional immunohistochemical DAB color development method. TSA technology also uses HRP labeled secondary antibodies, and also has corresponding "color rendering" steps to catalyze the addition of tyramine fluorescein substrate in the reaction system to produce an activated fluorescent substrate, which can covalently bind tyramine fluorescein and other residues on the antigen to make the sample covalently bind tyramine fluorescein. After that, the non-covalently bound primary - secondary -HRP complex was washed by thermal repair method, and the next primary -hrp secondary - antibody was repeated for the second round of incubation, so that multiple labeling could be achieved repeatedly.

## 2. Laboratory equipment and reagents

### 2.1 Laboratory equipment

| Equipment | Manufacturers | Model |
|---|---|---|
| Frozen microtome | DAKEWE | 6250 |
| Oven | Tianjin Leibo Terry Equipment Co., Ltd | GFL125 |
| Microwave oven | Midea | M1-L213B |
| Coverslips | Jiangsu Huida Medical Instruments Co., | 710510 |

| | | |
|---|---|---|
| | Ltd | |
| Glass microscope slides | Haimen Shenying Experimental Equipment Factory | 188109 |
| Rocker | Servicebio | SYC-Z100 |
| Vortex | Servicebio | MX-F |
| Micro-centrifuge | Servicebio | DS-S 100 |
| Microscope | NIKON | ECLIPSE E100 |
| Liquid Blocker PAP Pen | Gene tech | GT1001 |
| Pipettor | Dragon | KE003068 |
| 3D Imaging System | 3D HISTECH | Pannoramic SCAN |

**2.2 Laboratory reagents**

| Reagents | Manufacturers | Catlog |
|---|---|---|
| OCT embedding agent | Hangzhou Haoke Biotechnology Co., Ltd | HK2061 |
| Methyl alcohol | Sinopharm Chemical Reagent Co., Ltd | 67561 |
| EDTA Antigen repair solution (9.0) | Hangzhou Haoke Biotechnology Co., Ltd | HKI0004 |
| BSA | Hangzhou Haoke Biotechnology Co., Ltd | HKW2084 |
| PBS solution | Hangzhou Haoke Biotechnology Co., Ltd | HK0002 |
| Primary antibody | Hangzhou Haoke Biotechnology Co., Ltd | |
| Second antibody：HRP hypersensitive goat anti-rabbit secondary antibody | Hangzhou Haoke Biotechnology Co., Ltd | HKI0029 |
| FlareXXXX | Hangzhou Haoke Biotechnology Co., Ltd | |
| DAPI | Hangzhou Haoke Biotechnology Co., Ltd | HKI0005 |

## 3. Experimental procedure

**3.1 Frozen section fixation:** The frozen slices were taken out of the refrigerator and rewarmed, dried, fixed in pure methanol for 10min, then dried at 37 ℃ or room temperature, and washed three times on a decolorizing shaker in PBS (PH7.4) for 5min each time.

**3.2 Repair:** The tissue sections were placed in a repair box filled with EDTA antigen repair buffer (PH9.0) for antigen repair in a microwave oven. Low fire for 10min, during this process should prevent excessive evaporation of buffer, do not dry. After natural cooling, the slide was placed in PBS (PH7.4) and washed by shaking on the decolorizing shaker for 3 times, 5min each time. (Repair solution and repair strength are determined according to the tissue)

**3.3 Block endogenous peroxidase:** The sections were incubated with 3% hydrogen peroxide at room temperature for 25min. The slides were placed in PBS (PH7.4) and washed three times on a decolorizing shaker for 5min each time.

**3.4 Draw a circle around the tissue:** Use a histochemical pen to draw a small circle 3-4 mm apart from the tissue along the outer outline of the tissue, and then add enough PBS to ensure that the subsequent addition of blocking serum, primary antibody, secondary antibody, and color development agent can completely cover the tissue without flowing along the sections.

**3.5 Serum blocking:** After the section is slightly dried, draw a circle around the tissue with a histochemical pen (to prevent the antibody from flowing away), and add 3%BSA to the circle and seal it at room temperature for more than 30 minutes.

**3.6 Add primary antibody:** Gently shake off the sealing solution, add PBS to the slices with a certain proportion of primary antibody, and the slices are placed flat in a wet box at 4° C for overnight incubation. (Add a small amount of water to the wet box to prevent the antibody from evaporating)

**3.7 Add secondary antibody:** The sections were placed in PBS (PH7.4) and washed by shaking on the decolorizing shaker for 3 times, 5min each time. After the slices were slightly dried, the tissue covered by the second antibody of the corresponding species of the first antibody was added to the ring and incubated at room temperature for 50min away from light.

**3.8 TSA:** The sections were placed in PBS (PH7.4) and washed by shaking on the decolorizing shaker for 3 times, 5min each time. Add the corresponding color Flare signal amplification reagent, 3-5min. Place the slide in PBS (PH7.4), shake and wash it on the decolorizing shaker for 3 times, 5min each time.

**3.9 DAPI restaining nuclei:** The sections were placed in PBS (PH7.4) and washed by shaking on the decolorizing table for 3 times, 5 min each time. Blot the excess PBS in the ring and then add DAPI dye solution, and incubate at room temperature for 8min away from light.

**3.10 Sealing:** The sections were placed in PBS (PH7.4) and washed by shaking on the decolorizing table for 3 times, 5min each time. Remove the slices and seal them with anti-fluorescence quenching tablets.

**3.11 Microscopy photography:** Sections were observed under a fluorescence microscope and images were collected.

## 4. Interpretation of results

The nuclei stained by DAPI are blue under ultraviolet excitation, and the positive expression is the color labeled by the corresponding fluorescent reagent (refer to the table below for specific fluorescence channels).

| Flare Dye | Excitation wavelength | Emission wavelength |
| --- | --- | --- |
| DAPI | 350 | 420 |
| Flare480 | 450 | 480 |
| Flare520 | 490 | 520 |
| Flare570 | 550 | 570 |
| Flare620 | 590 | 620 |
| Flare690 | 630 | 690 |
| Flare780 | 750 | 780 |

## 5. Precautions

5.1 Don't let the slices dry out during the experiment；

5.2 Be careful of the injured tissue on the pipette suction head during experimental operation；

5.3 Pay attention to prevent peeling of frozen slices.