



石蜡切片 SweAMI-地高辛荧光原位杂交 (TSA) 双标实验报告

1. 实验器材及试剂

1.1. 实验器材

名称	厂家	型号
脱水机	DIAPATH	Donatello
载玻片	Servicebio	
盖玻片	江苏世泰实验器材有限公司	10212432C
病理切片机	上海莱卡仪器有限公司	RM2016
无酶离心管	Servicebio	EP-150-M
摇床(钟摆式)	Servicebio	TSY-B
涡旋混匀	Servicebio	MV-100
移液枪	Dragon	KE0003087/KA0056573
免疫组化笔	Servicebio	G6100
冰箱	青岛海尔股份有限公司	BCD-192TGN
正置荧光显微镜	日本尼康	NIKON ECLIPSE CI
成像系统	日本尼康	NIKON DS-U3
恒温箱	LABOTERY	GSP-70
高压灭菌锅	松下健康医疗	MLS-3751L-PC
包埋机	武汉俊杰电子有限公司	JB-P5
冻台	武汉俊杰电子有限公司	JB-L5
组织摊片机	浙江省金华市科迪仪器设备有限公司	KD-P

1.2. 主要实验试剂

试剂	厂家	货号	稀释比
DEPC	Amresco	E174	
4%多聚甲醛 (DEPC 水)	Servicebio	G1113	
石蜡	Sakura		
无水乙醇	国药集团化学试剂有限公司	100092683	
环保型脱蜡透明液	Servicebio	G1128	
PBS	Servicebio	G0002	
20×SSC 洗脱液	Servicebio	G3016-4	
BSA	Servicebio	G5001	

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蛋白酶 K	Servicebio	G1234
DAPI	Servicebio	G1012
抗荧光淬灭封片剂	Servicebio	G1401
杂交缓冲液	Servicebio	G3016-3
anti-DIG-HRP	Jackson	200-032-156
FITC-Tyramide	Servicebio	G1222
CY3-Tyramide	Servicebio	G1223

2. 石蜡切片地高辛荧光原位杂交实验步骤

- 2.1. **组织固定**: 组织取出洗净后立即放入固定液 (DEPC 水配制) 中固定 12h 以上。
- 2.2. **脱水**: 组织固定完成后经梯度酒精脱水后浸蜡, 包埋。
- 2.3. **切片**: 石蜡经切片机切片, 摊片机捞片, 62°烤箱烤片 2h。
- 2.4. **石蜡切片脱蜡至水**: 依次将切片放入脱蜡透明液 I 15min-脱蜡透明液 II 15min-无水乙醇 I 5min-无水乙醇 II 5min, 风干, DEPC 水浸泡。。
- 2.5. **消化**: 根据组织固定时间长短, 切片于修复液中煮沸 10 分钟, 自然冷却。后基因笔画圈, 根据不同组织不同指标特性, 滴加蛋白酶 K (20ug/ml) 37°消化___min。纯水冲洗后 PBS 洗 3 次×5min。
- 2.6. **阻断内源性过氧化物酶**: 滴加 3% 甲醇-H₂O₂, 室温避光孵育 15min, 将玻片置于 PBS (PH7.4) 中在脱色摇床上晃动洗涤 3 次, 每次 5min。
- 2.7. **预杂交**: 滴加预杂交液, 37°C 孵育 1h。
- 2.8. **杂交**: 倾去预杂交液, 滴加含探针___杂交液, 浓度 500nM, 恒温箱 42 度杂交过夜。
- 2.9. **杂交后洗涤**: 洗去杂交液, 2×SSC, 37°C 洗 10min, 1×SSC, 37°C 洗 2×5min, 0.5×SSC 室温洗 10min。若非特异杂交体较多, 可以增加甲酰胺洗涤。
- 2.10. **Imaging oligo (DIG) 孵育**: 滴加含 Imaging oligo (DIG) 杂交液, 稀释比 1: 400.42° 孵育 3h。后 2×SSC, 37°C 洗 10min, 1×SSC, 37°C 洗 2×5min, 0.5×SSC 37°洗 10min。
- 2.11. **滴加封闭液**: 滴加封闭血清 正常兔血清。室温 30min。
- 2.12. **滴加鼠抗地高辛标记过氧化物酶 (anti-DIG-HRP)**: 倾去封闭液, 滴加 anti-DIG-HRP。37°C 孵育 50min, 后 PBS 洗 3 次×5min。
- 2.13. **滴加 FITC-Tyramide**: 滴加 FITC-Tyramide 试剂, 避光室温反应 5min。后 TBST 洗 3 次×10min. PBS 洗 1 次×5min。
- 2.14. **杂交**: 倾去预杂交液, 滴加杂交液 (含探针 mir-195-5p 浓度 500nM), 42 度杂交过夜。
- 2.15. **杂交后洗涤**: 洗去杂交液, 2×SSC, 37°C 洗 10min, 1×SSC, 37°C 洗 2×5min, 0.5×SSC 37°洗 10min。若非特异杂交体较多, 可以增加甲酰胺洗涤
- 2.16. **Imaging oligo2 (DIG) 孵育**: 滴加含 Imaging oligo2 (DIG) 杂交液, 稀释比 1: 400.42° 孵



育 3h。后 2×SSC, 37°C 洗 10min, 1×SSC, 37°C 洗 2×5min, 0.5×SSC 37°C 洗 10min。

2.17.滴加封闭液: 滴加封闭血清 正常兔血清。室温 30min。

2.18.滴加鼠抗地高辛标记过氧化物酶 (anti-DIG-HRP): 倾去封闭液, 滴加 anti-DIG-HRP。37°C 孵育 50min, 后 PBS 洗 3 次×5min。

2.19.滴加 CY3-Tyramide: 滴加 CY3-Tyramide 试剂, 避光室温反应 5min。后 PBS 洗 3 次×5min。

2.20.DAPI 复染核: 切片滴加 DAPI 染液, 避光孵育 8min, 冲洗后滴加抗荧光淬灭封片剂封片。

2.21.镜检拍照: 切片于尼康正置荧光显微镜下观察并采集图像。(紫外激发波长 330-380nm, 发射波长 420nm, 发蓝光; FAM(488)绿光激发波长 465-495nm, 发射波长 515-555 nm, 发绿光; CY3 红光激发波长 510-560, 发射波长 590nm, 发红光。)

3.石蜡切片地高辛荧光原位杂交实验结果判读

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素 (488) 标记的绿光。CY3 标记的红光。mRNA 原位杂交显示结果理论为胞浆阳性, 少数核阳性属正常。micRNA 与 lncRNA 不同指标表达定位不同。根据表达量不同荧光亮度有强弱。

注: 上述涉及到的所有试剂, 仪器等在 RNA 原位杂交实验时都需使用 DEPC 处理后的 RNase free 环境。

附表 1 探针信息



Paraffin –DIG -TSA -ISH protocol

1. Apparatus and reagents

1.1 Apparatus

Name	Producer	Model
Dehydrator	DIAPATH	Donatello
Glass slide	Servicebio	
Coverslip	Citotest	10212432C
Pathologic microtome	Leica	RM2016
Enzyme-free centrifuge tubes	Servicebio	EP-150-M
Shaker	Servicebio	TSY-B
Vortex	Servicebio	MV-100
Pipettor	Dragon	KE0003087/KA0056573
Liquid Blocker PAP Pen	Servicebio	G6100
Microscopy	Nikon	NIKON ECLIPSE CI
Imaging system	Nikon	NIKON DS-U3
Incubator	LABOTERY	GSP-70
autoclave	PANASONIC	MLS-3751L-PC
Paraffin embedding machine	WHJJ	JB-P5
Frozen flat	WHJJ	JB-L5
Tissue spreader	Kedee	KD-P

1.2 Major reagents

reagent	manufacturer	article number
DEPC	Amresco	E174
4% of paraformaldehyde (DEPC water)	Servicebio	G1113
Paraffin wax	Sakura	G1113
Ethanol	SCRC	100092683
BioDewax and Clear solution	Servicebio	G1128
PBS	Servicebio	G0002
20×SSC solution	Servicebio	G3016-4
BSA	Servicebio	G5001
Proteinase K	Servicebio	G1234

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DAPI	Servicebio	G1012
Anti-fluorescence quenching sealing tablets	Servicebio	G1401
hybridization buffer	Servicebio	G3016-3
anti-DIG-HRP	Jackson	G3016-3
FITC-Tyramide	Servicebio	G1222
CY3-Tyramide	Servicebio	G1223

2. The steps of the experiment

2.1 Organization fixed: take out the organization, wash clean, then Immediately put in the fixed fluid (DEPC) above 12h.

2.2 Dehydration: The tissue is dehydrated by gradient alcohol, paraffin, embedding.

2.3 Section: the paraffin is sliced through the slicer, the piece of the slice machine and the 62 - degree oven roast for 2 hours.

2.4 Dewaxing and dehydration: soak sections in 2 changes of Dewaxing Transparent Liquid, 15 minutes each. Dehydrate in 2 changes of pure ethanol for 5 minutes each. Then, followed respectively by dehydrating in gradient ethanol of 85%and 75% ethanol 5 minutes for each. Wash in DEPC dilution.

2.5 Digestion: according to the tissue fixation time, the slices are boiled in the retrieval solution for 10-15 minutes and naturally cooled. Mark the objective tissue with liquid blocker pen, according to the characteristics of tissues, Add proteinase K(20 ug/ml) working solution to cover objectives and incubate at 37°C for___min. Washing with pure water, then wash three times with PBS (pH 7.4) in a Rocker device, 5 min each.

2.6 Block endogenous peroxidase: add 3% methanol-H₂O₂, incubate in dark at room temperature for 15min. Wash slides in PBS(PH7.4) three times for 5 min each with gentle agitation.

2.7 Pre-hybridization: add Pre-hybridization solution to each section and incubate for 1 h at 37°C.

2.8 Hybridization: remove the pre-hybridization solution, add the probe hybridization solution with concentration of ___, and incubate the section in a humidity chamber and hybridize overnight at___°C.

2.9 Washing: remove the hybridization solution. Wash sections with 2×SSC for 10 min at 37°C , Wash sections in 1×SSC two times for 5 min each at 37°C, and wash in 0.5×SSC for 10 min at room temperature. Formamide washing can be added if there are more non-specific hybrids.

2.10 Imaging oligo (DIG): Add the hybridization solution containing Imaging Oligo (DIG), Dilution ratio 1:400. 42 ° FOR 3 hours. Wash sections with 2×SSC for 5 min at 37°C , Wash sections in 1×SSC two times for 5 min each at 37°C, and wash in 0.5×SSC for 10 min at room



temperature.

2.11 **Blocking:** add blocking solution (rabbit serum) to the section and incubate at room temperature for 30 min.

2.12 Add the mouse anti-digoxigenin-labeled peroxidase (anti-DIG-HRP): remove the blocking solution and add anti-DIG-HRP. Incubate at 37 °C for 40 min, then Wash sections in PBS four times for 5 min each.

2.13 Tyramide **developing:** dry sections slightly, add fresh prepared Tyramide chromogenic reagent to marked tissue. Reaction in dark for 5 min at room temperature. Then wash sections in PBS three times for 10 min each.

2.14 **Stain cell nuclei (counter stain):** incubate with DAPI for 8min in the dark, and then mounting.

2.15 **Microscopic examination and photography:** to take photos with positive fluorescence microscope. DAPI glows blue by UV excitation wavelength 330-380 nm and emission wavelength 420 nm; FAM glows green by excitation wavelength 465-495 nm and emission wavelength 515-555 nm; CY3 glows red by excitation wavelength 510-560 nm and emission wavelength 590 nm.

3. Interpretation of the results

The nuclear stained by DAPI were blue under ultraviolet excitation, and the positive expression was a kind of fluorescence labeled by corresponding luciferin. FAM (488) appears green, cy3 appears red. The results of mRNA in situ hybridization were cytoplasmic positive and a few nuclear positive were normal. MicRNA and lncRNA were expressed differently. According to the expression, Different fluorescence brightness is strong or weak.

Note: All reagents, instrument need RNase free.

Attached table 1 probe information.