石蜡切片 SABER-DAB 显色原位杂交实验报告

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	wanwu	
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	wanwu	EP-150-M
摇床(钟摆式)	wanwu	TSY-B
涡旋混匀器	wanwu	MX-F
移液枪	Dragon	KE0003087/KA0056573
Gene tech pen	Gene tech	GT1001
冰箱	青岛海尔股份有限公司	BCD-192TGN
显微镜	CIC	XSP-C204
正置光学显微镜	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）	wanwu	G1113	
石蜡	Sakura		
无水乙醇	国药集团化学试剂有限公司	100092683	
二甲苯	国药集团化学试剂有限公司	10023418	
甲醇	国药集团化学试剂有限公司	40030561	
双氧水	国药集团化学试剂有限公司	10011208	
PBS 缓冲液（DEPC）	wanwu	G0020	
20×SSC 洗脱液	wanwu	G3016-4	

BSA	wanwu	G5001
蛋白酶 K	wanwu	G1205
苏木素染液中	wanwu	G1004
性树胶	国药集团化学试剂有限公司	10004160
杂交缓冲液	wanwu	G3016-3
anti-DIG-HRP	jackson	200-032-156
DAB 显色剂	wanwu	G1211

2.石蜡切片 DAB 显色原位杂交实验步骤

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：依次将切片放入二甲苯I15min-二甲苯II15min-无水乙醇I5min-无水乙醇II5min,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 20 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 linc00173/linc00173 TSV2 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。

2.10.二标孵育：滴加含二标探针杂交液，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°孵育 50min，后 PBS 洗 4 次×5min。

2.13．DAB 显色：切片稍甩干后，在圈内滴加新鲜配制的 DAB 显色液，显微镜下控制显色时间，阳性为棕黄色，纯水冲洗切片终止显色。

2.14．复染细胞核：Harris 苏木素复染 3min 左右，自来水洗，1%盐酸酒精分化数秒，自来水洗，氨水返蓝，流水冲洗。

2.15．脱水封片：将切片依次放入 75%酒精 6min-85%酒精 6min—100%酒精 I 6min—100%酒精 II 6min-正丁醇 6min—二甲苯透明，将切片从二甲苯中拿出稍晾干后，中性树胶封片。

2.16．显微镜检，图像采集分析。

3.石蜡切片 DAB 显色原位杂交实验结果判读

苏木素染细胞核为蓝色，DAB 显出的阳性表达为棕黄色。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。根据表达量显色有深浅。

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

linc00173 probe:

5’-CCAGCATTGGGGATGTGTAATTGATTGCCT

（ttt CATCATCAT ACATCATCAT）$_{30}$ -3’

linc00173 TSV2 probe:

5’-CCCTATCCCGCAAGCGTCCTCCCATTC

（ttt CATCATCAT ACATCATCAT）$_{30}$ -3’

二标探针：

5’-DIG-tt ATGATGATGT ATGATGATGT -3’

Paraffin –DIG -DAB -ISH protocol

1. Apparatus and reagents

1.1 Apparatus

Name	Producer	Model
Dehydrator	WHJJ	JJ-12J
Paraffin embedding machine	WHJJ	JB-P5
Pathologic microtome	Leica	RM2016
Frozen flat	WHJJ	JB-L5
Water Bath-Slide	Kedee	KD-P
RNase-freeGlass microscope slides	wanwu	
Micro-centrifuge	wanwu	D1008E
Rocker	wanwu	TSY-B
Vortex	wanwu	MX-F
Pipettor	Dragon	KE0003087/KA0056573
Microscopy	CIC	XSP-C204
Liquid blocker pen	Gene tech	GT1001
Refrigerator	HAIER	BCD-192TGN
incubator	LABOTERY	GSP-70
autoclave	PANASONIC	MLS-3751L-PC

1.2 Major reagents

reagent	manufacturer	article number
Ethanol	SCRC	100092683
Xylene	SCRC	10023418

PBS solution	wanwu	G0002
Proteinase K	wanwu	G1205
4% of paraformaldehyde（DEPC water）	wanwu	G1113
Methanol	SCRC	
Hydrogen peroxide	SCRC	
DEPC	Amresco	E174
20×SSC solution	wanwu	G3016-4
BSA	wanwu	G5001
Hematoxylin dye	wanwu	G1004
neutral balsam	SCRC	
hybridization buffer	wanwu	G3016-3
anti-DIG-HRP	Jackson	
DAB reagent	wanwu	G1211

2. The steps of the experiment

2.1 **Organization fixed**: Take out the organization, Wash clean,then Immediately put in the fixed fluid (DEPC) to fix 2-12h.

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 xylene, 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 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(20ug/ml) working solution to cover objectives and incubate at 37℃ 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-H_2O_2 , incubate in dark at room temperature for 15min. Wash slides in PBS(PH7.4) three times for 3 min each, with gentle agitation and protected from light.

2.7 **Pre-hybridization**: Add Pre-hybridization solution to each section and incubate for 1 h at 37℃.

2.8 **Hybridization**: Discard the pre-hybridization solution, add the probe hybridization solution ,concentration_____， and incubate the section in a humidity chamber and hybridize overnight at___℃.

2.9 **Washing**: Remove the hybridization solution. Wash sections with 2×SSC for 5 min at 37℃ ，

Wash sections in 1×SSC two times for 5 min each at 37℃, 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 **Blocking**: add blocking solution (Rabbit serum) to the section and incubate at room temperature for 30 min.

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

2.12 **DAB developing**: dry sections slightly, add fresh prepared DAB chromogenic reagent to marked tissue. Manage reaction time by observing in microscopy until positive expression appears brown-yellow. Then stop developing reaction by wash in running tap water.

2.13 Counterstain in nucleus with Hematoxylin staining solution for 3 min and wash in tap water. Treat with the differentiate solution for a few seconds, wash in running tap water. Back to blue by bluing solution, wash in running tap water.

2.14 Dehydrate successively in gradient ethanol of 75%, 85%, and 2 changes of pure ethanol, respectively, 6 min each. Clear in xylene for 6 min and mount with resin mounting medium.

2.15 Image the results using a brightfield fluorescence microscope. Store slides at RT.

3. Interpretation of the results

The nucleus stained with hematoxylin are blue, and the positive expression of DAB was brownish yellow. The results of in situ hybridization of mRNA were cytoplasmic positive and a few nuclear positive were normal. MicRNA and lncRNA were expressed differently. According to the expression of color depth.

Note: All reagents, instrument need RNase free.

Attached table 1 probe information.