冰冻切片 BCIP/NBT 显色原位杂交（CISH）实验报告

1.实验器材及试剂

1.1.实验器材

名称	厂家	型号
冰冻切片机	Thermo	Cryotome E
载玻片	Servicebio	
盖玻片	江苏世泰实验器材有限公司	10212432C
无酶离心管	Servicebio	EP-150-M
摇床(钟摆式)	Servicebio	TSY-B
涡旋混匀器	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
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

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	
OCT 包埋剂	Servicebio	G6059-110ML	
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	
蔗糖	Servicebio	G5031	
PBS	Servicebio	G0002	
20×SSC 洗脱液	Servicebio	G3016-4	
BSA	Servicebio	G5001	
蛋白酶 K	Servicebio	G1234	
核固红染液	Servicebio	G1035	

中性树胶	国药集团化学试剂有限公司	10004160
杂交缓冲液	Servicebio	G3016-3
Anti-DIG-AP	jackson	200-052-156
NBT/BCIP 显色剂	博士德	AR1023

2.冰冻切片 BCIP/NBT 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 4%多聚甲醛（DEPC 水配制）固定 12h 以上。

2.2.脱水：固定后组织放入 15%蔗糖溶液中 8h，后换 30%蔗糖溶液中过夜。

2.3.冰冻切片固定：冰冻切片室温晾干，置于 4%多聚甲醛（DEPC）固定 10min，于 PBS（PH7.4）中在脱色摇床上晃动洗涤 3 次，每次 5min。

2.4.消化：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____。纯水冲洗后 PBS 洗 3 次×5min。

2.5.预杂交：滴加预杂交液，37°C 孵育 1h。

2.6.杂交：倾去预杂交液，滴加含探针____杂交液,浓度____。恒温箱_____度杂交交过夜。

2.7.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。

2.8.滴加封闭液：滴加封闭血清__正常兔血清_____。室温 30min。

2.9.滴加鼠抗地高辛标记碱性磷酸酶（anti-DIG-AP）：倾去封闭液，滴加 anti-DIG-AP。37°C 孵育 50min，后 TBS 洗 4 次×5min。

2.10.BCIP/NBT 显色：滴加 BCIP/NBT 显色液，显微镜观察阳性。后纯水冲洗。

2.11.复染细胞核：滴加核固红染液，染核至合适程度后纯水冲洗。

2.12.封片：自然风干后，中性树胶封片。

2.13.显微镜检，图像采集分析。

3.冰冻切片 BCIP/NBT 显色原位杂交实验结果判读

核固红染细胞核为红色，BCIP/NBT 显出的阳性表达为蓝紫色。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

NBT/BCIP–DIG-ISH Protocol（Frozen Tissue）

1. Apparatus and Reagents

1.1 Apparatus

Name	Producer	Model
Frozen slicer	Thermo	Cryotome E
Glass slide	Servicebio	
Coverslip	Citotest	10212432C
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
Refrigerator	HAIER	BCD-192TGN
Microscopy	Nikon	NIKON ECLIPSE CI
Imaging system	Nikon	NIKON DS-U3
Incubator	LABOTERY	GSP-70
autoclave	PANASONIC	MLS-3751L-PC

1.2 Major reagents

Reagent	Manufacturer	Article Number
DEPC	Amresco	E174
4% of paraformaldehyde（DEPC water）	Servicebio	G1113
OCT embedding agent	Servicebio	G6059-110ML
Ethanol	SCRC	100092683
Xylene	SCRC	10023418
Sucrose	Servicebio	G5031
PBS	Servicebio	G0002
20×SSC solution	Servicebio	G3016-4
BSA	Servicebio	G5001
Proteinase K	Servicebio	G1234
Nuclear fast red dye	Servicebio	G1035

Glycerin-gelatin	Servicebio	G1402
Hybridization buffer	Servicebio	G3016-3
anti-DIG-AP	Jackson	200-052-156
NBT/BCIP reagent	Boster	AR1023

2. The Steps of the Experiment

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

2.2. **Tissue dehydration:** Transfer tissue to 15% sucrose solution for 8 h and were subsequently to a 30% sucrose solution overnight.

2.3. **Frozen section fixation:** The frozen sections were dried at room temperature, fixed in 4% paraformaldehyde (DEPC) for 10 min, and shaken on the shaker in PBS (pH 7.4) for three times, 5 min each.

2.4. **Digestion**: Mark the objective tissue with liquid blocker pen, according to the characteristics of different tissues and different indicators, add proteinase K (20 ug/ml) to cover tissues and incubate at 37℃ for____minutes. Washed with sterilized water and then washed three times in PBS for 5 min each time.

2.5. **Prehybridization:** add hybridization buffer onto specimen and incubate at 37℃ for 1h.

2.6. **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___℃.

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

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

2.9. **Add the Alkaline Phosphatase-conjugated IgG Fraction Monoclonal Mouse Anti-Digoxin Antibody**: (anti-DIG-AP): Remove the blocking solution and add anti-DIG-HRP. Incubate at 37 ℃ for 40 min, then Wash sections in TBS four times for 5 min each.

2.10. **NBT/BCIP developing**: dry sections slightly, add fresh prepared NBT/BCIP chromogenic reagent to marked tissue. Manage reaction time by observing in microscopy until positive expression appears shows blue-purple. Then stop developing reaction by wash in running tap water.

2.11. **Multicolor nucleus:** dripping with red dye, and then rinse with pure water.

2.12. **Mounting:** 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.

2.13. **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.