Manual non-radioactive RNA in situ hybridization

Materials:

1. 1x PBS[1]
2. 0.2 HCL[2]
3. PK- buffer[3]
4. proteinase K (Roche 3115828)
5. 4% PFA[4]
6. Hyb-Buffer[5]
7. DTT (1,4-Dithiothreitol) (Merch 11583786001)
8. Parafilm
9. SSC[6]
10. PBST[7]
11. 10% Sheep-Serum- inactivated (Equitech-bio SSA62) [8]
12. TMN buffer[9]
13. Levamisole 5mM (Merck 1359302-125MG)
14. Anti-DIG (AP) antibody (Merck 11093274910)
15. Glass slides and coverslips and mounting media (water based)
16. RNA probe
17. NBT (Merck- 11383213001)
18. BCIP (Merck 11383221001)

Method:

Manual non-radioactive RNA *in situ* hybridization

Tissue: fresh frozen tissue that are fixed in PFA, acetylated, and dehydrated [10]

Day 1				
Wash		PBS	5 min	RT
Denature	0.2 N	HCl	10 min	RT
Wash		PBS	2 x 5 min	RT

Deproteinate		PK-buffer + 5-10 µg/ml proteinase K[11]	2 x 10 min	RT
Wash		PBS	2 x 5 min	RT
Fix	4 %	PFA in PBS	15 min	RT
Wash		PBS	2 x 5 min	RT
Pre-hybridize		HyB-Buffer (add 1mg/ml DTT)	30 min	60 °C
Hybridize		HyB-Buffer + 0.5-2µg/ml probe (parafilmed)	o/n	60 °C
Day 2				
Wash	2x	SSC	15 min	60 °C
Wash	2x	SSC	5 min	RT
Wash	0.2x	SSC	2 x 30 min	60 °C
Wash	0.2x	SSC	2 min	RT
Wash		PBST (0.1% Tween 20 in PBS)	2 x 20 min	RT
Block	10 %	inactivated sheep serum (SS) in PBST	30 min	RT
Antibody	10 %	SS in PBST + anti-DIG (AP) antibody (1:1000)	90 min	RT
Wash		PBST	2 x 20 min	RT
Wash		TMN (pH 9.5; add 5mM Levamisole freshly)	2 x 5 min	RT
Stain		TMN + 1µl/ml NBT and 3.5µl/ml BCIP	> 120 min[12]	RT dark
Day 3				
Wash		PBS	2 x 5 min	RT
Mount		Hydro-Mount (cover slipped)	> 120 min	37 °C

Notes:

1. 1x PBS solution preparation-
   Phosphate-buffered saline (PBS) (cshlp.org)

2. 0.2HCL solution preparation- To make a 0.2M solution, slowly add 16.42mL of your stock 37% solution of HCLE to 250mL deionized H_2O. Adjust the volume of the solution to 1000mL with Deionized water.
   N.B. Always add the hydrochloric acid to the water, not the water to the hydrochloric acid!
   For other calculation you can use this calculator-
   Molarity Calculator & Normality Calculator for Acids & Bases | Sigma-Aldrich

3. PK (proteinase K) buffer-
   Proteinase K buffer (10X) (cshlp.org). After you prepare 10X dilute the solution to 1X.

4. 4% PFA solution preparation-
   Paraformaldehyde (PFA; 4%) (cshlp.org)

5. Hyb-buffer alternative to the commercially ready-made solutions:
   Hybridization buffer (A) (cshlp.org) + Denhardt's solution (100X) (cshlp.org)

6. SSC solution preparation- SSC (cshlp.org).

7. PBST solution preparation- PBST (cshlp.org)

8. To inactivate the sheep serum heating to 56°C for 30 minutes, then store -20°C

9. TMN buffer solution preparation protocol - TMN (Tris-MgCl2-NaCl buffer) (cshlp.org)

10. Check the Protocol for fixation, acetylation, and dehydration

11. The required Proteinase K concentration depends largely on the tissue type. By default, we use 5µl/100ml of proteinase K for E14.5 mouse embryo and 35-40 µl/100ml for adult mouse, monkey or human brain.

12. Be sure to visually inspect the slides from time to time for staining under the microscope.