

Whole Mount microRNA ISH Protocol (Really Short Version)

1. **Collect** embryos in ice-cold **saline (chick salt)**. Rinse several times with ice-cold saline.
2. Prep for fix: For HH 11 and below proceed to step 3.
 For HH 12-18, open extraembryonic and pericardial membrane.
 For HH 19+, remove extraembryonic and vitellin membranes; open pericardium.
3. **Fix** (HH3-18) flat with fresh ice-cold **4% paraformaldehyde (PFA)**. Change to fresh **PFA, O/N@4°C**
4. (HH 19+) place up to 30 embryos in 15 ml fresh **PFA**, mix, replace with fresh **PFA, O/N@4°C**
5. Transfer into **PBT** and trim; open cavities.
6. **Dehydrate** through a **graded MeOH series** and freeze in fresh 100% MeOH at -20 1h to 3 days.
7. **Rehydrate** through a **graded MeOH series** 5-15 minutes or until embryos sink. change to **PBT**.
8. **Digest** embryos: **Proteinase K** in a 20 ml volume as follows:

a. Days of incubation	HH stages	treatment
1	7 and below	none
2	8 to 13	10 min in 10 μ g/ml Proteinase K in PBS
3	14 to 18	20 min in 10 μ g/ml Proteinase K in PBS
4	19 to 24	20 min in 20 μ g/ml Proteinase K in PBS
9. Rinse 5 min with 20 ml of **PBT**, Post-fix For 20 min in **4% PFA with 0.2% gluteraldehyde**.
10. Rinse twice in **PBT** for 5 minutes each.
11. **Store** in **prehyb** at -20°C until ready to use for ISH:
12. **Prehybridization:** move to 1 ml of fresh **prehyb** and incubate at the annealing temperature for 2 hours.
13. **Hybridization:** Add 1 μ l of a 5 μ M DIG-labeled LNA **working stock** per ml of **prehyb** [5nM final concentration] and incubate at the annealing temperature overnight.
14. **Wash** 3x20 min with prewarmed **2x SSC with 0.1% CHAPS** at the annealing temperature.
15. Wash 3x20 min with prewarmed **0.2x SSC with 0.1% CHAPS** at the annealing temperature.
16. Rinse 2x10 min at room temperature with **KTBTw**.
17. Block embryos in 20% sheep serum in **KTBTw** for 2-3 hours or longer at 4°C.
18. Incubate with 1:4000 of anti-DIG-AP in 20% sheep serum in **KTBTw** overnight at 4°C.
19. Wash 5x1 hour in **KTBTw** on a shaker or nutator at room temperature. (May continue overnight at 4°C).
20. Rinse 2x15 minutes in fresh **NTMTw**.
21. Add **color reagent**, incubate in the dark at RT. React until optimized. May repeat steps 19-22.
22. Stop color reaction with 2 brief rinses in **KTBTw**, then **PBS**.

KTBTw:

50 mM Tris-HCl (pH 7.5)	10 ml of 1M stock
150 mM NaCl	6 ml of 5M stock
10 mM KCl	1 ml of 2M stock
1% Tween 20	10 ml of 20% stock
MilliQ water	173 ml
total	200 ml

NTMTw: (make fresh)

100 mM NaCl	2 ml of 5M
100 mM Tris (pH 9.5)	10 ml of 1M
50 mM MgCl ₂	2.5 ml of 2M
0.1% Tween 20	500 μ l of 20% stock
MilliQ water	84.5 ml
total	100 ml