Whole Mount microRNA ISH Protocol (Shorter Version)
Adapted from Nieto, Patel and Wilkinson (1996)

Embryo Processing

1. Embryo collection:
   Collect embryos in ice-cold saline (chick salt). Rinse several times with ice-cold saline.

2. Prepare embryos for fixation as follows:
   For HH (Hamburger & Hamilton) stages 11 and below proceed to step 3.
   For HH stages 12-18, open the extraembryonic and pericardial membrane.
   For HH stages 19 and above, remove the extraembryonic and vitellin membranes and open the pericardial membrane.

3. Fix embryos as follows:
   For HH stages 18 and below, spread embryos flat by gently adding fresh ice-cold 4% paraformaldehyde (PFA) drop wise. After all embryos have been flattened in PFA, carefully remove this saline/PFA mixture and add fresh PFA drop wise.

   For HH stages 19 and above, since membranes have been removed, place up to 30 embryos drop wise into a 15 ml conical tube of fresh PFA. After 5 minutes of gentle mixing, replace this solution with fresh PFA. Fix overnight at 4°C (or 2 hours at RT).

4. Trim embryos as follows:
   Transfer embryos into a clean 100 mm petri dish containing PBT and trim off excess membrane.

   For HH stages 15 and above, major cavities are poked. This may include any or all of the following: forebrain, hindbrain, eye, neural tube, and allantois.

5. Dehydrate embryos:
   Group embryos by stage and transfer into 6 well plate containing mesh baskets. Dehydrate through a graded MeOH series, by lifting mesh well insert into the next MeOH dilution. Typical time between changes is 5 minutes.

6. Freeze embryos:
   Transfer embryos into a scintillation vial containing 100% MeOH. Freeze in at least 10 ml of 100% MeOH at −20°C for at least 1 hour but less than a week.

7. Rehydrate embryos:
   Rehydrate embryos through a graded MeOH series in a scintillation vial. Time between changes is 5-15 minutes or until embryos sink. This is followed by a change in PBT.
8. Digest embryos:
   Using **Proteinase K** in a 20 ml volume as follows:

<table>
<thead>
<tr>
<th>Days of incubation</th>
<th>HH stages</th>
<th>treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7 and below</td>
<td>none</td>
</tr>
<tr>
<td>2</td>
<td>8 to 13</td>
<td>10 min in 10 µg/ml Proteinase K in PBS</td>
</tr>
<tr>
<td>3</td>
<td>14 to 18</td>
<td>20 min in 10 µg/ml Proteinase K in PBS</td>
</tr>
<tr>
<td>4</td>
<td>19 to 24</td>
<td>20 min in 20 µg/ml Proteinase K in PBS</td>
</tr>
</tbody>
</table>

9. Rinse once for 5 minutes with 20 ml of **PBT**.

10. Post-fixation of embryos:
   For 20 minute in at least 10 ml of 4% **PFA with 0.2% gluteraldehyde**.

11. Rinse twice in **PBT** for 5 minutes each.

12. Thoroughly remove **PBT** and add **prehyb** with sufficient volume to allow embryos to sink.
    Switch to fresh prehyb. Go to step 13 or (optional) store at –20°C until ready to use for ISH.

### Prehybridization and Hybridization

13. Prehybridation:
    Incubate embryos in **prehyb** at the annealing temperature for 2 hours.

14. Hybridization:
    Add 1 µl of a 5 µM DIG-labeled LNA **working stock** per ml of **prehyb** (5nM final concentration, see page 7 of 8 of the long protocol for the dilution directions) and incubate at the annealing temperature overnight.

### Post-hybridization Washes

15. Transfer embryos into 6 well plate containing mesh baskets.
    Wash 3x20 min with prewarmed 2x **SSC with 0.1% CHAPS** at the annealing temperature.

16. Wash 3x20 min with prewarmed 0.2x **SSC with 0.1% CHAPS** at the annealing temperature.

17. Rinse 2x10 min at room temperature with **KTBTw**.

### Antibody

18. Transfer embryos into a 24 well plate. Block embryos in 20% sheep serum in **KTBTw** for 2-3 hours or longer at 4°C.
19. Preabsorb the anti-DIG-AP Fab fragment (Roche cat # 11 093 274 910) with 3mg chick embryo powder/µl concentrated antibody in 0.5 ml 20% sheep serum in KTBTw.

20. Incubate with 1:4000 dilution of anti-DIG-AP Fab fragment in 20% sheep serum in KTBTw overnight at 4˚C.

21. Transfer back into 6 well baskets to allow for increased wash volumes. Wash 5x1 hour in KTBTw on a shaker or nutator at room temperature. (May continue overnight at 4˚C).

**Color Reaction**

22. Rinse 2x15 minutes in fresh NTMTw.

23. Add color reagent and incubate in the dark at room temperature. React until signal to background is optimized.

24. Stop color reaction with 2 brief rinses in KTBTw, then PBS.

**Photography**

25. Dehydrate embryos in a graded MEOH series. (optional)

26. Rehydrate embryos in a graded MEOH series, followed by a change in PBT for photography.

Solutions: see the full length protocol