

Whole Mount microRNA ISH Protocol (Full Version)

Adapted from Nieto, Patel and Wilkinson (1996)

microRNAs (miRNAs) are detected using DNA oligos in which locked nucleic acids (LNAs) have been substituted for some nucleotides. Compared to RNA probes, probes containing LNAs exhibit superior hybridization kinetics and enhanced biostability, dramatically enhanced affinity towards both DNA and RNA. LNA probes to detect miRNAs can be obtained from several commercial sources, including Exiqon S/A [<http://www.exiqon.com>]. LNA probes can be obtained 3' or 5' end-labeled with DIG from Exiqon (3' worked better for us) or unlabeled probe can be end labeled in the laboratory using the Roche DIG Oligonucleotide 3'-End 2nd Generation Labeling Kit (cat# 03 353 575 910). Details on probe labeling can be found in the solutions section of this protocol.

For the most part, hybridizations are conducted using standard protocols for detection of mRNAs. Following extensive testing and optimizations, we have identified the following as important to obtaining successful hybridizations:

1. DIG labeled LNA probes are used at a final concentration of 5nM and hybridized at 22°C below the calculated probe:RNA duplex melting temperature (1-2°C higher or lower usually doesn't make a difference and allows several probes to be run at one temperature). Successful hybridizations are obtained over a surprisingly narrow probe concentration window.
2. We observed better signal quality when Triton X-100 was replaced in all standard solutions with the same concentration of Tween-20.
3. Although overnight hybridizations are usually effective, longer hybridization times (up to 5 days) will give higher signal. For long hybridizations, wells or hybridization tubes should be tightly sealed to prevent evaporation.
4. Many signal patterns are not detectable during the first few hours of color reaction. Signal to noise can be maximized by performing the first color reaction until background color becomes just visible, then returning the embryos to an over night wash in KTBT, followed by a second color reaction and washing cycle. This can be repeated daily for several days. Prolonged washing in KTBT following the last staining reaction can sometimes reduce residual background. Dehydration through a methanol series will also help to remove background.
5. Although some in situ hybridization protocols indicate that embryos can be stored for up to several months in methanol, we have found that storage in methanol for longer than 7 days significantly reduces hybridization signal for detection of both mRNAs and miRNAs.
6. Embryos should not be allowed to cool while being transferred from hybridization solution to the first hot wash or between hot washes. All wash buffers for these steps should be prewarmed.

Embryo Collection

1. Collect embryos in ice cold **saline (chick salt)**. Rinse embryos several times with cold saline to remove yolk and red blood cells.
2. Under the microscope, using a pair of fine forceps:
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 vitelline membranes and open the pericardial membrane.
3. Using a wide mouth pipette, drop wise place embryos individually into a 12 well dish using the smallest volume possible.

For HH stages 18 and below: Spread embryos flat by gently adding fresh ice cold **4% paraformaldehyde (PFA)** drop wise. Flatten embryos using small volumes. This minimizes curling of extraembryonic membranes that obscure embryos during post-ISH visualization. After all embryos have been flattened in **PFA**, carefully remove this saline/PFA mixture and add fresh **PFA** drop wise to sufficiently cover the embryo yet minimize membrane curling.

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**.

4. Fix overnight at 4°C (or 2 hours at RT) in **4% PFA**.

(NOTE: It is recommended that the time from the first egg cracked to the last embryo fixed should not exceed 20 minutes. In other words, an embryo should be fixed within 20 minutes from the time it was cracked. All solutions should be kept ice cold throughout the entire process.)

5. Using a pair of blunt forceps, pick embryos up by their extraembryonic membranes and transfer into a clean 100 mm petri dish containing **PBT**. Trim off excess membrane using a scalpel.

(NOTE: Excess membrane, which is membrane more than twice the width of the embryo is removed to minimize the amount of tissue exposed to probe and maximize post hybridization wash efficiency.)

For HH stages 15 and above, major cavities are poked with fine sharp forceps to prevent trapping of *in situ* reagents, specifically probe and antibody. Presence and size of cavities is age dependent and may include any or all of the following: forebrain, hindbrain, eye, neural tube, and allantois. When possible, these are all poked on one side, leaving the other side pristine for photography.

For high throughput washes, trimmed embryos are separated by day of incubation into mesh well inserts in a 6 well plate containing **PBT**. (6 well plates with mesh well inserts consist of 24 mm Netwell Inserts from Corning, Inc., cat # 3479 that fit into a tissue culture plate.) These mesh wells eliminate pipetting during solution changes, which is time consuming and can damage embryos.

6. Rinse twice with **PBT**.
7. Dehydrate through a **graded MeOH series**, by lifting mesh well insert into the next MeOH dilution in the 6 well plate. Typical time between changes is 5 minutes. Embryos are removed from mesh wells using a blunt pair of forceps and transferred into a scintillation vial containing 100% MeOH.
8. Freeze in at least 10 ml of 100% MeOH for at least 1 hour but not more than a week at -20°C .

(NOTE: Minimizing time in MeOH is preferable. Some protocols state that embryos are stable stored in MeOH for months at -20°C , but our results indicate that signal falls off after 7 or more days in MeOH. We presently process embryos (steps 7-13) and then store in **prehyb** for up to 10 days only.)

9. 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**.
10. Transfer embryos by pouring from scintillation vial to 50 ml conical tube containing **PBS**. Remove **PBS** and digest embryos with **Proteinase K** in a 20 ml volume as follows:

<u>Days of incubation</u>	<u>HH stages</u>	<u>treatment</u>
1	7 and below	none
2	8 to 13	10 min in 10 $\mu\text{g/ml}$ Proteinase K in PBS
3	14 to 18	20 min in 10 $\mu\text{g/ml}$ Proteinase K in PBS
4	19 to 24	20 min in 20 $\mu\text{g/ml}$ Proteinase K in PBS

11. Rinse once for 5 minutes with 20 ml of **PBT** followed by a 20 minute fixation in at least 10 ml of **4% PFA** with 0.2% gluteraldehyde. Rinse twice in **PBT** for 5 minutes each.
12. Transfer embryos back to scintillation vials, thoroughly remove **PBT**, and add **prehyb** with sufficient volume to allow embryos to sink. Store at -20°C until ready to use for ISH (max 10 days).

Hybridization

13. Prehybridization and hybridization steps are carried out in a 24 well plate in a hybridization oven, or in small, capped vials (Fisher cat # 03-339-25B, 15x44mm 1 dram vial) in a shaking (preferred) water bath. Embryos are added to 1 ml of **prehyb** and incubated at **LNA probe** annealing temperature for at least 2 hours. (Annealing temperatures used for LNA ISH are 22°C below the melting temperature for the particular probe used.)
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 for dilution information) and incubate at the annealing temperature overnight.

15. Wash 3x20 minutes with prewarmed **2x SSC with 0.1% CHAPS** at the annealing temperature. If using plates, warm up a sufficient number of 6-well plates with mesh well inserts filled with the next wash solution. Move embryos into prewarmed wash solution. Mesh well inserts and 6 well plates are used for all subsequent wash steps to maximize wash volume and minimize embryo handling and damage for high-throughput screening.

(NOTE: Keeping the embryos warm, that is, transferring the embryos into wash solution that is at the annealing temperature reduces background.)

16. Wash 3x20 minutes with prewarmed **0.2x SSC with 0.1% CHAPS** at the annealing temperature.
17. Rinse 2x10 minutes with **KTBTw** at room temperature.

Antibody

18. Transfer embryos into cleaned 24 well plates to conserve reagents. Block embryos in 20% sheep serum in **KTBTw** for 2-3 hours or longer at 4°C.
19. During this blocking period, preabsorb the anti-DIG-AP Fab fragment (Roche cat # 11 093 274 910) with 3mg **chick embryo powder**/μl concentrated antibody in 0.5ml 20% sheep serum in **KTBTw**. Spin and use supernatant for antibody dilution in step 20 (antibody currently at 1:500 dilution).
20. Incubate with 1:4000 dilution of anti-DIG-AP Fab fragment (Roche cat # 11 093 274 910) in 20% sheep serum in **KTBTw** overnight at 4°C.
21. Transfer into 6-well plates with mesh well inserts for increased wash volumes. Wash in **KTBTw** on a shaker or nutator at room temperature 5x1 hour, (may continue overnight at 4°C).

Color Reaction

22. Rinse 2x15 minutes in fresh **NTMTw**. Longer times in **NTMTw** for stage 20 and above embryos may improve penetration of color reagents.
23. Add **color reagent** and incubate for 20 minutes in the dark at room temperature. After this point, light does not matter. Reaction can proceed at room temp (faster) or at 4°C (slower) for up to several days depending on signal and background.
24. Stop color reaction and decrease background with 2 rinses in **KTBTw**.

(NOTE: Color reaction may be stopped and started several times by repeating steps 21 through 23. React until signal to background is optimized. See note on page 1.)

25. After final rinses in **KTBTw**, stop color reaction using **PBS**.

(NOTE: Don't go directly to **PBS** from **NTMTw** or crystals may form on the embryos and in the solution, making photography difficult.)

Photography

26. Dehydrate embryos in a **graded MEOH** series (optional). Rinses in 100% MEOH can reduce some background.
27. Rehydrate embryos in a **graded MEOH** series, followed by a change in **PBT** for photography. Store embryos for several months at 4°C in **PBS** with 0.1% sodium azide.
28. On the dissecting scope, our embryos are photographed in a large depression slide (Columbia special watch glass, Thomas Scientific #9790R15) with both matte transmitted and direct (horizontal) illumination. Gastrula stage embryos are sometimes gently cover-slipped to flatten and photographed with direct vertical illumination on a white background.

Solutions and Reagents for microRNA ISH

Saline (Chick Salt):

123 mM NaCl consisting of 71.9 g NaCl per liter of milliQ water. Cool to 4°C before use.

PBS:

10x liquid concentrate (EMD Chemicals, OmniPur® 10X PBS Liquid Concentrate, cat # 6505) is diluted to 1x with DEPC treated water; cool to 4°C before use.

It can also be made: 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na₂HPO₄, and 0.2 g/L KH₂PO₄ OR 137mM NaCl, 2.7mM KCl, 10mM Phosphate buffer (pH7.3-7.5). If you make your own, sterilize by filtration and store at 4°C.

PBT:

1x PBS containing 0.1% Triton X-100. Add 0.5 ml of 20% Triton X-100 stock into 100 ml 1x PBS. (Triton as a 20% stock dissolves faster and is easier to pipette accurately.) 0.1% Tween20 can be substituted for 0.1% Triton X 100 with comparable results.

4% Paraformaldehyde (PFA):

In a 2 liter beaker, warm 750 mL of DEPC treated water to approximately 60°C. (Beaker should be warm to touch but not hot.) In a fume hood, add 40 g of PFA prills (Sigma Aldrich cat # 441244) and 2 NaOH pellets. Stir until PFA is in solution, add 100 mL of 10x PBS then add HCl dropwise until pH is 7.2. Bring up to 1 liter with DEPC treated water. Transfer to 50 mL conical tubes. Store in -20°C, thaw just before use (within 24 hours).

Graded MeOH Series:

25% MeOH: 25ml MeOH/75ml PBT
50% MeOH: 50ml MeOH/50ml PBS
75% MeOH: 75ml MeOH/25ml DEPC H₂O
100% MeOH: 100ml MEOH 2x.

Dehydration runs from low to high MeOH. Rehydration from high to low MeOH.

Proteinase K:

100 mg Proteinase K (ISC BioExpress cat # 0706)
10 ml DEPC treated water
Make 150 µl aliquots. Store at -20°C until just before use.

Prehybridization Solution (Prehyb):

<u>Final Concentration in Prehyb</u>	<u>Stock Concentration</u>
50% deionized formamide (ISC BioExpress cat # 0606)	25 ml
5x SSC	12.5 ml of 20x stock
2% blocking powder (Roche cat # 1 096 176)	1 g
0.1% Tween 20	250 µl of 20% stock
0.1% CHAPS (ISC BioExpress cat # 0465)	500 µl of 10% stock
50 µg/ml yeast RNA* (Calbiochem cat # 55714)	250 µl of 10 mg/ml stock
5 mM EDTA	500 µl of 0.5M pH 8.0 stock
50 µg/ml heparin (Sigma cat # H3400)	50 µl of 50 mg/ml stock
DEPC treated water	11 ml
<hr/>	
Total	50 ml

- Nieto **prehyb** protocol calls for 1 mg/mL tRNA, we substitute yeast RNA (Calbiochem cat# 55714) and it works well and is a lot cheaper.

LNA Probe Synthesis:

Locked Nucleic Acid probes ordered from Exiqon (Denmark www.exiqon.com) labelled with 3' DIG (if ordered unlabeled, end labelled with 3'DIG with Roche DIG Oligonucleotide 3'-End Labeling Kit 2nd Generation Labeling Kit: cat # 03 353 575 910.)

Working Stock:

LNA concentration as received from Exiqon is typically 25 µM, which is diluted to a **working stock** by taking 4 µl of 25 µM stock into 16 µl of **prehyb**. From this **working stock**, 1 µl is removed and added to each ml of **prehyb** for ISH with embryos. Annealing temperatures used for ISH are 22°C below the melting temperature for the particular probe used.

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
<hr/>	
total	200 ml

Chick embryo Powder

Homogenize day 3-5 chick embryos in a minimum volume of PBS. Add 4 volumes of ice-cold acetone, mix and ice for 30min. Spin at 10,000 g for 10 min; remove supernatant, wash pellet with icy acetone and re-spin. Remove supernatant and spread pellet out to air dry in a clean mortar. Grind dry pellet to a fine powder and store in an air-tight tube at 4°C.

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 µl of 20% stock
MilliQ water	84.5 ml
<hr/>	
total	100 ml

20x SSC:

3.0 M NaCl, 175 g/L
0.3 M Sodium Citrate, 88 g/L
adjust to pH 7.0 with 3 drops of conc HCl per liter

SSC with CHAPS:

2x SSC with 0.1% CHAPS:

2x SSC	10 ml of 20x SSC
0.1% CHAPS	1 ml of 10% CHAPS
distilled water	89 ml
<hr/>	
total	100 ml

0.2x SSC with 0.1% CHAPS:

0.2x SSC	1 ml 20x SSC
0.1% CHAPS	1 ml 10% CHAPS
distilled water	98 ml
<hr/>	
total	100 ml

Color Reagent:

3 µl NBT (ISC BioExpress cat # 0329) @ 75mg/ml in 70% Dimethylformamide (DMF)
2.3 µl BCIP (ISC BioExpress cat # 0885) @ 50 mg/ml in DMF
per 1 ml NTMTw (scale up as necessary)