

Whole Mount In Situ Hybridization Protocol for mRNA Detection

Adapted from Nieto, Patel & Wilkinson (1996)

Embryo Processing

1. Collect embryos in room temperature (RT) **123mM NaCl (chick salt)**. For young embryos, remove yolk gently with a hair tool if necessary.
2. For embryos up to HH stage 18, using a wide mouth pipette place up to half a dozen embryos into a 60mm dish using the smallest volume possible. Separate and spread embryos flat, then carefully removing saline. Gently add fresh **4% paraformaldehyde (PFA)** drop-wise directly onto each embryo. Remove PFA, and then add fresh **PFA** drop-wise to sufficiently cover the embryos. For older embryos, clean off extraembryonic regions (unless you wish to assay for gene expression in these membrane) and place embryos in PFA fixative.

NOTE: Fixing embryos flat minimizes curling of embryos and extraembryonic membranes that obscures embryo during post-ISH visualization.

3. Fix 2 hours at RT or overnight at 4°C.
4. Rinse twice with **PBT** to remove fixative. Trim away opaca region as desired. At this point we transfer embryos to a small screw top vial.
5. Dehydrate through a **graded MeOH series**, ending in two changes of 100% MeOH, 10 min each.
6. Freeze in at least 10 ml of 100% MeOH for at least 1 hour but less than a week at -20°C. We typically freeze embryos overnight.
7. Rehydrate embryos through a **graded MeOH series**. Change solutions every ten minutes for younger embryos, or for older embryos when they sink. Transfer embryos to **PBT**.
8. Digest embryos with **Proteinase K** in a 20 ml volume as follows:

<u>Days of incubation</u>	<u>HH stages</u>	<u>Proteinase treatment</u>
1	7 and younger	none
2	8 to 10	5 min in 10 µg/ml Proteinase K in PBS
2	11 to 12	10 min in 10 µg/ml Proteinase K in PBS
3	14 to 18	20 min in 10 µg/ml Proteinase K in PBS
4	19 to 24	20 min in 20 µg/ml Proteinase K in PBS

NOTE: Proteinase K activity varies from lot to lot. It is important to determine the best concentration/time parameters for your lot.

9. Rinse for 10 minutes in **PBT** followed by a 20 minute fixation in **4% PFA**. Rinse 2x10 minutes in **PBT**.

Hybridization

10. Remove PBT, add 1 ml of **prehyb** and allow embryos to sink. Replace with fresh prehyb and incubate at **65°C** for 2 hours.

NOTE: Embryos can be stored at -20°C prior to ISH, although signal intensity falls off with increasing time in storage. We recommend storing embryos for a week or less. Following storage, remove plates or vials from the freezer and warm to 65°C before continuing. Hybridization steps can be performed in a 24-well plate covered with foil (VWR #60941-074) in a hybridization oven, or in small capped vials (Fisher cat # 03-339-25B, 15x44mm 1 dram vial) in a water bath (shaking is not necessary).

11. Hybridization: Add 50-1000 ng antisense probe per ml of **prehyb** and incubate at 65°C for 48 hours.

NOTE: The optimal probe concentration will vary between probes. Higher concentrations are not necessarily better. To obtain the best results, run a probe dilution series to determine the optimal concentration for each probe.

NOTE: Lengthening the hybridization time beyond 48 hours will result in modest though progressively increasing signal intensity. We have hybridized for up to five days with excellent results.

12. Wash 3x20 minutes in **2x SSC with 0.1% CHAPS** that has been prewarmed to 65°C. Embryos can be transferred to 6-well plates for larger washing volumes.
13. Wash 3x20 minutes in **0.2x SSC with 0.1% CHAPS** at 65°C.
14. Rinse 2x10 minutes with **KTBT** at RT.

Antibody Detection of DIG

15. Block embryos in **KTBT** containing 20% sheep serum for 2 hours to overnight at 4°C.
16. Preabsorb the anti-DIG-AP Fab fragment (Roche cat # 11 093 274 910) by diluting 1:500 in 20% sheep serum, then adding 3mg **chick embryo powder** per 500ul of antibody solution. Incubate for 30 minutes at RT. Spin and remove supernatant for antibody incubation in the next step (presently at 1:500 dilution).
17. Incubate embryos overnight at 4°C in 1:2000 to 1:4000 final dilution of DIG antibody solution in **KTBT-20% sheep serum**.
18. Wash 5 x 1hr with **KTBT** on a shaker or nutator at RT, then overnight at 4°C.

Color Reaction d

19. Rinse 2 x15 minutes in fresh **NTMT**.
20. Add **color reagent** and incubate in the dark. Staining can proceed at RT (faster) or at 4°C (slower).
21. When hybridization signal is optimal, or when general background color begins to appear, stop the reaction by washing embryos in **KTBT**.

NOTE: Embryos can be washed overnight with **KTBT** and the staining reaction repeated, or staining can continue 4°C for extended time periods. Staining at 4°C or staining/washing/restaining be continued for a week or more if necessary. Many genes show highly variable expression levels in different cells. We frequently identify expression patterns that do not become visible until several rounds of staining and washing or prolonged standing at 4°C.

When the staining has proceeded to your satisfaction, permanently stop the reaction by washing embryos in **PBS**. The lower pH of the PBS will darken the reaction product and remove some of the background

NOTE: Avoid transferring embryos directly from **NTMT** to **PBS** as crystals will form on the embryos and in the solution.

22. Dehydrate embryos in a **graded MEOH** series. Rinse one to several times in 100% methanol to reduce background, then rehydrate to PBS. Embryos can be stored at 4°C in **PBS** with 0.1% sodium azide.

NOTE: Although this last step is not required, methanol darkens the reaction product to a deep purple and also helps to remove background staining. If embryos are still too dark, they can be partially destained in PBS containing 0.1% Triton X-100.

Solutions and Reagents for mRNA ISH

123mM Saline (Chick Salt):

123 mM NaCl consisting of 71.9 g NaCl per liter of milliQ water

PBT:

1x PBS containing 0.1% Triton X-100. Add 0.5 ml of 20% Triton X-100 stock into 100 ml 1x PBS.

4% Paraformaldehyde (PFA):

In fume hood, add 40grams of PFA prills to approximately 900mls PBS. Heat to 65°C with stirring until dissolved. If prills do not completely dissolve, add approximately 3 drops of 10N NaOH. Dilute to 1L with PBS. The pH should be 9.1-9.3. Aliquot and store at -20°C. Thaw and bring to room temp immediately before use.

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

Prepare 150 µl aliquots (10mg/ml). Store at -20°C until just before use.

10x NTP-DIG Mix:

Roche NTP set cat # 11277057001 20 µmol in 200 µl = 100mM

Roche DIG-11-UTP cat # 11209256910 250 nmol in 25ul = 10mM

7.14 µl of 100 mM = 10 mM rATP

7.14 µl of 100 mM = 10 mM rCTP

7.14 µl of 100 mM = 10 mM rGTP

4.64 µl of 100 mM = 6.5 mM rUTP

25.00 µl of 10 mM = 3.5 mM DIG-11-UTP

20.37 µl water

71.4 µl total

71.4 µl 10xNTP DIG mix (made this way to use all of the DIG-11-UTP in a single mix)

RNA Probe Synthesis:**Probe Preparation:**

To a microfuge tube containing 0.1–0.5 µg of plasmid template linearized at the 5' end of the cDNA, add:

- 2 µl 10x NTP-DIG mix
- 2 µl 10x Transcription Buffer
- 1 µl RNase Inhibitor (Roche cat # 3 335 399)
- water to 19ul
- 1 µl RNA Polymerase*

Incubate @ 37°C for 2 hours.

***RNA Polymerases:**

- T7 polymerase, Roche cat # 10 88 176 700
- T3 polymerase, Roche cat # 11 031 163 001
- SP6 polymerase, Roche cat # 108 102 74 001

NOTE: We often run a 40ul reaction.

Prehybridization Solution (Prehyb):**Final Concentration**

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% Triton X-100	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
water	11 ml
Total	50 ml

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

KTBT:

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% Triton X-100	10 ml of 20% stock
MilliQ water	173 ml
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 10,000x 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.

NTMT (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% Triton X-100	500 µl of 20% stock
MilliQ water	84.5 ml
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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 concentrated 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
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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
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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

1 ml NTMT (scale up as necessary)

This protocol was prepared by the GEISHA In Situ Hybridization Project (<http://geisha.arizona.edu>).

For questions or comments, contact:

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