

Whole Mount In Situ Hybridization Protocol for mRNA Detection

Adapted from Hervé, Wilkinson and Nieto (2008), and Streit and Stern (2001)

This protocol incorporates several recent modifications that have significantly improved hybridization results. These include:

1. Method of preparing paraformaldehyde fixative; using fixative within three days of preparation.
2. Adding 0.1% glutaraldehyde to the postfix following proteinase K treatment.
3. Careful attention to probe cleanup following synthesis to remove template DNA.
4. Denaturing probe before adding to hybridization solution.

Embryo Processing

1. Collect embryos in room temperature (RT) 123mM NaCl (chick salt) or calcium-magnesium free PBS. For young embryos, if necessary gently remove yolk using a hair tool. Minimize the time between embryo dissection and fixation.
2. For embryos up to HH stage 18, use a wide mouth pipette to place up to half a dozen embryos into a 60mm dish using the smallest volume possible. Separate and spread embryos flat, then carefully remove saline. Gently add fresh 4% paraformaldehyde-2mM EGTA (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 membranes) and place embryos in PFA fixative. To minimize trapping of reagents, the brain cavities can be opened with forceps. Fix for five hours at room temperature (RT) or overnight at 4°C.

NOTE: Fixing young embryos flat minimizes curling at later steps in the protocol. Use freshly prepared paraformaldehyde (see preparation instructions are at the end of this protocol).

3. Rinse twice with PBT to remove fixative. Trim embryos as desired. At this point we transfer embryos to a glass vial.
4. Transfer embryos directly to 100% MeOH followed by 2 quick changes of MeOH. Store embryos at 100% MeOH overnight. This step is necessary to improve probe penetration.

Note: Embryos can be stored in methanol at -20°C for up to several days, though it is preferable to process embryos through to the prehybridization step as soon as possible following the overnight methanol step. Longer storage in methanol results in reduced hybridization signal.

5. Rehydrate embryos through a graded MeOH series into PBT. Change solutions every ten minutes for younger embryos, or for older embryos when they sink. Wash 2x 10 min in fresh PBT.

Note: at this point older embryos can be bleached in 6% H₂O₂ for 30min-1hr to remove eye pigmentation, then washed 3x 10 min in PBT.

6. 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	6 and younger	0-15 min in 10 µg/ml Proteinase K in PBS
2	7 to 10	30 min in 10 µg/ml Proteinase K in PBS
2	11 to 12	30 min in 10 µg/ml Proteinase K in PBS
3	14 to 18	30 min in 10 µg/ml Proteinase K in PBS
4	19 to 24	30 min in 20 µg/ml Proteinase K in PBS

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

7. Rinse 2x 10 min in PBT followed by 30 min fixation in 4% PFA/2mMEGTA plus 0.1% glutaraldehyde. Rinse 2x briefly in PBT.

Hybridization

8. 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: Several protocols recommend prehybridizing and hybridizing at 70°C. Streit and Stern (2001) recommend 70°C for probes longer than 400nt, and 65°C for shorter probes, especially if they are A/T-rich.

NOTE: Embryos can be stored in prehyb at -20°C prior to ISH, although it is our experience that signal intensity falls off with prolonged storage times. 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).

9. Hybridization: Add 100-1000 ng antisense probe per ml of prehyb and incubate at 65°C for 48 hours.

NOTE: The optimal probe concentration will vary. 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.

10. Wash 3x 20 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.
11. Wash 3x 20 minutes in 0.2x SSC with 0.1% CHAPS at 65°C.
12. Rinse 2x 10 minutes with KTBT at RT.

Antibody Detection of DIG

13. Block embryos in KTBT containing 20% heat inactivated (55°C for 30 min) sheep serum for 3hrs at room temperature or overnight at 4°C.

Note: Some protocols add 1mg/ml BSA to the blocking solution, though we have not noticed a difference with or without.

14. 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 2 hours at RT while rocking. Spin and dilute supernatant to final concentration of 1:2000 to 1:4000 DIG AB solution/20% sheep serum in KTBT.
15. Incubate embryos in DIG AB solution 3hr at RT or overnight at 4°C.

16. Wash 5x 1hr with KTBT on a shaker or nutator at RT, then overnight at 4°C.

Color Reaction

Rinse 2x15 minutes in fresh NTMT.

17. Add color reagent and incubate in the dark. Staining can proceed at RT (faster) or at 4°C (slower).
18. 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 in KTBT and the staining reaction (in NTMT) repeated, or staining can continue 4°C for extended time periods. Staining at 4°C or staining/washing/restaining can be continued for a week or more if necessary. Many genes show highly variable expression levels in different cells/tissues. Detection of low expression levels can be missed if staining reactions are terminated when high expressing cells become visible. We frequently identify expression patterns that do not become visible until several rounds of staining and washing or prolonged staining 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.

NOTE: Additional background staining can be removed by dehydrating embryos through a graded MeOH series to 100% MeOH. This step darkens the reaction product to deep purple. Destaining with PBT can also reduce background signal.

Refixing in 4% PFA/CMF/EGTA following staining may increase tissue integrity when proceeding to embryo sectioning.

Solutions and Reagents

123mM Saline (Chick Salt):

123 mM NaCl consisting of 7.19 g NaCl per liter of sterile DI 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 (w/v), 2mM EGTA in CMF-PBS:

Dissolve the appropriate amount of paraformaldehyde in distilled H₂O at 65–70°C by continuous stirring and adjusting the pH to 7.2–7.4 with 1 N NaOH (usually 2–4 drops per 100 ml). Add the appropriate amount of 10X CMF–PBS and EGTA stock solutions and adjust the final volume. Cool down on ice before use. This solution can be used for 2–3 days if stored at 4°C in the dark.

Graded MeOH Series:

25% MeOH: 25ml MeOH/75ml PBT

50% MeOH: 50ml MeOH/50ml PBS

75% MeOH: 75ml MeOH/25ml 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

These volumes are used because the 10mM DIG-11UTP is sold in 25ul aliquots.

RNA Probe Synthesis:

I. Probe Preparation:

To a microfuge tube containing 0.1–0.5 µg of plasmid template linearized at the 5' end of the cDNA, or 200ng of PCR generated probe template, add:

4 µl 10x NTP-DIG mix

4 µl 10x Transcription Buffer

2 µl RNase Inhibitor (Roche cat # 3 335 399)

water to 38ul

4 µl RNA Polymerase*

Incubate @ 37°C for 3 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

II. DNase and Cleanup

1. At the end of the probe synthesis reaction, add 1ul ribonuclease-free DNase I (Invitrogen) and Incubate at 37°C for 20 min.

2. At this point 1ul can be removed to run on an agarose gel.

3. Add 5.0ul 4M LiCl plus 150ul 100% Ethanol. Mix, incubate at -20 overnight.

4. Spin at 13Krpm, 4°C for 15 min. Remove the EtOH, then add 300ul of ice-cold 70% EtOH.

5. Vortex 30 seconds to dislodge pellet. Spin at 7Krpm, 4°C for 5 min. Remove EtOH, then add 50ul cold 100% EtOH.
6. Spin at 7Krpm, 4°C for 2 min. Gently remove as much EtOH as possible. Evaporate off the remaining EtOH by incubating for 3 min at 42°C.
7. Add 20ul water, incubate at 42°C for 15 min, agitating gently every 5 min.
8. Incubate at 95°C for 3 min to denature the probe, then transfer directly to an ice bath for 5 min.
9. Remove 1ul for to assess probe concentration using a nanodrop.
10. Add the appropriate amount of probe directly to hybridization solution. Mix well.

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

* The Nieto prehyb protocol uses 1 mg/mL tRNA, we substitute yeast RNA (Calbiochem cat# 55714) because it works well for us and is less expensive.

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 ice-cold 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
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)

References:

Hervé A., Wilkinson, D.G., and Nieto, A.M. (2008) In situ hybridization analysis in whole mount and tissue sections. *Methods in Cell Biology* 87: 169-185

Streit, A., and Stern, C.D. (2001) Combined Whole-Mount and in situ hybridization and immunohistochemistry in avian embryos. *Methods* 23:339-344.

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

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