Preparation of Probe:

Note: Work has to be done using gloves and sterile tubes and buffers.

1. Prepare DNA:
   
   Linearize 5 µg of DNA by digesting with the appropriate restriction enzyme for 2h.
   Stop the reaction using first a mix of phenol/chloroform and then chloroform.
   Precipitate the DNA with Ethanol, centrifuge, and wash with RNAse free 70% Ethanol.
   Resuspend the DNA in 10 mM Tris and 1 mM EDTA.
   Test an aliquot on Agarose gel.

2. Synthesis of the antisense RNA probe. Incubate 2h at 37°C in transcription mix:

   **Transcription mix:**
   
   1 µg linearized DNA  
   Transcription buffer (T3 or T7 RNA polymerase) 4 µl  
   NTP DIG RNA (Boehringer) 2 µl  
   RNase inhibitor (35 units/µl) 1 µl  
   T3/T7 RNA polymerase (20 units/µl, Stratagene) 1 µl  
   Sterile water to 20 µl total

3. Digest the template DNA by adding 10 µl RNase free DNAse for 15 minutes at 37°C.

4. Stop the synthesis reaction and precipitate the RNA for 30 minutes with:
   
   1 µl EDTA 0.5M pH 8  
   2.5 µl LiCl 4M  
   75 µl Ethanol 100% at 70°C

5. Centrifuge at 4°C for 30 minutes at 12,000 rpm

6. Wash with 70% ethanol, dry and resuspend in 20 µl sterile DEPC water.

7. Test 1 µl on Agarose gel (generally 1 µl will be used for the hybridization).

Fixation and storage of embryos:

1. Remove chorions by pronase treatment (for embryos older than 18 somites) or manually (for earlier stages).

2. Fix embryos in 4% paraformaldehyde (PFA) in PBS overnight at 4°C.

3. Transfer embryos into 100% Methanol (MeOH), store them at 20°C (2h several months).

In situ Day 1:

Rehydration: Transfer embryos into small baskets and rehydrate by successive incubations in:

- 75% MeOH 25% PBS for 5 minutes
- 50% MeOH 50% PBS for 5 minutes
- 25% MeOH 75% PBS for 5 minutes
- 100% PBT (PBS/Tween20 0.1%) 4 x 5 minutes

Digest with Proteinase K (10 µg/ml):

- blastula and gastrula: 30 seconds
- early somitogenesis: 1 minute
- late somitogenesis (14 to 22 somites): 5 minutes
- 24 hpf embryos: 15 minutes
- 36/48 hpf embryos: 30 minutes

Refix in 4% PFA PBS, 20 minutes.

Wash in PBT, 5 x 5 minutes.

Preabsorb the anti DIG antibody (Boehringer) in a 1:1000 dilution in PBT sheep serum 2% BSA (2mg/ml) for several hours at room temperature with a batch of previously fixed embryos. Use about 500 embryos for 10 ml of antibody.
Prepare the Prehybridization and Hybridization mix:

**Prehyb and Hybridization mix (HM):**

- Formamide 50 65%
- 5 x SSC
- Tween20 0.1%
- Citric acid to pH 6.0 (460 µl of 1M for 50 ml)
- Heparin 50 µg/ml
- tRNA 500 µg/ml

**NOTE:** Add tRNA and Heparin to the prehybridation and hybridization only (not the wash solutions). Vary the % of formamide according to the desired stringency.

7. Prehybridize embryos in 800 µl of hybridization mix, 2 to 5 hours at 70°C.

8. Remove prehybridization mix, discard, and replace with 200 µl of hybridization mix containing 100 200 ng of antisense RNA probe. Hybridize overnight in a water bath at 70°C.

**In situ Day 2:**

**Washes:**

1. 100% HM at 70°C, very brief wash
2. 75% HM/25% 2 x SSC at 70°C, 15 minutes
3. 50% HM/50% 2 x SSC at 70°C, 15 minutes
4. 25% HM/75% 2 x SSC at 70°C, 15 minutes
5. 2 x SSC at 70°C, 15 minutes
6. 0.2 x SSC, 50% formamide (for normal stringency) or 0.05 x SSC, 65% formamide (for high stringency), 2 x 30 minutes
7. 75% 0.2 (or 0.05) x SSC/25% PBT at room temperature, 10 minutes
8. 50% 0.2 (or 0.05) x SSC/50% PBT at room temperature, 10 minutes
9. 25% 0.2 (or 0.05) x SSC/75% PBT at room temperature, 10 minutes
10. PBT at room temperature, 10 minutes
11. PBT/2% sheep serum/2mg/ml BSA at room temperature, several hours

**Incubation with anti DIG antiserum:**

Incubate in antibody solution overnight with agitation at 4°C.

**Anti DIG antibody solution:**

Preabsorbed anti DIG, 1:5000 dilution (final concentration) in PBT

2% sheep serum
2mg/ml BSA

**In situ Day 3:**

**Washes:**

Remove antiserum, discard, and then wash extensively:

- PBT at room temperature, very brief wash
- PBT at room temperature, 6 x 15 minutes
- Staining buffer (100 mM Tris HCl pH9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20), 3 x 5 minutes

**Staining:**

1. Incubate embryos in staining solution at room temperature and monitor with a dissecting microscope.

**Staining solution:**

NBT 50 mg/ml 225 µl
BCIP 50 mg/ml 175 µl
Staining buffer 50 ml

**NBT stock:**
50 mg Nitro Blue Tetrazolium in 0.7 ml of Dimethylformamide anhydrous + 0.3 ml H2O.

**BCIP stock:**

50 mg of 5 Bromo 4 Chloro3Indolyl Phosphate in 1 ml anhydrous Dimethylformamide

2. Stop the reaction by removing the staining solution and washing the embryos in:

**Stop solution:**

PBS pH5.5
EDTA 1mM

3. Store the embryos in stop solution at +4°C in the dark.

**Mounting**

1. For observation using a dissecting microscope, mount embryos directly in stop solution and methylcellulose.

2. For observation using a compound microscope, mount embryos in 100% glycerol.

3. For embryos at early development stage (up to 18h), dehydrate in 100% methanol, clear for a few minutes in methyl salicylate, and mount in Permount.

**Materials and supplies:**

PFA: paraformaldehyde (Sigma)
10 x PBS
MeOH: methanol
Tween20 (Sigma P1379)
Proteinase K (Boehringer 1000144)
Anti DIG antibody alkaline phosphatase Fab fragment (Boehringer 1 093 274)
BSA fraction V protease free (Sigma A 3294)
Formamide (deionized, high purity grade)
20 x SSC
Heparin at 5 mg/ml (Sigma H3393)
RNase free tRNA (Sigma R7876, 50 mg/ml resuspended in H2O and extensively extracted several times in Phenol/CHCl3 to remove protein)
Citric acid 1M
Normal Sheep serum (Jackson Immunoresearch 013 000 121)
Tris HCl pH9.5 1M
MgCl2 1M
NaCl 5M
NBT 50 mg/ml (made from powder, Sigma N6876)
BCIP 50 mg/ml (made from powder, Sigma B8503)
PBS pH5.5
EDTA 0.5M
Glycerol 100%
Methyl salicylate (Sigma M6752)
Permount (Fisher SP15 100)

This protocol is adapted from:


If there are any questions, comments, or suggestions to improve this protocol, please contact:

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