Whole-Mount *in situ* Hybridizations of Zebrafish Embryos

Reagents

1 M Tris-HCl, pH 7.5
SDS
Phenol/Chloroform/Isoamyl Alcohol (50:48:2)
Chloroform
3 M NaOAc, pH 5.2
Ethanol
RNA-free H_2O
Paraformaldehyde
10X PBS
Methanol
Tween 20
Proteinase K (Roche)
Formamide
Mixed Bed Ion Exchange Resin (for de-ionizing formamide; Sigma)
20X SSC
Heparin (Sigma H3393)
RNase-free tRNA
1 M Citric Acid
Boehringer Mannheim Blocking Reagent (BMB; Roche)
Normal Sheep Serum (heat inactivated at 56 °C for 30 minutes)
1 M Maleic Acid Buffer, pH 7.5
Anti-Digoxigenin Fab Fragment, Alkaline Phosphatase Conjugate (Roche)
1 M Tris-HCl, pH 9.5
1 M $MgCl_2$
5 M NaCl
NBT stock in DMF (Roche)
BCIP stock in DMF (Roche)
1 M sodium phosphate, dibasic
1 M sodium monophosphate, monobasic
0.5 M EDTA, pH 8
Methylcellulose
Glycerol
Methylsalycilate (Sigma M6752)
Permount (Fisher SP15-100)
Benzyl Alcohol/Benzyl Benzoate (2:1)

Procedure

DNA Linearization

1) Linearize 5 μg of DNA by digesting with the appropriate restriction enzyme for 2 hours.

1) Add an equal volume of 2X proteinase K buffer (200 mM Tris-HCl, pH 7.5, 25 mM EDTA, 200 mM NaCl, 2% SDS) and proteinase K to a final concentration of 10 ng/μL and incubate for 15 minutes at 37 °C.
1) Add one volume of phenol/chloroform/isoamyl alcohol and vortex mixture. Centrifuge at 14,000 rpm for 5 minutes at room temperature**.**
1) Transfer DNA-containing upper aqueous layer to a new microfuge tube. Add one volume of chloroform, vortex, and centrifuge at 14,000 rpm for 5 minutes at room temperature.
1) Transfer upper aqueous layer to a new microfuge rube. Add 0.1 volume of 3 M NaOAc, pH 5.2. Add 2.25 volumes (relative to aqueous layer plus 3 M NaOAc solution) of ethanol. Centrifuge at 14,000 rpm for 30 minutes at 4 °C.
1) Remove supernatant. Wash DNA pellet with 70% ethanol by centrifuging for 5 minutes at 4 °C and removing supernatant. Air dry pellet.
1) Resuspend pellet in 20 μL 10 mM Tris-HCl, pH 7.5.
1) Analyze an aliquot of this DNA solution on an agarose gel.

Digoxigenin-Labeled RNA Preparation

1) Incubate the following transcription mixture for 2 hours at 37 °C.

   1 μg linearized DNA
   T3, T7, or SP6 RNA polymerase transcription buffer (4 μL)
   NTP-DIG-RNA (Roche) (2 μL)
   RNase inhibitor (35 units/μL) (1 μL)
   T3, T7, or SP6 RNA polymerase (20 units/μL, Strategene) (1 μL)
   RNase-free H_2O to 20 μL total volume

1) Remove 1 μL of this completed reaction mixture for analysis on an agarose gel.
1) Digest the template DNA by adding 1 μL RNase-free DNase for 15 minutes at 37 °C.
1) Purify the RNA on a Centrisep spin column according to the manufacturer's instructions. Add RNase-free H_2O to a final volume of 100 μL. Typically 1-5 μL will be used for hybridization.

Fixation and storage of embryos

1) Remove chorions for embryos older than 18 somites.
1) Fix embryos in 4% paraformaldehyde in PBS overnight at 4 °C.
1) Dechorionate embryos that are younger than 18 somites.
1) Transfer embryos into 100% methanol and sore them at –20 °C (2 hours to several months).

In situ Day One

1) Rehydration. Transfer embryos into microfuge tubes or mesh-containing microplates and rehydrate them by successive incubations in the following solutions (room temperature, ~500 μL/tube):

75% MeOH/25% PBS for 5 minutes (no agitation)
50% MeOH/50% PBS for 5 minutes (no agitation)
25% MeOH/75% PBS for 5 minutes (no agitation)
100% PBT (PBS containing 0.1% Tween 20) for 4 x 5 minutes (rocking agitation)

2) Digest with Proteinase K (10 μg/mL in PBT, 500 μL/tube).

Less than 22 somites: none
24 h embryos: 5 minutes
36h/48h embryos: 15-30 minutes

3) Refix in 4% paraformaldehyde in PBS (if Proteinase K digestion was necessary) for 20 minutes at room temperature (500 μL/tube).
3) Wash embryos in PBT for 5 x 5 minutes (500 μL/tube).
3) Prepare the hydridization mix (HM) with the appropriate stringency (20 mL)

	50%	60%	65%
Formamide (freshly de-ionized)	10 mL	12 mL	13 mL
20X SSC	5 mL	5 mL	5 mL
20% Tween 20	100 μL	100 μL	100 μL
1 M Citric Acid	184 μL	184 μL	184 μL
5 mg/mL Heparin	200 μL	200 μL	200 μL
50 mg/mL tRNA	200 μL	200 μL	200 μL
H_2O	4.3 mL	2.3 mL	1.3 mL

3) Incubate embryos in 800 μL HM for 2 hours at 70 °C.
3) Remove HM and replace with 200 μL HM containing 100 – 200 ng of antisense digoxigenin-labeled RNA probe (typically 1-2 μL of stock). Hybridize overnight at 70 °C.

In situ Day Two

1) Washes at 70 °C without agitation (500 μL/tube):

100% HM at 70 °C, very brief wash
75% HM/25% 2X SSC for 15 minutes
50% HM/50% 2X SSC for 15 minutes
25% HM/75% 2X SSC for 15 minutes
2X SSC for 15 minutes
0.2X SSC (for normal stringency, 50% formamide) or 0.05X SSC (for high stringency, 65% formamide), for 2 x 30 minutes

1) Washes at room temperature with rocking agitation (500 μL/tube):

75% 0.2 (or 0.05)X SSC/25% PBT for 10 minutes

50% 0.2 (or 0.05)X SSC/50% PBT for 10 minutes
25% 0.2 (or 0.05)X SSC/75% PBT for 10 minutes
PBT for 10 minutes

2) Block in antibody blocking buffer (450 μL/tube; 20% sheep serum, 2% BMB, 150 mM NaCl, 100 mM maleic acid, pH 7.5) for 2 hours at room temperature with rocking agitation. This blocking solution is prepared by dissolving BMB in the maleic acid/NaCl solution with heating, then adding the sheep serum upon cooling.
3) Dilute the anti-digoxigenin antibody 1:500 into blocking buffer. Add 50 μL of this solution to each tube for a final dilution of 1:5000. Incubate overnight at 4 °C with rocking agitation.

In situ Day Three

1) Washes at room temperature with rocking agitation:

PBT, very brief wash
PBT for 6 x 15 minutes
Staining Buffer (100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20) for 3 x 5 minutes

2) Staining. Transfer the embryos to a 9-spot depression plate and remove as much staining buffer as possible. Replace with Staining Buffer containing NBT (2.25 μL of DMF stock/mL) and BCIP (3.5 μL of DMF stock/mL; 0.5 mL/spot). Incubate at room temperature or at 37 °C depending on the probe and expression level. Incubation times can be a few minutes or several hours depending on conditions.
3) When the desired staining is achieved, stop the reaction by removing the substrate-containing Staining Buffer and washing the embryos in Stop Solution (0.1 M PBS, pH 5.5, EDTA 1 mM; this can be made by mixing 0.418 mL 1 M dibasic, 9.58 mL 1 M monobasic, 0.2 mL 0.5 M EDTA, pH 8, and 89.8 mL H_2O).
4) Store the embryos in the 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 developmental stage (up to 18 hpf), dehydrate in 100% methanol (1 hour), clear for a few minutes in methylsalycilate, and mount in Permount.
4) Embryos can also be dehydrate in 100% methanol (1 hour), cleared in benzyl alcohol/benzyl benzoate (1:1, v/v; several minutes), and mounted in Permount.