Whole-Mount In Situ Hybridization for *Astyanax* embryos

Fixation

1. Fix embryos with 4% paraformaldehyde in PBS overnight at 4°C.

2. Wash twice in PBS, 5 minutes each, at room temperature.

3. Remove the embryos from their chorions using watchmaker forceps (easiest at this point).

4. Dehydrate embryos in an increasing methanol series in PBS and store at -20°C.

25% Meth in PBS for 5min

50% Meth in PBS for 5min

75% Meth in PBS for 5min

Three times in 100% Meth for 5min/each

Store embryos at -20°C

5. Rehydrate embryos in a decreasing methanol series in PBST

100% Meth in PBST for 5min

75% Meth in PBST for 5min

50% Meth in PBST for 5min

25% Meth in PBST for 5min

4 times in PBST for 5min/each

6. Fix the rehydrated embryos in 4% paraformaldehyde in PBST for 20min at room temperature.

7. Rinse twice in PBST for 5 minutes each.

Proteinase digestion and post-fixation

8. Digest the embryos with proteinase K (10 µg/ml in PBST) at room temperature for 5 to 12 minutes (depends on the stage; younger stages are more sensitive; depends also on the batch of enzyme).

9. Rinse briefly in PBST to dilute the proteinase, wash once with PBST for 5min.

10 Post fix for 20 min with 4% paraformaldehyde in PBST.

11, Wash 5 times with PBST (5 min each).

Prehybridization

12. Transfer the embryos into small glass bottles with approximately 300µl of HYB-.

13. Incubate 5 minutes at 60ºC without shaking; afterwards replace HYB- with 300µl of HYB+.

14. Prehybridize at 60ºC for 4h or overnight (over night preferred for best results) with gentle shaking, then store the embryos at -20°C.

Hybridization

15. Remove as much of the preHYB+ as possible without letting the embryos touch air (in fact, never let embryos contact air throughout the procedure), and replace with 1 ng/ul sense or antisense probe in HYB+. Heat the probe in HYB+ for 5 minutes at 68ºC before adding to the embryos.

16. Incubate at 60°C overnight with gentle shaking.

Probe removal

17. Wash embryos 2 times for 30 minutes each at 60°C in 50% formamide in 2xSSCT.

18. Rinse 15 minutes at 60°C in 2xSSCT.

19. Rinse 2 times for 30 minutes each at 60°C in 0.2xSSCT.

20. Transfer embryos to Eppendorf tubes and wash twice with MABT for 5 min each at room temperature.

Detection

21. Incubate the embryos with blocking solution (MABT, 2% blocking reagent) for 4 hours at room temperature with rocking.

22. Incubate the embryos with Anti-DIG-AP Fab fragments (1:5,000; Roche) in blocking solution overnight at 4 C with gentle rocking.

23. Wash the embryos once with MABT containing 10% sheep serum at room temperature for 25 min.

24. Wash the embryos eight more times (45–60 min each) with MABT at room temperature with gently shaking (the last time can wash overnight at 4C).

25. Wash the embryos with PBS and incubate in BM Purple AP Substrate (Roche) at room temperature in the dark.

26. Terminate the reaction by rinsing the embryos several times in PBS after the signal developed.

Clearing

Process embryos for clearing through an increasing glycerol series in PBS (30–50–80%) and image by microscopy.

Solutions:

10XPBS
80g NaCl
2g KCl
14.4g Na_2PO_4
2.4g KH_2PO_4
adjust to pH 7.4 with HCl and bring volume to 1 liter with water

PBST
1XPBS plus 0.1% Tween-20

SSCT: saline sodium citrate (SSG) plus 0.1% Tween

HYB* (50% formamide, 5xSSC , 0.1% Tween-20)

HYB+ (HYB* with 50 µg/ml heparin, 5 µg/ml torula (yeast) RNA,
(The RNA is precipitated and dissolved in DEPC-treated water. HYB* and HYB+ should be kept at -20°C)

50% formamide/2XSSCT (For 20ml)
formamide 10ml
20XSSC 2ml
10% Tween-20 0.2ml
DEPC-H_2O to 20ml

2XSSCT (for 10ml)
20XSSC 1ml
10% Tween-20 0.1ml
DEPC-H_2O 10ml

0.2XSSCT (for 20ml)
20XSSC 0.2ml
10% Tween-20 0.1ml
DEPC-H_2O to 20ml

MABT (150mM maleic acid, 100mM NaCl, PH7.5, 0.1% Tween-20)
(for 250ml)
Maleic acid 4.355g
Adjust PH with NaOH to 7.5
NaCl 1.461g
10% Tween-20 2.5ml
DEPC-H_2O to 250ml