Whole Mount In Situ Hybridization

Probe synthesis

1) **Enzyme digestion**

   Cut ~10μg of plasmid DNA with appropriate restriction enzyme (choose enzymes that create blunt end or 5'-overhang; 10U/μg DNA). Cut to completeness (>4 hours) to avoid rolling circle transcripts. Clean up RE reaction with PCR purification kit/Gel extraction kit (Qiagen). Dissolve in 30-50μl DEPC-dH_20.

2) **Probe synthesis**

   Mix following components and incubate at 37°C for 2-3 hours

	Final concentration
Linearized plasmid	~1μg
10x transcriptional buffer (Vortex well, to redissolve the precipitate of DTT)	1x
10x DIG RNA labeling mix	1x
10x RNA polymerase (T7, T3, SP6)	1x
RNase inhibitor	1μl
DEPC-dH_2O	
Total volume	20-30μl

   Add 5μl of DNase I (RNase free) and incubate at 37°C for 30~60 minutes.
   Stop reaction by add 1μl 0.5M EDTA pH 8.0
   Precipitate the RNA with 1/10 volume of 3M NaOAC, pH5.2, and 2.5x volume of cold 100% ethanol (or 1x volume of isopropanol) at -70°C for 30 minutes. Spin down the RNA pellet at 14000 rpm for 30 minutes, wash with 1 ml of cold 70% ethanol, dry pellet and dissolve RNA in 50 μl DEPC-dH_2O.

Preparation of embryos

1) Remove pigments: add 0.003% phenylthiocarbamide (PTU) between bud stage and 24hpf.
2) Fix embryos in fresh 4% PFA/PBS at 4°C for overnight (Store the fresh 4% PFA at 4°C, and it can be used in one week.).

Dehydrate with Methanol (MeOH) at **RT**

DEPC-PBST	5 min*4 times
25% MeOH /75% DEPC-PBST	5 min

50% MeOH /50% DEPC-PBST	5 min
75% MeOH /25% DEPC-PBST	5 min
100% MeOH (Change MeOH several times)	

Embryos are stored at –20°C for several months

***In situ* DAY 1:**

Rehydration:

75% MeOH/ 25% DEPC PBST	5 min
50% MeOH/ 50% DEPC PBST	5 min
25% MeOH/ 75% DEPC PBST	5 min
DEPC PBST	5 min*4~5 times

Proteinase K digestion (stock: 10 mg/ml) (dilute in DEPC PBST)

Stage	[PKμg/ml]	Time (min.)	PK stock vol.(μl/ml)	temperature
<24hr	--	--	--	RT(25℃)
24hr	10	12~15	1	RT(25℃)
36hr	15	25	1.5	RT(25℃)
48hr	25	25	2.5	RT(25℃)
60hr	25	30	2.5	RT(25℃)
72hr	50	25	5	RT(25℃)
84hr	50	30	5	RT(25℃)
96hr	100	30	10	RT(25℃)
>96hr	100	40	10	RT(25℃)

Wash with DEPC PBST for 5 min *2 times at RT

Re-fix embryos with 4% PFA/PBS for 20~30 min at RT

Wash with DEPC PBST for 5 min*5 times at RT

Incubate in Hyb-B Buffer for 5 min at 65°C.

Prehybridize in Hyb^+ Buffer at least for 2 hours at 65°C$^{\#}$.

Heat RNA probe in Hyb^+ Buffer for 5 minutes at 68°C and chill on ice.

Replace with 300 μl Hyb^+ Buffer containing 150ng RNA probe and incubate at 65°C overnight.

($^{\#}$hybridization temperature is varied due to different probe)

Preabsorption of the anti-DIG-alkaline phosphatase antibody

1) Making blocking solution- 2mg/ml BSA and 5% serum in PBST.
2) Embryos used for preabsorption have been gone through all treatments including

prehybridization in Hyb-B buffer at 65°C for 2 h and wash with PBST for 5 min*4 times at RT.

3) Preabsorption of the antibody- Add 1:400 (2.5μl) anti-DIG-AP in 1 ml blocking solution.
4) Incubate at 4°C with rotation for overnight.

DAY 2:

Remove probe (if necessary, it can be kept at -20°C for reuse)
Wash away the excess probe at **65°C**: (preheated solutions)

75% Hyb-B Buffer/ 25% 2x SSC	10 min
50% Hyb-B Buffer/ 50% 2x SSC	10 min
25% Hyb-B Buffer/ 75% 2x SSC	10 min
2x SSC	1 hour
0.2x SSC/0.1% Tween20 at **70°C**	30 min*4 times

75% 0.2x SSC/ 25%PBST	5 min	RT
50% 0.2x SSC/ 50%PBST	5 min	RT
25% 0.2x SSC/ 75%PBST	5 min	RT
PBST	5 min	RT

Incubate embryos in blocking solution for 2 hours (or 1 hour) at RT
Incubate embryos in blocking solution containing 1:10000 dilution[##] of anti-DIG-AP antibody for overnight at 4°C (or 3 hours at RT then quickly wash with PBST*3 times→4°C overnight.)
([##]antibody dilution is varied due to different probe and stage)

Note: For probes or early stage embryos having background problem, one can choose using anti-DIG-AP incubation at RT for 3 h method (This give less background staining).

DAY 3~ :

Remove antibody
Wash embryos with PBST for **15-20** minutes at least 8 times at RT
([###]remove PBST as clean as possible before adding staining buffer to prevent dark blue precipitate stuck on embryos)

Equilibrate embryos in freshly made staining buffer for 5min *3 times at **RT**

Stain embryos with 1ml staining buffer containing 3.5μl of 100 mg/ml NBT and 3.5μl of 50 mg/ml X-phosphate.

Staining buffer: 0.1M Tris-HCl pH **9.5**
50 mM $MgCl_2$
0.1M NaCl
0.1% tween 20

Stop reaction with quickly rinse in PBST for 5 min*3 times

Wash embryos with MeOH for 20 min*1~3 times at **RT**

Rinse with PBST 1~2 times

Re-fix embryos with 4% PFA/PBS for >20 min

Wash with PBST for several times

Put embryos in 70% glycerol and take pictures (Embryos can be stored at 4°C)

References:

Chen, J.-N. and Fishman, M.C. (1996). Zebrafish tinman homolg demarcates the heart field and initiates myocardial differentiation. Development 122: 3809-3816.

Thisse and Thisse (2008). High-resolution *in situ* hybridization to whole-mount zebrafish embryos. Nature Protocols 3: 59-69.