Two color in situ (mouse)

Day1

1. Move embryos through the wells

75%MeOH, 5min	50%MeOH, 5min	25%MeOH, 5 min	PBT, 5min	PBT, 5 min	Prot K
PBT, rinse	Post fix, 20min	PBT, 5min	PBT, 5min	Hyb:PBT 1:1, 5min	Hyb, 5 min

25%, 50%, 75% MeOH in PBT (PBS, 0.1% Tween20),
Pot K - 1 ug/ml (time depends on embryo age)
Post fix 4%PFA, 0.025% GA in PBS

Hyb. Buffer 50% FA
0.75M NaCl
1X PE
0.1% BSA
1% SDS
0.1% Tween20
0.1mg/ml tRNA

10X PE 10mM EDTA, 100mm PIPES

2. Block in Hyb buffer for 1h or longer @ 70C, add both probes, leave o/n @70C

Day2

3. Rinse in Hyb buffer, wash in Hyb buffer 2 times, 30 min @70C
4. Wash in Hyb:TBST (1:1), 30 min @55C
5. Set a plate

TBST, rinse	TBST, rinse	TBST, rinse, 15min	Buffer A, 1h	Buffer B, 1h	

Buffer A 2% BBR in TBST
Buffer B 2% BBR, 20% goat serum in TBST
TBST -1X TBS, 0.1% Tween 20
10XTBS -

6. Incubate with AP antibody (I did anti fluorescein first, 1:2000) in buffer B o/n

Day 3

7. Wash 3 times in TBST, each wash is 1h.
8. Wash once in NTMT buffer, 5min
9. Move embryos to NBT+BCIP in NTMT/PVA, watch for color to develop

NBT (Sigma, T# 4000) 75 mg/ml in DMF,
BCIP (Sigma, B-8503) 50mg/ml in DMF.
Mix 0.45ul of NBT and 3.5ul of BCIP in 0.5ml of 2XNTMT and 0.5ml 10%PVA

1X NTMT	2X NTMT
100mm Tris pH 9.5	200mM Tris, pH9.5
50mM $MgCl_2$	100mM $MgCl_2$
100mmNaCl	200mmNaCl
0.1% Tween20	0.2% Tween20

10. Wash embryos in TBST 2 times, 5 min
10. Fix embryos in 4% PFA for 20min,
11. Heat inactivate AP @65C for 30 min.
12. Rinse in Buffer B, block in Buffer B for 1h
13. Add anti DIG –AP antibody (Roche, 1:2000), incubate o/n @4C

Day 4

14. Wash in TBST 3 times, 1h each wash
15. Wash once in NTMT buffer, 5min
16. Move embryos to INT+BCIP in NTMT/PVA, watch for color to develop

INT (Sigma, I-8377), 55mg/ml in DMF)
Mix 4.5ul of INT and 3.5ul of BCIP with 0.5ml of 2XNTMT and 0.5ml 10%PVA (Sigma)