WISH

Tissue Collection:

1. Dissect embryos in cold RNAse free PBS.
2. Fix in 4% PFA in PBS for two hours at 4C.
3. Wash 3x5 in RNAse free PBST at RT.
4. Dehydrate for 5min through 25%, 50%, 75% in MeOH/PBST, and x2 in 100% MeOH at RT.
5. Store in 100% MeOH at -20C.

Day1

1. Re-hydrate through 75%, 50%, and 25% MeOH for 5min each at RT.
2. Wash 3x5 min in PBST at RT.
3. Bleach embryos for 1hr in fresh 6% H2O2 in PBST.
4. Wash 3x5 min in PBST at RT.
5. Incubate in 10µg/ml proteinase K in PBST at RT as follows:

E6.5	4 min
E7.5	5 min
E8.5	6 min
E9.5	7 min
E10.5	8-10 min

6. Wash in 2mg/ml glycine in PBST at RT for 5 min.
7. Wash 3x5 min in PBST at RT.
8. Fix in 4% PFA/0.2% glutaraldahyde in PBST for 20 min.
9. Wash 2x5 min in PBST at RT.
10. Separate embryos in to appropriate wells of a 12 well plate.
11. Wash in 1:1 PBST/hybe solution for 10 min.
12. Replace wash with RT hybe solution, place plate into a 70C oven, allow embryos to warm to 70C (~15 min), and incubate for 2 hrs at 70C.
13. Replace pre-hybe with 3ml hybe/probe at an absolute probe amount of ~75-150ng and incubate at 70C O/N while shaking gently.

Day2

1. Rinse embryos at 70C in pre-warmed Wash Solution I for 2 min.
2. Wash 2x30 min at 70C in pre-warmed Wash Solution I
3. Wash for 30 min at 65C in pre-warmed Wash Solution I
4. Wash 3x30 min at 65C in pre-warmed Wash Solution II
5. Let embryos cool to RT (about ~20 min)
6. Wash in 3ml of MABT for 3x5 min
7. Block embryos in 10% HISS/10%BBR (Roche 1096176) in MABT at RT for at least 90 min.
8. Pre-absorb antibody:

a) Add 0.5mg of embryo powder to 1ml 2%BBR/MABT and incubate at 70C for 30 min, vortexing briefly every five minutes.

b) Vortex for 3 min and cool on ice for 2 min.
c) To the embryo powder/MABT mix add 5µl of HISS and 1µl of anti-DIG AP-conjugate for **each well** of embryos you will have and nutate for at least 1hr at 4C
(e.g. 5 wells = 25µl HISS and 5µl Ab).
d) Quick spin to pellet embryo powder and remove the supernatant

9. Prepare antibody mix:

a) Add 2%BBR/MABT to a 15ml or 50ml tube so you have enough to fill each well to 2.5ml (e.g. 5 wells = 12.5ml).
b) To this add 20µl of HISS for every well (e.g. 5 wells = 100µl)
c) Finally, add the full volume of pre-absorbed antibody. This will result in a final [HISS] of 1% and an [AB] of 1:2500.

10. Replace blocking solution with 2.5ml/well of antibody mix and incubate O/N at 4C on a nutator.

Day3

1. Wash embryos 3x5 with 3ml of MABT at RT
2. Wash all day in MABT at RT changing the MABT every hour or so
3. Wash O/N with MABT at 4C
4. Do another day of washes if necessary

Day4

1. Wash 3x10 min in AP buffer/1% Levamasole at RT
2. Incubate embryos in staining solution at RT in the dark until desired signal appears
3. Stop reaction with 2mM EDTA for at least 5 min.
4. Fix in 4% PFA/0.1% glutaraldehyde in PBST for 1hr at RT or O/N at 4C
5. Wash 3x5 min in 2mM EDTA/PBST
6. Clear embryos in 80% glycerol/PBST and store at 4C

Solutions:

Stop Buffer
1% SDS
20mM EDTA
20mM Tris pH 7.5
100mM NaCl

PFA (80ml):
4% PFA (10ml 32% Electron Microscopy Sciences paraformaldehyde)
70ml PBS

PFA (40ml):
4% PFA(10ml 16% Electron Microscopy Sciences paraformaldehyde)
30ml PBS

10X PBS (1L):
80g NaCl
2g KCl

6.1g Na_2HPO_4 (dibasic,anhydrous)
2g KH_2PO_4
800ml dH_2O
pH 7.4 via NaOH or HCl
DEPC treat and autoclave

PBST (1L):
100ml 10X PBS
10ml 10% Triton X-100
bring to volume via DEPC H_2O

1M Triethanolamine pH 8.0 (500ml):
300ml DEPC dH_2O
66.5ml triethanolamine (sigma T1377 à stock is 7.5M)
pH 8.0 via HCl
final volume 500ml via DEPC treated dH2O

0.1M Triethanolamine pH 8.0 / 0.25 % acetic anhydride(50ml):
45ml DEPC dH2O
5ml 1M triethanolamine pH 8.0
125µl acetic anhydride

Hybridization Buffer (500ml)
250ml formamide
0.1% SDS (2.5ml of 20%)
89ml DEPC H_2O
50µg/ml Yeast tRNA (0.5ml 50mg/ml)
125ml 20X SSC
50µg/ml Heparin (0.5ml 50mg/ml)
60mM Citric Acid (30ml 1M Citric Acid)
0.1% Tween 20 (0.5ml 100%)

20X SSC (1L):
3M NaCl (175.3g)
0.3M $C_6H_5Na_3O_7 \cdot 2H_2O$ (dihydrous sodium citrate) (88.23g)
pH 7.0 via HCl
Final volume 1L autoclaved dH_2O
DO NOT AUTOCLAVE

5X MABT (1L):
58g Maleic Acid
43.5g NaCl
pH 7.5 with Tris Base (need ~100g)
final volume 1L via autoclaved dH2O

Alkaline Phosphatase Buffer (100ml):
100mM Tris pH 9.5 (10ml of 1M)
50mM $MgCl_2$ (5ml of 1M)
100mM NaCl (2ml of 5M)
0.1% Tween 20 (1ml of 10%)

Wash Solution I (500ml):
250ml formamide
25ml 20% SDS
70ml DEPC H_2O
125ml 20X SSC
30ml 1M Citric Acid

Wash Solution II (500ml):
250ml formamide
5ml 20% SDS
182.5ml DEPC H_2O
50ml 20X SSC
12ml 1M Citric Acid
0.5ml Tween 20

12 well plates are from EMS (cat# 71549-08)