Version 4

# Protocol for Whole-mount *in situ* Hybridization

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## I. Tissue Preparation

1. Dissect embryos in chilled PBS.
2. Fix embryos in an adequate amount (> 50 vol.) of **fresh** fixative (4% paraformaldehyde in PBS) with rocking for 2-12 hr at 4°C.
3. Wash with PBS containing 0.1% Tween-20 (PBT). Dehydrate embryos through ascending series of methanol (50-75-100-100%) and store at -20°C.

## II. Pretreatment & Hybridization

1. Bleach the embryos in 5:1 methanol/30% hydrogen peroxide for 1 hour at R/T (embryos can be stored again in methanol at -20°C). Rehydrate the embryos through graded series of methanol/PBT (75-50%) at R/T. Then wash through several changes of PBT.
2. Treat samples with 10 $\mu$g/ml proteinase K in PBT for 10 min at R/T.
3. Wash **gently** but quickly with PBT twice.
4. Fix the embryos in **fresh** 0.2% glutaraldehyde/4% paraformaldehyde in PBS at R/T for 20 min.
5. Wash embryos three times with PBT.
6. Transfer embryos into tubes for hybridization with 1:1 mixture of hybridization buffer and PBT. Incubate until samples sink to the bottom.
7. Equilibrate once with hybridization buffer, and prehybridize for at least 1 hour at 65°C.
8. Replace hybridization buffer, and add briefly heated (at 65°C for 1 min) probe solution to 0.2-2 $\mu$g/ml. Hybridize overnight at 63-65°C.

## III. Washing & Probe Detection

1. Wash twice with prewarmed Wash 1 for 30 min each with rocking.
2. Wash twice with prewarmed Wash 2 for 30 min each at 50°C with rocking.
3. Wash once with RNase buffer. Treat with 100 $\mu$g/ml RNase A (and with 100 U/ml RNase T1) in RNase buffer for 1 hr at 37°C. Wash once with RNase buffer.

4. Wash with Wash 3 for 30 min at 50°C with gentle rocking.
5. Wash with Wash 4 for 30 min at 50°C with gentle rocking.
6. Wash twice with Wash 5, then place embryos in a heating block at 70°C for 20 min.
7. Wash embryos twice in MABT.
8. Incubate the samples with blocking solution (2% BBR, 10% heat-inactivated serum in MABT) for at least 1 hour at R/T.
9. Incubate samples with anti-Dig Fab (1/2000-5000), or anti-fluorescein antibodies (1/1000-2000) for O/N at 4°C.
10. Wash 5-8 times with MABT for 1 hour each at R/T with rocking.
11. Soak samples with freshly prepared NTMT containing 2mM levamisole (0.5 mg/ml **freshly added**) for 10-20 min at R/T.
12. Start reaction with color reagents (4.5 $\mu$l/ml NBT, 3.5 $\mu$l/ml BCIP and 2 mM levamisole in NTMT). Rock the tubes for the first 5 min of reaction, then transfer to a small dish, and incubate at R/T **without** rocking.
13. Stop the reaction by washing twice with PBT containing 1mM EDTA to prevent further alkaline phosphatase activity. Postfix the embryos with 4% paraformaldehyde/0.1% glutaraldehyde in PBS for 1 hr, wash several times in PBT.
14. Dehydrate samples (50-70-100-100% methanol) to intensify signals and remove excess background. Rehydrate samples to PBT. When necessary, clear the specimens through 50-80-100% glycerol/PBT. Store the specimens at 4°C.

# Appendix

## I. Solutions

1. Hybridization Buffer
   - 50% formamide
   - 500mM NaCl
   - 1x PE
   - 100 $\mu$g/ml tRNA
   - 0.5 mg/ml heparin
   - 0.5% CHAPS
   - 0.1% Tween 20
2. Washing Buffers

   Wash 1
   - 300mM NaCl
   - 1x PE
   - 0.1% Tween 20

   Wash 2
   - 50mM NaCl
   - 1x PE
   - 0.1% Tween 20

   Wash 3
   - 50% formamide
   - 300mM NaCl
   - 1x PE
   - 0.1% Tween 20

   Wash 4
   - 50% formamide
   - 150mM NaCl
   - 1x PE
   - 0.1% Tween 20

   Wash 5
   - 500mM NaCl
   - 1x PE
   - 0.1% Tween 20

| | |
|---|---|
| 3. NTMT | 100mM Tris-Cl (pH 9.5) |
| | 100mM NaCl |
| | 50mM $MgCl_2$ |
| | 0.1% Tween 20 |
| 4. RNase Buffer | 10 mM PIPES (pH 7.2) |
| | 500mM NaCl |
| | 0.1% Tween-20 |

## II. Probe Preparation

1. Mix the following reagents:

| Reagent | Volume |
|---|---|
| Linear template (1 μg/μl) | 1 μl |
| Distilled water (**NON-DEPC-treated**) | 3.5 μl |
| 5x Transcription buffer | 4 μl |
| 100 mM DTT | 2 μl |
| 10mM ATP, GTP, CTP | 2 μl each |
| 10mM UTP | 1.2 μl |
| 10mM Dig-UTP | 0.8 μl |
| RNase inhibitor (40 U/μl) | 0.5 μl |
| RNA polymerase (40 U/μl) | 1 μl |
| Total | 20 μl |

2. Incubate for 2 hr at 37°C with occasional spin down.
3. Take 1 μl of an aliquot, mix with loading buffer, heat breafly at 65°C, and check on a 1% agarose/TAE gel.
4. Add 80 μl of stop buffer (20mM Tris pH7.5, 20mM EDTA, 100mM NaCl, 1% SDS).
5. Purify probes through Sephadex G-50 column (DEPC-treated, washed and autoclaved in 0.3M NaOAc/0.1% SDS). Prepack RNase-free columns are available.
6. Add 1/10 vol. of 3M NaOAc (pH5.2) and 2 vol. of EtOH, chill at -80°C. Precipitate transcripts.
7. Dissolve in **DEPC**-water, heat for a second at 65°C, add formamide to 50%, and store at -80°C.

## IV. Reagents

Distilled water (autoclaved, filtered through nitro cellulose with 0.2 $\mu$m)

DEPC-treated water

*Make 10% DEPC in EtOH, mix vigorously with distilled water, and leave overnight at R/T. Autoclave thoroughly until it doesn't smell.

5M NaCl

1M $MgCl_2$

0.5M EDTA (pH8.0)

10 mg/ml yeast tRNA

10% Boehringer Blocking Reagent

Heat inactivated serum (70°C, 20 min. Any kinds)

10% Tween-20

10% CHAPS

200 mg/ml heparin (x400)

2M Tris-Cl pH 9.5

1 mg/ml Proteinase K (in PBS)

20 mg/ml RNase A (in TE8.0)

10x PE (100mM PIPES pH 6.0, 10mM EDTA)

10x PBS (80 g NaCl, 2 g KCl, 11.5 g $Na_2HPO_4$, 2 g $KH_2PO_4$/ 1 l)

5x MAB (pH7.5) (29.0 g Maleic Acid, 21.75 g NaCl/500 ml)

*Adjust pH before adding NaCl.

75 mg/ml NBT (nitroblue tetrazolium salt) in 70% dimethylformamide

50 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate, **toluidine salt**) in 100% dimethylformamide