

in situ hybridization

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* Reaction is carried out at RT, unless described otherwise

A. Fixation

Fix embryos with 4% paraformaldehyde (PFA) in PBS O/N at 4 °C.

- if > 15 somites, dechorionate prior to fixation.

Wash with methanol (MeOH) for 15 min.

Replace with MeOH, store them at -20°C (2h to several months).

B. Rehydration

Wash with 75% MeOH in PBST for 5 min.

Wash with 50% MeOH in PBST for 5 min.

Wash with 25% MeOH in PBST for 5 min.

Wash 2x with PBST for 5 min each.

C. Permeabilization

* Permeabilization is recommended for gastrula stage embryos because it helps prevent the yolk membrane from rupturing.

Wash in DEPC-H₂O for 5 min.

Incubate in pre-chilled acetone at – 20 °C for 7 min.

Wash in DEPC-H₂O for 5 min.

Wash 2x in PBST for 5 min each.

Wash in PBST for 5 min.

D. Proteinase Digestion and Post-fixation

Incubate embryos in Proteinase K (working: 10 µg/ml; stock: 20 mg/ml; 1:2,000 dilution of stock) for the following time depending on the stage:

1 cell to high:

1 min

30% epiboly to 10 somite:	2 - 3 min
10 - 20 somite:	3 - 4 min
24 - 32 hpf:	5 – 6 min
40 - 50 hpf:	10 – 15 min

Wash 2x shortly in 2 mg/ml glycine (pH 2.2) in PBST to kill Proteinase K.

Post-fix with 750 μ l of 4% PFA in PBS for 20 min.

Wash 3x in PBST for 5 min each.

E. Prehybridization

Wash embryos with Hybridization Buffer (HB) briefly.

Replace with fresh HB and incubate on rocker at 65 °C for at least 2 hr.

F. Hybridization

Heat DIG-labeled riboprobe in HB (0.5 – 5.0 ng/ μ l) to 80 °C for 5 min and then place on ice.

Replace prehybridization buffer with 500 μ l riboprobe in HB and incubate on rocker at 70 °C O/N.

G. Wash

Remove DIG-labeled probe and store at - 20 °C for later use.

Preheat 50% Formamide/2x SSC-Tween, 2x SSC-Tween and 0.2x SSC Tween in 65 °C oven.

Wash 2x in 50% Formamide/2x SSC-Tween at 65 °C for 5 min.

Wash in 2x SSC Tween at 65 °C for 15 min.

Wash 2x in 0.2x SSC Tween at 65 °C for 30 min.

H. Antibody Application

Block in 2% Roche blocking reagent for at least 1 hr on orbital shaker.

Replace with anti-DIG-AP in 2% Roche blocking reagent (diluted 1:2,000) and incubate at RT for 2 hr (or at 4 °C O/N).

Wash briefly in PBST at RT.

Wash in PBST for 5 min.

Wash 2x in PBST for 20 min.

Transfer embryos to 24-well plate

Wash in PBST at 4 °C O/N.

I. Alkaline Phosphatase Staining

Wash 2x in PBST for 10 min.

Prepare fresh NTMT, enough for 2.25 ml per sample.

Wash 2x in NTMT (pH 9.5) on orbital shaker for 5 min.

Prepare alkaline phosphatase substrate solution as described below. Note that ratio could be adjusted depending on the abundance of mRNA.

BM Purple: NTMT (pH 9.5) = 1 : 7

Replace with AP substrate solution.

Incubate on orbital shaker in dark at RT until staining is satisfactory. In case of low abundant mRNA, it could take days.

Wash 2x in PBST for 5 min.

Wash in PBST at 4 °C O/N.

Post-fix in 4% PFA in PBS at 4 °C O/N.

Store stained embryos in fix at 4 °C, if need be.

Stock Solutions

PBST (PBS + 0.1% Tween 20)

10x PBS	100 ml
Tween 20	1 ml
DEPC-H ₂ O	900 ml

NTMT

	Stock	Final	Total 7 ml	Total 20 ml	Total 50 ml
NaCl	5 M	100 mM	140 µl	400 µl	1 ml
Tris (pH 9.5)	1 M	100 mM	700 µl	2 ml	5 ml
MgCl ₂	2 M	50 mM	175 µl	500 µl	1.25 ml
Tween-20		0.1%	7 µl	20 µl	50 µl
H ₂ O			6 ml	17 ml	42.7

Hybridization Buffer (HB)

	Stock	Final	Total 500 ml
Formamide		50%	250 ml
20x SSC, pH 7.0, RNase free		5x	125 ml
Yeast tRNA		5 mg/ml	2.5 g
Heparin	50 mg/ml	50 µg/ml	500 µl
Tween 20		0.1%	500 µl
DEPC-H ₂ O			124 ml

Blocking Solution

	Stock	Final	Total 500 ml
PBS	10x	1x	50 ml
Tween 20		0.1%	0.5 ml
Blocking Reagent (Roche)		2%	10 g
H ₂ O			450 ml

- Heat to 60 C while stirring until Blocking Reagent is mostly dissolved. Adjusting pH to 7 – 8 with NaOH expedites this process.
- Cool solution and aliquot into 50 ml Falcon tubes.
- Store at – 20 °C.

50% Formamide/2x-SSC-Tween

	Stock	Final	Total 100 ml
Formamide			50 ml
20x SSC		2x	10 ml
Tween 20			100 µl
DEPC-H ₂ O			40 ml

2x SSC-Tween

	Stock	Final	Total 500 ml
20x SSC		2x	50 ml
Tween 20			0.5 ml
DEPC-H ₂ O			450 ml

0.2x SSC-Tween

	Stock	Final	Total 500 ml
20x SSC		0.2x	5 ml
Tween 20			0.5 ml
DEPC-H ₂ O			495 ml

Chemicals

BM Purple (AP substrate precipitating) (Roche : #11442074001)

Anti-Digoxigenin-AP (antibody) (Roche : #11093274910)

Yeast tRNA (Sigma: #R-3629)

Blocking Reagent (50 g): Roche 11096176001