Embryo Whole Mount *in situ* Hybridization with Digoxigenin-labeled RNA Probes Followed by Tyramide Signal Amplification™ (Perkin Elmer NEL701A – Fluorescein)

For the detection of transcripts that cannot be observed directly with fluorescently conjugated probes.

Fixation

Embryos fixed via standard methanol or methanol/formaldehyde fixation protocol. Embryos stored in methanol at –20˚ C.

Hybridization

1. Rehydrate embryos through methanol series: 90% MeOH, 75% MeOH, 50% MeOH and finally in PBT. Rehydrate in PBT for 20 min at room temperature with gentle rocking.
2. Thaw Fixative 10 min at 70˚ C. Rinse embryos in 1 ml PBT.
3. Post-fix embryos in 1:1 PBT: Fixative (250 µl PBT + 250 µl Fixative) for 15 min with gentle rocking.
4. Rinse 4× with PBT.
5. Incubate 1 min in 1 ml PBT + 2.5 µl Proteinase K (~0.005 mg/ml). Rock vials at room temperature for 30 sec and let the embryos settle ~30 sec. Stop immediately by proceeding to step 6.
6. Rinse 2× with PBT.
7. Post-fix in 1:1 PBT:Fixative for 15 min with gentle rocking.
8. Rinse 4× with PBT.
9. Rinse with embryos with 1:1 PBT:Hyb-B Buffer (250µl PBT: 250µl Hyb-B); rinse with 250 µl Hyb-B.
10. Prehybridize embryos in 250 µl Hyb-A Buffer for 1 hour at 60˚ C.
11. Heat 1 µl (~100 ng) RNA probe/sample at 80˚ C for 10 min and place on ice. Add 30 µl/sample Hyb-A Buffer.
12. Remove as much of the Hyb-A Buffer from the embryos as possible. Add probe + Hyb-A solution (~31 µl) to the embryos.
13. Hybridize overnight at 60˚ C.
14. Add 500 µl room temperature Hyb-B Buffer. Incubate embryos at 60˚ C for 15 min while they settle.
15. Exchange Hyb-B Buffer 2 times, incubate embryos at 60˚ C for 15 min each while they settle.
16. Dilute at room temperature by adding 500 µl PBT.
17. Rinse 3× with 1 ml PBT. Wash 20 min with PBT. Wash 30 min with TNB Buffer.

TSA™ (Tyramide Signal Amplification) Protocol

18. Incubate with gentle agitation for 2 hours at room temperature with Anti-DIG POD antibody 1:100 recommended starting dilution in TNB Buffer (It may be necessary to optimize the antibody concentration.)

19. Wash 3× for 5 min each in PBST at room temperature with agitation.

20. Dilute Tyramide Stock Solution 1:50 in 1× Amplification Diluent. Pipet 200 µl of this working solution to each sample. Incubate at room temperature for 10 min (can be incubated up to 30 min - 20 min works well for most applications). (Optional) Samples may be simultaneously stained with TOTO3. (0.15 µl TOTO3 (100 µM stock); 0.2 µl RNase ONE/ 200 µl sample). Incubate 20 min at room temperature with gentle rocking.

21. Wash 3× for 5 min each in PBST Buffer at room temperature with gentle rocking.

22. Mount embryos with mounting media (9:1, Glycerol:10× PBS + p-phenylenediamine dihydrochloride).

Solutions and Reagents

PBT

Reagent	Quantity
10X PBS	50 ml
Tween 20	500 µl

- Adjust volume to 500 ml with dH_2O
- Add 0.5 ml DEPC/500 ml, shake; place in the hood overnight with the cap loosened; autoclave (Solution will appear cloudy until it cools)

10× PBS

Reagent	Quantity	10× Concentration
NaCl	74.8 g	1.28 M
KCl	1.5 g	0.02 M
KH_2PO_4 (MW136.1)	2.7 g	0.08 M
$Na_2HPO_4 \bullet 7H_2O$ (MW 268.07)	21.4 g	0.02 M

- pH ~7.2-7.4
- Adjust volume to 1 L with dH_2O
- Add 1 ml DEPC/1 L, shake; place in the hood overnight with the cap loosened; autoclave

20X SSC

Reagent	Quantity
NaCl	87.65 g
Sodium Citrate	44.1 g

- pH to 7.0, Adjust volume to 500 ml with dH_2O.
- Add 0.5 ml DEPC/500 ml, shake; place in the hood overnight with the cap loosened; autoclave

Fixative

Reagent	Quantity	Concentration
16% Paraformaldehyde	10 ml	10%
10× PBS	1.6 ml	1×
0.5M EGTA	1.6 ml	50 mM
dH_2O DEPC	2.8 ml	

- Store Solution at -20˚ C

Hybridization Buffer A (Hyb-A)

Reagent	Quantity (10 ml)	Concentration
Formamide	5 ml	50%
20× SSC	2.5 ml	5×
DNA from Salmon testes (10 mg/ml) Heat denatured (boiled for 10 min)	0.2 ml	0.2 mg/ml
tRNA (20 mg/ml)	50 µl	0.1 mg/ml
Heparin (100 mg/ml)	5 µl	0.05 mg/ml
dH_2O DEPC	2.245 ml	

- Aliquot and store at –20˚ C.

Hybridization Buffer B (Hyb-B)

Reagent	Quantity (10 ml)	Concentration
Formamide	5 ml	50%
20× SSC	2.5 ml	5×
dH_2O DEPC	2.5 ml	

- Prepare fresh

PBST

Reagent	Quantity	Concentration
10× PBS	5 ml	1×
Triton X-100 (or 10% Triton X-100)	25 µl (250 µl 10% Triton X-100)	0.05%

- Adjust volume to 50 ml with dH_2O.

TNB

Reagent	Quantity (10 ml)	Concentration
Tris-HCl, pH 7.5 1M	1 ml	0.1 M
NaCl 5M	0.3 ml	0.15 M
Blocking Reagent (provided in TSA kit)	0.05 g	0.5%
dH_2O DEPC	8.7 ml	

Mounting Medium w/1,4-Phenylenediamine dihydrochloride

Reagent	Quantity
p-Phenylenediamine Dihydrochloride	100 mg
10× PBS	10 ml
Glycerol	90 ml

- Store in aliquots at -20°C (discard when solution appears dark purple/brown)

Reagent	Vendor	Catalog Number
Paraformaldehyde 16% EM Grade (10 X 10 ml)	Electron Microscopy Sciences	15710
DNA from Salmon Testes for Hybridization (10 mg/ml) Heat denatured- boiled 10 min. store –20˚C	Sigma	D9156
tRNA	Roche	109541
Heparin (100 mg/ml –20˚C)	Sigma	H3393
Formamide	Sigma	F7503
Proteinase K (solution ~15-20 mg/ml)	Roche	1373196
Diethyl Pyrocarbonate (DEPC)	Sigma	D5758
Tween 20®	Bio-Rad	170-6531
Anti-DIG POD	Roche	11207733910
TSA™Fluorescein System	Perkin Elmer	NEL701A
10% Triton X-100	Roche	1332481
1,4-Phenylenediamine dihydrochloride	Sigma	P1519

Sources:

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