

Whole-mount in situ hybridization on zebrafish embryos

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Synthesis of DIG-labelled RNA probes (DIG-RNA) by in vitro transcription.

In order to prevent RNase contamination and contact with harmful chemicals, work must be done using gloves, sterile tubes and solutions with caution.

1. Preparing the DNA template from cDNA clones:

Linearize 10 μ g of DNA for each probe by digesting with the appropriate restriction enzyme in a total volume of 100 μ l. Take 1 μ l to test on 1% agarose gel.

Purify the DNA template from the digestion mix with Phenol:Chlorform (1:1) extraction and Ethanol precipitation. Dissolve the DNA in 20 μ l DEPC-water, take 1 μ l to test on 1% agarose gel

Determine the concentration of the template on a spectrometer.

2. Transcription reaction: follow the suggestions of the supplies, an example of using Promega system is given below.

| | |
|---------------------------------------|-------------|
| linearized DNA template | 1 μ g |
| Transcription buffer (5X) | 4 μ L |
| DTT (100 mM) | 2 μ l |
| DIG-RNA Labeling Mix (Roche 10X) | 2 μ L |
| RNase inhibitor (40 units/ μ L) | 0.5 μ L |
| RNA polymerase (10-20 units/ μ L) | 1 μ L |
| DEPC-water to total | 20 μ L |

Incubate the mixture for 1 hour at 37^oC. Take 1 μ L to test on a clean 1.3% agarose gel.

Add 1 μ L Dnase1 (Rnase free) to the rest of the mix and incubate in 37^oC for 15 minutes.

3. Purification of the DIG-RNA probes:

| | |
|------------------------------|----------------------|
| add 0,5M EDTA (pH 8.0) | 1 μ L (Optional) |
| add 4M LiCl | 2.5 μ L |
| add pre-chilled 100% Ethanol | 75 μ L |

Mix, and incubate at -80^oC for 30 minutes (Never incubate for more than 1 hour)

Centrifuge at 13000 G for 15 minutes at 4^oC.

Discard the ethanol and wash the pellet twice with 70% ethanol (pre-chilled), air dry and dissolve in 100 μ L pre-hybridization buffer.

Preparation of the zebrafish embryos.

Sort the embryos at the stages required for the experiment. If necessary, remove chorions manually with needle.

Fix the embryos in 4% paraformaldehyde (PFA) in 1xPBS overnight at 4^oC.

Rinse with PBST several times, then dehydrate the embryos as follow:

- 25% MeOH / 75% PBST for 5 minutes
- 50% MeOH / 50% PBST for 5 minutes

- 75% MeOH / 25% PBST for 5 minutes
- 100% MeOH 2x5 minutes

Transfer the embryos to Methanol and store them at -20°C until use. The embryos can be stored this way for up to 1 year.

Whole mount in situ hybridization.

Day 1

1. Rehydrate the embryos by successive washes as follow:

75% MeOH / 25% PBST for 5 minutes
50% MeOH / 50% PBST for 5 minutes
25% MeOH / 75% PBST for 5 minutes
100% PBST 2x 10 minutes

2. Permeabilization

This step is not necessary for zebrafish embryos younger than 24 hpf. For older embryos proteinase K digestion is needed to make them permeable for probe. The conditions (concentration, duration and temperature) of proteinase K treatment are crucial for the final result of in situ and need to be optimized for different batch of Proteinase K. Usually, 3dpf embryos need to be digested in 20 µg / ml Proteinase K solution (In PBST) for 18-22 minutes. After the proteinase K treatment, the embryos are post-fixed in 4% PFA for 30 minutes, followed by two 10 minutes PBST wash.

3. Prehybridization

Incubate embryos in pre-hybridization⁺ buffer at 68 °C with gentle shaking for 2 hours.

4. Hybridization

Replace hybridization buffer with probe (pre-hybridization⁺ buffer containing DIG-labelled antisense RNA of appropriate concentration) and incubate at 68°C overnight with gentle shaking

Day 2

5. Post-hybridization washes:

- Remove probe and collect for re-use
- Wash 2 X 20 min in 2 X SSCT/50% Formamide (pre-warm) at 68°C(shake gently)
- Wash 1 X 20 min in 2 X SSCT/25% Formamide (pre-warm) at 68°C(shake gently)
- Wash 2 X 20 min in 2 X SSCT (pre-warm) at 68°C(shake gently)
- Wash 3 X 30 min in 0.2 X SSCT (pre-warm) at 68°C (shake gently)
- Wash 2 X 5 min in PBST at RT (shake gently)

6. Antibody incubation

-Blockking: incubate embryos in blocking buffer (5 % lamb/goat serum diluted in PBST) for two hours with gentle shaking at RT

-Incubate in anti-DIG-AP antibody (1:5000 dilution in blocking buffer) overnight with gentle shaking at 4°C

Day 3

7. Signal detection

-Replace antibody (collect for re-use) with PBST and wash briefly at RT

-Wash 2 X 10 min in 0.5% lamb serum (diluted in PBST) at RT

-Wash 3 X 30 min in 0.5% lamb serum (diluted in PBST) at RT

-Wash 3 X 10 min in Buffer 9.5 T

-Stain in dark in NBT/BCIP staining solution until signal becomes satisfactory (temperature and duration may vary upon different requirement)

-wash 2 X 5 min in PBST

-wash 15 min in 4% PFA

-Wash 2 X 5 min in PBST and then mount in 80% glycerol to take image.

It is recommended to take image with a few hours of mounting in glycerol. After taking image, the embryos can be stored in fridge for future reference. However, they usually become unsuitable for taking high quality image after long time storage.

Recipes (all solutions are prepared with sterile RO water).

1x PP
10x PBS (DEPC treated)
0,1% Tween 20

Hybridization solution (Hyb+):
50% Formamide
5x SSC
0.1% Tween 20
Adjust the pH to 6,0 with citric acid
50 μ g/mL Heparin
1 mg/mL tRNA

2xSSCT:
1:10 of 20xSSC
0,1% Tween 20

0.2xSSCT:
1:100 of 20xSSC
0,1% Tween 20

Blocking solution:
5% lamb serum diluted in in PBST

Buffer 9.5 T:
100 mM Tris HCl pH 9,5
100 mM NaCl
0.1% Tween 20

Staining solution:
To make 10 ml, dissolve, 50 μ l NBT solution (Roche) and 37.5 μ l BCIP solution (Roche) in Buffer 9.5T
Note: prepare fresh just before use.