In situ Hybridisation protocol for Trochophore and Veliger larvae of the Mediterranean mussel *Mytilus galloprovincialis*

I. Sample preparation and fixation method.

1. Retrieve the larvae at the desired developmental stage from your culture vessel using a 40 µm mesh filter;
2. Gently collect larvae with a 1ml plastic pipette and transfer them to a 1.5ml collection tube with 50 µL PFA 4% in PBS1X/ASW ;
3. Gently centrifuge the tube (trochophore stages) or wait for the larvae to pellet in the bottom of the tube (Veligers);
4. Remove the supernatant and add 1ml of PFA 4% in PBS1X/ASW;
5. Fix overnight at 4°C or for 1h at RT;
6. Remove the excess of fixative with 3 washes in PBS-Tween (0.1%);
7. Remove the supernatant, only the larvae should remain in the tube;
8. Transfer the larvae in a new 1.5ml collection tube previously filled with 100% methanol;
9. Once the larvae have settled, remove the supernatant and replace it with new methanol 100%;
10. Preserve at -20°C.

II. Rehydration and Hybridisation

1. Rehydrate the larvae serially with PBS1X/ASW 25%, 50%, 75% and finally 100%;
2. Perform an extra wash in PBS-Tween (0.1%)
3. Incubate for 25’ at 27°C (trochophores) and 37°C (Veligers) in Proteinase K (10ug/ml) in PBS1X;
4. Remove the Proteinase K with two washes in PBS 1X;
5. In order to make the trochophore stages more transparent, wash with 0.1 M glycine in PBS for 5 minutes and then wash twice with PBS-Tween (0.1%);
6. Fix the larvae in PFA 4% in PBS1X for 40 minutes at RT;
7. Remove the fixative with three washes in PBS-Tween (0.1%);

8. Remove the supernatant and transfer the larvae in a new 1.5mL collection tube previously filled with the Hybridisation Buffer[a];
9. Let the tubes at RT for 15 minutes;
10. Aliquot the larvae in the multi-well and pre-hybridize at 65 °C for at least 2 hours;
11. Add the probes (diluted in the Hybridisation buffer) and let hybridise at 65 °C for at least 18 hours.

III. Probes' removal and anti-DIG antibody incubation

1. Remove the probes with the hybridisation buffer and preserve them in a new tube (probes can be used up to 3 times);
2. At 65°C: Wash the larvae with 2 X 30 minutes washes in Solution 1[b];
3. At 65°C: Wash the larvae for 30 minutes with Solution 2[c];
4. At 65°C: Wash the larvae for 30 minutes with Solution 3[d];
5. At 65°C: Wash the larvae for 30 minutes with Solution 4[e];
6. Wash the larvae in PBS-Tween (0.1%) if trochophores or PBS-Triton 1% if Veligers at RT;
7. Incubate the larvae with the Blocking buffer[f] for 1 hour at RT;
8. Incubate the larvae with anti-DIG antibody (1:4'000 in the blocking buffer) overnight at 4°C;
9. Remove the excess of antibody with 5 X 1 hour washes in TBS-Tween (0.1%)
10. Let the larvae in TBS-Tween (0.1%) overnight at 4°C.

IV. Revelation with NBT/BCIP

1. Wash one last time the larvae in TBS-Tween (0.1%);
2. Incubate the larvae with the NBT/BCIP revelation buffer[g] for 10 minutes at RT;
3. Prepare a solution with NBT and BCIP in their buffer and add it to the larvae (for 1 mL of Revelation Buffer: 3.5 µL BCIP 50 mg/mL, 6.75 µL NBT 50 µg/mL);
4. Let the signal reveal at a constant temperature (16-25°C, the lower is the temperature the lower is the risk to obtain unspecific signal around the shell margins), resuspend the solution every 30 minutes or place the larvae on a shaker and protect from light;
5. Renew the solution every 3 hours in the case of Veligers;
6. Block the reaction with 3 X 5 minutes washes in TBS-Tween (0.1%) and EDTA 50mM;

7. Perform other 5/6 washes in TBS-Tween (0.1%) and let overnight in PBS-Tween (0.1%) at 4°C. Extra washes and incubation time in PBS-Tween will help in obtaining a good quality of signal by making the larvae more transparent and the color more evident;
8. Soak the larvae in glycerol 80% in PBS1X and let them overnight at 4°C;
9. Let the larvae for 10/20 minutes at RT prior to mounting the slide and/or imaging.

V. Solutions

a. **Hybridisation buffer**: 50% Formamide (25 mL), 6X SSC (15 mL 20X), 5X Denhards (2.5mL 100X), 1mg/ml yeast RNA/tRNA (2.5 mL 20mg/mL), 0.1% Tween 20 (500µL, Tween 10%), to 50 mL with H_2O DEPC.

b. **Solution 1**: Solution 50% formamide (25mL), 5XSSC (12.5mL of 20X), 1%SDS** (2.5mL of 20%) to 50mls with H_2O DEPC.

c. Solution 2: 50% Formamide (25mL), 2XSSC (5mL of 20X), 1%SDS** (5mL of 10%) to 50mL with H_2O DEPC.

d. Solution 3: 2XSSC (5mL of 20X), 0.1% tween 20 (500µL, Tween 10%) to 50mls with H_2O DEPC.

e. Solution 4: 0.2XSSC (0.5mL of 20X), 0.1% tween 20 (250µL, Tween 10%) to 50mls with H_2O DEPC.

f. Blocking buffer for trochophores: 0.1M Tris (5mL of 1M; pH 7.5); 0.15M NaCl (1.5mL of 5M); 1% BBR (5mL BBR 10%), 0.1% tween 20 (500µL, Tween 10%), to 50 mL with H_2O DEPC.

g. Blocking buffer for Veligers: 0.1M Tris (5mL of 1M; pH 7.5); 0.15M NaCl (1.5mL of 5M); 0.5% BBR (2.5mL BBR 10%), 1% Triton-X (500µL), to 50 mL with H_2O DEPC.

h. NBT/BCIP revelation buffer: 0.1M Tris (5mL Tris 1M, pH: 9.5), 50mM $MgCl_2$ (2.5mL $MgCl_2$ 1M), 100mM NaCl(1mL NaCl 5M), 0.1% tween 20 (500µL, Tween 10%), to 50 mL with H_2O DEPC.