

Multiplexed mRNA *in-situ* Hybridization Chain Reaction (HCR) in whole-mount *Mytilus galloprovincialis* embryos

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– PROTOCOL –

Day 1 – Sample preparation

This protocol refers to samples that have been already fixed and stored in pure methanol at -20 °C. Mind to protect samples from light if they are marked with MitoTracker.

Check if the detergent solution need to be stirred and heated to ~40 °C to dissolve SDS.

1. Wash samples for 4 × 5 min with 1 mL of **PBST** at RT.

5 µL of embryo suspension collected from the pellet should be enough.

2. Incubate samples for 10 min with 1 mL of **PBST** at RT.
3. Permeabilize samples for 30 min with 500 µL of **detergent solution** at RT.

Pre-heat probe-hybridization buffer to 37 °C (you will need 400 µL per tube [200 µL for the pre-hybridization, and 200 µL for the probe solution]).

4. Wash samples for 2 × 5 min with 1 mL of **PBST** at RT.

Day 1 – HCR detection stage

1. Pre-hybridize samples for 30 min with 200 µL of **pre-warmed probe hybridization buffer** at 37 °C.

If reusing probes, pre-warm probe solution to 37 °C, then skip to Step 3.

At this step, samples can be stored in probe hybridization buffer at -20 °C for several months.

2. Prepare probe solution by adding 0.8 µL (0.8 pmol) of each **probe set 1 µM stock solution** to 200 µL of **probe hybridization buffer** (final concentration of 4 nM) at 37 °C.

Note: if using 500 µL of probe solution, add 2 µL (2 pmol) of each probe set.

3. Incubate samples for >12 h (overnight) with 200 µL of **pre-warmed probe solution** at 37 °C.

Pre-heat probe wash buffer at 37 °C (you will need 500 µL per tube per 4 washes).

Day 2 – HCR amplification stage

Pre-equilibrate amplification buffer to room temperature (you will need 600 µL per tube [500 µL for pre-amplification, and 100 µL for the hairpin solution]).

1. Wash samples for 4 × 20 min with 500 µL of **probe wash buffer** at 37 °C.

Probe solution can be saved and reused for 2–3 times; store at –20 °C.

2. Wash samples for 3 × 5 min with 500 µL (or 1 mL) of **5× SSCT** at RT.
3. Pre-amplify samples for 30 min with 500 µL of **amplification buffer** at RT.
4. Snap cool in separate tubes 2 µL of **hairpin H1** (15 pmol) and 2 µL of **hairpin H2** (15 pmol) **3 µM stock solutions**: heat at 95 °C for 90 s and cool to RT in the dark for 30 min.

If reusing hairpin solutions, heat at 95 °C and cool at RT, then skip at Step 6.

5. Prepare hairpin solution by adding **snap-cooled H1 hairpins** and **snap-cooled H2 hairpins** to 100 µL of **amplification buffer** (final concentration of each hairpin of 60 nM) at RT.

Note: if using 200 µL of amplification buffer, add 4 µL (30 pmol) of each hairpin.

Note: if using 500 µL of amplification buffer, add 10 µL (75 pmol) of each hairpin.

6. Incubate samples for >12 h (overnight) with 200 µL of **hairpin solution** at RT.

Day 3 – HCR conclusion

1. Wash samples for 2 × 5 min, 2 × 30 min, and 1 × 5 min with 500 µL (or 1 mL) of **5× SSCT** at RT.

Hairpin solution can be saved and reused for 2–3 times; store at –20 °C.

At this step, samples can be stored in the dark in 5× SSCT for several days at room temperature.

Day 3 – Sample mounting

1. Incubate samples for 30–60 min with 1 mL of **50 % glycerol in 1× PBS** at RT.
2. Incubate samples for 30–60 min with 1 mL of **75 % glycerol in 1× PBS** at RT.

Maintaining samples at pH 7.40 is critical for the stability of fluorophores and long-term storage of mRNA in-situ HCR samples.

At this step, samples can be stored in the dark in 75 % glycerol for several month at 4 °C.

3. Collect a small amount of samples (~1.5–2 µL) from the 75 % glycerol solution and place them on a cleaned (bridged, if necessary) slide.
4. Add ~15–20 µL of VECTASHIELD® PLUS Antifade Mounting Medium with DAPI [H-2000] directly on samples.

Adjust the amount of embryo suspension and mounting medium according to the cover glass dimensions, the quantity of samples, and the thickness of the bridge.

5. Seal the slide with nail polish and store in the dark at 4 °C.