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 dissolove 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× SSCTfor 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.