Multiplexed mRNA *in-situ* Hybridization Chain Reaction (HCR) in *Pleurobrachia pileus* adult tissues

First created: Mar 25th, 2025 *Last update*: Apr 3rd, 2025

– PROTOCOL –

Day 1 – Sample preparation

> *In each step, samples can be rocked on a nutator or on an orbital (horizontal) shaker.*

1. Dissect the desired adult tissue in 100% methanol.
2. Wash samples for 15 min each with a graded series of 500 µL (or 1 mL) of **methanol (60 and 30 %) in PBST**.
3. Wash samples for 15 min with 500 µL (or 1 mL) of **PBST** at RT.
4. Rock samples for 2 min with 500 µL (or 1 mL) of **2.6 µg/mL proteinase K** at RT.
5. Wash samples for two times without incubation with 500 µL (or 1 mL) of **PBST** at RT.
6. Post-fix samples for 30 min with 500 µL (or 1 mL) of **3.7 % PFA** at RT.
7. Wash samples for 2 × 10 min with 500 µL (or 1 mL) of **PBST** at RT.

> *Pre-heat probe-hybridization buffer to 37 °C (you will need 500 µLper tube [250 µL for the pre-hybridization, and 250 µL for the probe solution]).*

Day 1 – HCR detection stage

1. Pre-hybridize samples for 30 min with 500 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 µLper tube per 4 washes).*

Day 2 – HCR amplification stage

> *Pre-equilibrate amplification buffer to room temperature (you will need 700 µL per tube [500 µL for pre-amplification, and 200 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 4 µL of **hairpin H1** (30 pmol) and 4 µL of **hairpin H2** (30 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 200 µL of **amplification buffer** (final concentration of each hairpin of 60 nM) at RT.
   Note: if using 100 µL of amplification buffer, add 2 µL (15 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.

dsDNA can be stained on the second 30 min wash by using a 1:500 DAPI solution.

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 samples from the 75 % glycerol solution and place them on a cleaned (bridged, if necessary) slide.
4. Add ∼15–20 µL of mounting medium (or VECTASHIELD® PLUS Antifade Mounting Medium with DAPI [H-2000]) directly on samples.

Adjust the amount of 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.