

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

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## – 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  $\mu$ L (or 1 mL) of **methanol (60 and 30 %)** in **PBST**.
3. Wash samples for 15 min with 500  $\mu$ L (or 1 mL) of **PBST** at RT.
4. Rock samples for 2 min with 500  $\mu$ L (or 1 mL) of **2.6  $\mu$ g/mL proteinase K** at RT.
5. Wash samples for two times without incubation with 500  $\mu$ L (or 1 mL) of **PBST** at RT.
6. Post-fix samples for 30 min with 500  $\mu$ L (or 1 mL) of **3.7 % PFA** at RT.
7. Wash samples for 2  $\times$  10 min with 500  $\mu$ L (or 1 mL) of **PBST** at RT.

*Pre-heat probe-hybridization buffer to 37 °C (you will need 500  $\mu$ L per tube [250  $\mu$ L for the pre-hybridization, and 250  $\mu$ L for the probe solution]).*

### Day 1 – HCR detection stage

1. Pre-hybridize samples for 30 min with 500  $\mu$ L 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  $\mu$ L (0.8 pmol) of each **probe set 1  $\mu$ M stock solution** to 200  $\mu$ L of **probe hybridization buffer** (final concentration of 4 nM) at 37 °C.

*Note: if using 500  $\mu$ L of probe solution, add 2  $\mu$ L (2 pmol) of each probe set.*

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

*Pre-heat probe wash buffer at 37 °C (you will need 500  $\mu$ L per tube per 4 washes).*

### Day 2 – HCR amplification stage

*Pre-equilibrate amplification buffer to room temperature (you will need 700  $\mu$ L per tube [500  $\mu$ L for pre-amplification, and 200  $\mu$ L for the hairpin solution]).*

1. Wash samples for 4  $\times$  20 min with 500  $\mu$ L 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× 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 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.

**– RECIPES –****1× PBS with 0.1 % Tween 20 (PBST or PTw)**

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 50 mL)
Tween 20	50.0 µL	1,227.54	0.01 %
20× PBS	5.0 mL	–	1×
Ultrapure water	–	–	–

1. Add 5 mL of 20× PBS to a graduated cylinder.
2. Add 50 µL of Tween 20.
3. Fill up to 50 mL with ultrapure water.

**5× SSC 0.1 % Tween 20 (SSCT)**

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 50 mL)
Tween 20	50.0 µL	1,227.54	0.01 %
20× SSC	12.5 mL	–	5×
Ultrapure water	–	–	–

1. Add 12.5 mL of 20× SSC to a graduated cylinder.
2. Add 50 µL of Tween 20.
3. Fill up to 50 mL with ultrapure water.

**Proteinase K working solution**

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 10 mL)
20 mg/mL proteinase K stock solution	1.30 µL	–	2.6 µg/mL
PBST	–	–	–

1. Add 1.3 µL of 20 mg/mL proteinase K stock solution to 8 mL of PBST.
2. Fill up to 10 mL with PBST.

**1:500 DAPI staining solution**

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 500 µL)
DAPI	1.0 µL	–	1:500
1× PBS	499.0 µL	–	–

## – RESOURCES –

### 1. ISH/IHC in *Mnemiopsis leidyi*.

- Burkhardt, P., Colgren, J., Medhus, A., Digel, L., Naumann, B., Soto-Angel, J. J., ... & Kittelmann, M. (2023). Syncytial nerve net in a ctenophore adds insights on the evolution of nervous systems. *Science*, 380(6642), 293-297. [10.1126/science.ade5645](https://doi.org/10.1126/science.ade5645)
- Sachkova, M. Y., Nordmann, E. L., Soto-Àngel, J. J., Meeda, Y., Górski, B., Naumann, B., ... & Burkhardt, P. (2021). Neuropeptide repertoire and 3D anatomy of the ctenophore nervous system. *Current Biology*, 31(23), 5274-5285. [10.1016/j.cub.2021.09.005](https://doi.org/10.1016/j.cub.2021.09.005)