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

First created: Mar 25th, 2025 *Last update*: May 15th, 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** at RT.
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.

> *Pre-heat probe-hybridization buffer to 37 °C (you will need 700 µL per tube [500 µL for the pre-hybridization, and 200 µL for the probe solution]).*

7. Wash samples for 2 × 10 min with 500 µL (or 1 mL) of **PBST** at RT.

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 µL per 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.

– 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	0.1 %
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	0.1 %
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.

Urea-based probe hybridization buffer

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 10 mL)
Urea	2.40 g	60.06	4 M
20× SSC	2.50 mL	–	5×
1 M citric acid	90.00 µL	192.12	9 mM
Tween-20	10.00 µL	1,227.54	0.1 %
50× Denhardts's solution	200.00 µL	–	1×
50 % dextran sulfate	2.00 mL	–	10 %
10 mg/mL heparin	50.00 µL	–	50 µg/mL

1. Add 2.4 g of urea to a becker.

2. Combine all the other components fresh.
3. Stir thoroughly to allow complete solubilization of urea.
4. Fill up to 10 mL with ultrapure water.

Urea-based probe wash buffer

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 10 mL)
Urea	2.40 g	60.06	4 M
20× SSC	2.50 mL	–	5×
1 M citric acid	90.00 µL	192.12	9 mM
Tween-20	10.00 µL	1,227.54	0.1 %
10 mg/mL heparin	50.00 µL	–	50 µg/mL

1. Add 2.4 g of urea to a becker.
2. Combine all the other components fresh.
3. Stir thoroughly to allow complete solubilization of urea.
4. Fill up to 10 mL with ultrapure water.

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*.**
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   - Mitchell, D. G., Edgar, A., Mateu, J. R., Ryan, J. F., & Martindale, M. Q. (2024). The ctenophore *Mnemiopsis leidyi* deploys a rapid injury response dating back to the last common animal ancestor. *Communications Biology*, *7*(1), 203. 10.1038/s42003-024-05901-7
   - Pang, K., & Martindale, M. Q. (2008). Ctenophore whole-mount *in situ* hybridization. *Cold Spring Harbor Protocols*, *2008*(11), pdb-prot5087. 10.1101/pdb.prot5087
   - 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
2. **ISH with urea-based buffers**
   - Aguillon, R., Rinsky, M., Simon-Blecher, N., Doniger, T., Appelbaum, L., & Levy, O. (2024). CLOCK evolved in cnidaria to synchronize internal rhythms with diel environmental cues. *Elife*, *12*, RP89499. 10.7554/eLife.89499.4
   - Sinigaglia, C., Thiel, D., Hejnol, A., Houliston, E., & Leclère, L. (2018). A safer, urea-based in situ hybridization method improves detection of gene expression in diverse animal species. *Developmental biology*, *434*(1), 15-23. 10.1016/j.ydbio.2017.11.015