

Multiplexed mRNA *in-situ* Hybridization Chain Reaction (HCR) in *Sycon ciliatum*

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– PROTOCOL –

Day 1 – Sample preparation (version with xylene clearing)

This protocol refers to samples that have been already fixed and stored in pure methanol at -20 °C.

1. Wash samples for 15 min with 500 µL (or 1 mL) of **100 % ethanol** at RT.
2. Clear samples for 30 min with 500 µL (or 1 mL) of **50 % xylene in ethanol** at RT.

Always use xylene in glass containers (as it dissolves plastic) and under a chemical safety hood.

3. Wash samples for 15 min with 500 µL (or 1 mL) of **ice-cold 100 % ethanol** at 4 °C.
4. Wash samples for 15 min each with a graded series of 500 µL (or 1 mL) **ice-cold ethanol (75, 50 and 25 %) in 0.25× HS** at 4 °C.
5. Wash samples for 10 min with 500 µL (or 1 mL) of **ice-cold 0.25× HS** at 4 °C.
6. Wash samples for 10 min with 500 µL (or 1 mL) of **ice-cold 1× PBS** at 4 °C.
7. Post-fix samples for 30 min with 500 µL (or 1 mL) of **ice-cold fixative solution** at 4 °C.

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]).

8. Wash samples for 4 × 10 min with 500 µL (or 1 mL) of **PBST** at 4 °C.

Day 1 – Sample preparation (version with proteinase-K permeabilisation)

This protocol refers to samples that have been already fixed and stored in pure methanol at -20 °C.

1. Wash samples for 15 min with 500 µL (or 1 mL) of **ice-cold 100 % ethanol** at 4 °C.
2. Wash samples for 15 min each with a graded series of 500 µL (or 1 mL) **ice-cold ethanol (75, 50 and 25 %) in PBST** at 4 °C.
3. Rock samples for 10 min with 500 µL (or 1 mL) of **7.5 µg/mL proteinase K in PBST** at 37 °C.
4. Wash samples for 10 min with 500 µL (or 1 mL) of **PBST** at RT.
5. Quench samples for 10 min with 500 µL (or 1 mL) of **2 mg/mL glycine in PBST** at RT.
6. Wash samples for 3 × 10 min with 500 µL (or 1 mL) of **PBST** at RT.
7. Acetylate samples for 15 min each with a graded series of 500 µL (or 1 mL) **0.1 M triethanolamine containing 0, 1.5 and 3 µL/mL acetic anhydride** at RT.
8. Wash samples for 2 × 10 min with 500 µL (or 1 mL) of **PBST** at RT.

9. Post-fix samples for 30 min with 500 μ L (or 1 mL) of **fixative solution** at RT.

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

10. Wash samples for 4 \times 10 min with 500 μ L (or 1 mL) of **PBST** at RT.

Day 1 – HCR detection stage

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

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

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

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

Day 2 – HCR amplification stage

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

1. Wash samples for 4 \times 20 min with 500 μ 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 \times 5 min with 500 μ L (or 1 mL) of **5 \times SSCT** at RT.
3. Pre-amplify samples for 30 min with 500 μ L of **amplification buffer** at RT.
4. Snap cool in separate tubes 4 μ L of **hairpin H1** (30 pmol) and 4 μ L of **hairpin H2** (30 pmol) **3 μ M 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 μ L of **amplification buffer** (final concentration of each hairpin of 60 nM) at RT.

Note: if using 100 μ L of amplification buffer, add 2 μ L (15 pmol) of each hairpin.

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

6. Incubate samples for >12 h (overnight) with 200 μ L 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. Wash samples for 3 × 5 min with 500 µL (or 1 mL) of **1× PBS** at RT.

If using VECTASHIELD® PLUS Antifade Mounting Medium with DAPI (H-2000), skip directly to Step 4.

2. Incubate samples for 20 min with 500 µL (or 1 mL) of **DAPI staining solution** at RT.
3. Wash samples for 3 × 5 min with 500 µL (or 1 mL) of **1× PBS** at RT.
4. Incubate samples for 30–60 min with 1 mL of **50 % glycerol in 1× PBS** at RT.
5. 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.

6. Collect precipitated samples from the 75 % glycerol solution and place them on a cleaned (bridged, if necessary) slide.
7. Add ~15–20 µL of mounting medium (or 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.

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

– RECIPES –**Fixative solution**

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 100 mL)
36–38 % formaldehyde	3.7 mL	30.03	3.7 %
25 % glutaraldehyde	0.2 mL	100.12	0.05 %
10× PBS	10.0 mL	–	1×
Ultrapure water	–	–	–

1. Add 3.7 mL of 36–38 % formaldehyde to a graduated cylinder.
2. Add 0.2 mL of 25 % glutaraldehyde to a graduated cylinder.
3. Fill up to 100 mL with ultrapure water.
4. Store the fixative solution at 4 °C.

1× Holtfreter's solution (HS)

Compound	Quantity	Molar mass (g/mol)	Final concentration (for 1 L)
NaCl	3.46 g	58.44	0.05900 M
KCl	0.05 g	74.55	0.00067 M
CaCl ₂	0.10 g	110.98	0.00076 M
or CaCl ₂ · 2 H ₂ O	0.13 g	120.04	0.00090 M
NaHCO ₃	0.20 g	84.00	0.00240 M
Ultrapure water	–	–	–

1. Dissolve solutes in 800 mL of ultrapure water, by continuous stirring.
2. Fill up to 1 L with ultrapure water.
3. Store the 0.25× HS indefinitely at RT.

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	3.75 µL	–	7.5 µg/mL
PBST	–	–	–

1. Add 3.75 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. mRNA fluorescent *in-situ* HCR in *Sycon* spp.

- *Fortunato, S., Adamski, M., Bergum, B., Guder, C., Jordal, S., Leininger, S., ... & Adamska, M. (2012). Genome-wide analysis of the sox family in the calcareous sponge *Sycon ciliatum*: multiple genes with unique expression patterns. *EvoDevo*, 3, 1-11. [10.1186/2041-9139-3-14](https://doi.org/10.1186/2041-9139-3-14)
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2. mRNA *in-situ* HCR in other sponges.

- Kojima, C., & Funayama, N. (2022). *In situ* hybridization to identify stem cells in the fresh-water sponge *Ephydatia fluviatilis*. In: Blanchoud, S., & Galliot, B. (eds.), *Whole-Body Regeneration*, 335-346. [10.1007/978-1-0716-2172-1](https://doi.org/10.1007/978-1-0716-2172-1)
- Larroux, C., Fahey, B., Adamska, M., Richards, G. S., Gauthier, M., Green, K., & Degnan, B. M. (2008). Whole-mount *in situ* hybridization in *Amphimedon*. *CSH protocols*, pdb-prot5096. [10.1101/pdb.prot5096](https://doi.org/10.1101/pdb.prot5096)
- Musser, J. M., Schippers, K. J., Nickel, M., Mizzon, G., Kohn, A. B., Pape, C., & Arendt, D. (2021). Profiling cellular diversity in sponges informs animal cell type and nervous system evolution. *Science*, 374(6568), 717-723. [10.1126/science.abj2949](https://doi.org/10.1126/science.abj2949)
- Funayama, N., Nakatsukasa, M., Hayashi, T., & Agata, K. (2005). Isolation of the choanocyte in the fresh water sponge, *Ephydatia fluviatilis* and its lineage marker, *Ef annexin*. *Development, growth & differentiation*, 47(4), 243-253. [10.1111/j.1440-169X.2005.00800.x](https://doi.org/10.1111/j.1440-169X.2005.00800.x)