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

First created: Dec 5th, 2024 *Last update*: Dec 12th, 2024

– 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 µLfor the pre-hybridization, and 200 µL for the probe solution]).*

8. Wash samples for 2 × 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** ar 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 µL (or 1 mL) of **fixative solution** at RT.
10. 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 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.

> *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. 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	30.031	3.7 %
25 % gluteraldehyde	0.2 mL	100.120	0.05 %
10× PBS	10.0 mL	–	1× PBS
Distilled water	86.1 mL	–	–

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– RESOURCES –

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