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

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– PROTOCOL –

Day 1 – Sample preparation

> *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. Wash 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 – 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.

Day 3 – Sample mounting

– RECIPES –

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– RESOURCES –

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