

Multiplexed mRNA *in-situ* Hybridization Chain Reaction (HCR) in *Drosophila melanogaster* adult brain

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– PROTOCOL –

Day 1 – Sample preparation

For fixation and all subsequent steps, samples can be placed in Eppendorf tubes or in wells of a spot plate.

In each step, samples can be rocked on a nutator or on an orbital (horizontal) shaker.

1. Dissect adult brains in Schneider's *Drosophila* medium or in nuclease-free 1× PBS.
2. Fix samples for 20 min with 800 µL of **4% paraformaldehyde in PBST** at RT.

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

3. Rinse samples for 3 times with 500 µL of **PBST** at RT.
4. Wash samples for 3 × 15 min with 500 µL of **PBST** at RT.

Day 1 – HCR detection stage

1. Pre-hybridize samples for 30 min with 250 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 1 µL (1 pmol) of each **probe set 1 µM stock solution** to 250 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 450 µL per tube [250 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 250 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× 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 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 *Drosophila* brains.

- Ferreira, A. A., Sieriebriennikov, B., & Whitbeck, H. (2021). HCR RNA-FISH protocol for the whole-mount brains of *Drosophila* and other insects. <https://slack.protocols.io:8443/view/hcr-rna-fish-protocol-for-the-whole-mount-brains-o-bzh5p386.html> (accessed on Feb 11th, 2024)