

HCR™ RNA-FISH (v3.0) protocol for whole-mount fruit fly embryos

This protocol has not been optimized for all stages and should only be used as a template.

Technical Support

support@molecularinstruments.com

Safety Data Sheets (SDS)

www.molecularinstruments.com/safety-v3

Patents

www.molecularinstruments.com/patents

Ordering for Multiplex Experiment

Order one HCR™ RNA-FISH (v3.0) kit per target RNA

Example 2-Plex Experiment

- HCR™ RNA-FISH (v3.0) kit for target mRNA1
 - HCR™ Probe (v3.0): target mRNA1 for use with amplifier B1
 - HCR™ Amplifier (v3.0): B1-647
 - HCR™ RNA-FISH Buffers (v3.0): HCR™ Probe Hybridization Buffer (v3.0), HCR™ Probe Wash Buffer (v3.0), HCR™ Amplifier Buffer (v3.0) (for use with all kits)
- HCR™ RNA-FISH (v3.0) kit for target mRNA2
 - HCR™ Probe (v3.0): target mRNA2 for use with amplifier B2
 - HCR™ Amplifier (v3.0): B2-488

Storage conditions

- Store HCR™ Probes (v3.0), HCR™ Amplifiers (v3.0), HCR™ Probe Hybridization Buffer (v3.0), and HCR™ Probe Wash Buffer (v3.0) at -20 °C.
- Store HCR™ Amplifier Buffer (v3.0) at 4 °C.
- On the bench top, keep stock solutions on ice.
- Make sure all solutions are well mixed before use.

Preparation of fixed whole-mount fruit fly embryos

1. Collect fly embryos and incubate with yeast paste (food source) until they reach stage 4–6 (approximately 3 h).
2. Rinse embryos into a mesh basket and wash excess yeast paste using DI H₂O.
3. Immerse the mesh basket into 100% bleach and wash for 2 min to dechorionate embryos.
4. Rinse the basket with DI H₂O.
5. Transfer embryos to a scintillation vial containing 8 mL of 4.5% formaldehyde fixation solution.
CAUTION: use formaldehyde with extreme care as it is a hazardous material.
6. Gently rock embryos in scintillation vial for 25 min on an orbital rocker.
NOTE: Rocking does not have to be very vigorous. However, embryos must move continuously within the heptane-fixative interphase to be exposed uniformly to the solvent and fixative.
7. Remove the bottom liquid phase in the vial.
8. Add 8 mL of methanol (MeOH) and shake the vial hard for 1 min. Devitellinized embryos will sink to the bottom of the vial.
9. Remove all liquid and rinse 2 times in 1 mL of MeOH to remove debris.
NOTE: Embryos can be stored in 1 mL of MeOH at -20 °C before use.
10. For each sample, transfer 50 µL of embryos (using a cut pipet tip) to a 1.5 mL tube.
11. Rinse embryos 4 times by quickly adding 1 mL of ethanol (EtOH), inverting the tube, and aspirating the supernatant.
12. Add 500 µL of EtOH and 250 µL of xylene and invert the tube.
CAUTION: use xylene with care as it is a hazardous material.
13. Add an additional 250 µL of xylene and invert the tube.
14. Add another 250 µL of xylene again and invert the tube.
NOTE: The tube should now contain 500 µL of EtOH and 750 µL of xylene.
15. Rock the tube at room temperature for 45–60 min. Longer rocking time is acceptable.
16. Aspirate the supernatant.
17. Rinse embryos once and wash 3 × 5 min with EtOH.
NOTE: All washes have a volume of 1 mL unless specified. All washes before pre-hybridization (step 1) are performed with rocking.
18. Rinse embryos once and wash 2 × 5 min with MeOH.

19. Wash with 50% MeOH / 50% PBST for 5 min.
20. Wash 1×10 min and 2×5 min with PBST.
21. Rock embryos in 1 mL of 4 $\mu\text{g/mL}$ proteinase K solution at room temperature for 7 min.
NOTE: Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage.
22. Rinse embryos 2 times and wash 2×5 min with PBST.
23. Rock embryos in 4% formaldehyde post-fixation solution at room temperature for 25 min.
24. Rinse embryos and wash 5×5 min with PBST.

Multiplexed HCR™ RNA-FISH (v3.0) protocol

Detection stage

1. Pre-hybridize embryos in 200 μ L of HCR™ Probe Hybridization Buffer (v3.0) for 30 min at 37 °C.
CAUTION: HCR™ Probe Hybridization Buffer (v3.0) contains formamide, a hazardous material.
2. Prepare probe solution by adding 0.8 pmol of each HCR™ Probe (v3.0) (e.g. 0.8 μ L of 1 μ M stock) to 200 μ L of HCR™ Probe Hybridization Buffer (v3.0) at 37 °C.
NOTE: For single-molecule RNA imaging, use higher probe concentration (e.g., 16 nM) to increase probe hybridization yield. If desired, this approach can also be used to increase signal for qHCR imaging.
3. Remove the pre-hybridization solution and add the probe solution.
4. Incubate embryos overnight (>12 h) at 37 °C.
5. Remove excess probes by washing embryos 4 \times 15 min with 1 mL of HCR™ Probe Wash Buffer (v3.0) at 37 °C.
CAUTION: HCR™ Probe Wash Buffer (v3.0) contains formamide, a hazardous material.
NOTE: pre-heat HCR™ Probe Wash Buffer (v3.0) to 37 °C before use.
6. Wash samples 2 \times 5 min with 1 mL of 5 \times SSCT at room temperature.

Amplification stage

1. Pre-amplify embryos with 1 mL of HCR™ Amplifier Buffer (v3.0) for 10 min at room temperature.
NOTE: equilibrate HCR™ Amplifier Buffer (v3.0) to room temperature before use.
2. Separately prepare 6 pmol of hairpin h1 and 6 pmol of hairpin h2 by snap cooling 2 μ L of 3 μ M stock (heat at 95 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
NOTE: Hairpins h1 and h2 are provided in hairpin storage buffer ready for snap cooling. h1 and h2 should be snap cooled in separate tubes.
3. Prepare hairpin solution by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 100 μ L of HCR™ Amplifier Buffer (v3.0) at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate the embryos overnight (>12 h) in the dark at room temperature.
NOTE: For single-molecule RNA imaging, amplify for a shorter period of time to ensure single-molecule dots are diffraction-limited.
6. Remove excess hairpins by washing with 1 mL of 5 \times SSCT at room temperature:
 - (a) 2 \times 5 min
 - (b) 2 \times 30 min
 - (c) 1 \times 5 min
7. Samples can be stored at 4 °C protected from light before microscopy.

Buffer recipes

4.5% formaldehyde fixation solution

4.5% formaldehyde
0.5× PBS
25 mM EGTA
50% heptane

For 8 mL of solution

975 μ L of 37% formaldehyde
400 μ L of 10× PBS
76 mg of EGTA
4 mL of heptane
Fill up to 8 mL with ultrapure H₂O

Proteinase K solution

4 μ g/mL proteinase K

For 2 mL of solution

0.4 μ L of 20 mg/mL proteinase K
Fill up to 2 mL with PBST

4% formaldehyde post-fixation solution

4% formaldehyde

For 2 mL of solution

216 μ L of 37% formaldehyde
Fill up to 2 mL with PBST

PBST

1× PBS
0.1% Tween 20

For 50 mL of solution

5 mL of 10× PBS
500 μ L of 10% Tween 20
Fill up to 50 mL with ultrapure H₂O

5× SSCT

5× sodium chloride sodium citrate (SSC)
0.1% Tween 20

For 40 mL of solution

10 mL of 20× SSC
400 μ L of 10% Tween 20
Fill up to 40 mL with ultrapure H₂O

NOTE: avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the embryos.

S1 HCR™ Technology Citation Notes

For citation, please select from the list below as appropriate for your application:

- **HCR™ RNA-ISH**

HCR™ RNA in situ hybridization (RNA-ISH) offers unmatched performance, robustness, and versatility imaging RNA targets in diverse organisms and sample types (Choi et al., 2010, Choi et al., 2014, Choi et al., 2018):

- **HCR™ RNA-FISH**

HCR™ RNA-FISH enables 10-plex, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) with automatic background suppression throughout the protocol for dramatically enhanced performance (signal-to-background, quantitative precision, single-molecule fidelity) and ease-of-use (no probe set optimization for new targets and organisms).

- **Enzymatic HCR™ RNA-CISH/RNA-FISH**

Enzymatic HCR™ RNA-ISH integrates enzymatic signal amplification to enable extreme-sensitivity RNA imaging using either chromogenic or fluorescent staining (RNA-CISH/RNA-FISH). In tissue sections, entirely protease-free workflows preserve sample morphology and maintain protein target integrity, enabling seamless compatibility with existing immunohistochemistry (IHC)/immunofluorescence (IF) assays. HCR™ RNA-CISH offers the convenience of brightfield microscopy and the option of archival staining.

- **10-Plex HCR™ Spectral Imaging**

HCR™ RNA-FISH/IF enables quantitative high-resolution imaging of 10 RNA and/or protein targets with 1-step HCR™ signal amplification for all targets simultaneously. The method is suitable even for whole-mounts and delicate samples as it requires no repeated staining, imaging, registration, or stripping (Schulte et al., 2024).

- **HCR™ RNA-FISH/IF**

HCR™ RNA-FISH/IF enables a unified approach to multiplex, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) and protein immunofluorescence (IF), with quantitative 1-step enzyme-free signal amplification performed for all RNA and protein targets simultaneously (Schwarzkopf et al., 2021).

- **HCR™ IF**

HCR™ IF enables multiplex, quantitative, high-resolution protein immunofluorescence (IF) in highly autofluorescent samples (e.g., FFPE brain tissue sections) (Schwarzkopf et al., 2021).

- **Subcellular Quantitative RNA and Protein Imaging**

HCR™ RNA-FISH enables analog relative quantitation of RNA and/or protein targets with subcellular resolution in the anatomical context of thick autofluorescent samples (e.g., whole-mount vertebrate embryos) (Trivedi et al., 2018, Choi et al., 2018, Schwarzkopf et al., 2021).

- **Single-Molecule Quantitative RNA Imaging**

HCR™ RNA-FISH enables digital RNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah et al., 2016, Choi et al., 2018).

- **Read-Out/Read-In Analysis Framework**

The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (Trivedi et al., 2018).

- **Protocols in Diverse Sample Types**

Protocols for HCR™ RNA-FISH and/or IF in diverse sample types are adapted from the zoo paper ([Choi et al., 2016](#)):

- bacteria in suspension
- FFPE human tissue sections
- generic sample in solution
- generic sample on a slide
- mammalian cells on a slide
- mammalian cells in suspension
- whole-mount chicken embryos
- whole-mount fruit fly embryos
- whole-mount mouse embryos
- whole-mount nematode larvae
- whole-mount sea urchin embryos
- whole-mount zebrafish embryos and larvae

- **HCR™ RNA Flow Cytometry**

HCR™ RNA Flow Cytometry enables analog RNA relative quantitation for high-throughput expression profiling of mammalian cells and bacteria without the need to engineer reporter lines ([Choi et al., 2018](#)).

- **HCR™ Northern Blots**

HCR™ Northern Blots enable simultaneous quantification of RNA target size and abundance with automatic background suppression throughout the protocol ([Schwarzkopf & Pierce, 2016](#)).

- **HCR™ Amplifiers**

HCR™ Amplifiers enable multiplex, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings ([Dirks & Pierce, 2004](#)).