HCR™ RNA-FISH (v3.0) protocol for whole-mount nematode larvae

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 nematode larvae

1. Wash nematode larvae off plate with 1 mL of M9 buffer and transfer to a 1.5 mL tube.
2. Centrifuge at 200 × g for 2 min to bring larvae to the bottom of the tube and remove the M9 buffer.
3. Wash larvae 3 times with 1 mL of M9 buffer each. Centrifuge at 200 × g for 2 min between washes.
4. Centrifuge and remove ≈ 800 μL of M9 buffer.
5. Aliquot larvae sufficient for a reaction into 1.5 mL tubes.
6. Add 1 mL of 4% paraformaldehyde (PFA).
   CAUTION: *use PFA with extreme care as it is a hazardous material.*
   NOTE: *use fresh PFA to avoid increased autofluorescence.*
7. Immediately freeze sample at -80 °C overnight before use.
   NOTE: *Larvae could stay in -80 °C freezer for long-term storage.*
8. Fix larvae by thawing at room temperature for 45 min.
9. Wash larvae 2 times with 1 mL of PBST each. Bring larvae to the bottom of the tube with centrifugation at 200 × g for 2 min in between washes.
10. Treat larvae with 1 mL of proteinase K (100 μg/mL) for 10 min at 37 °C.
    NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage.*
11. Wash larvae 2 times with 1 mL of PBST each.
12. Incubate in 1 mL of 2 mg/mL of glycine solution for 15 min on ice.
13. Wash larvae 2 times with 1 mL of PBST each.

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

Detection stage

1. Incubate larvae in 1 mL of 50% PBST / 50% HCR™ Probe Hybridization Buffer (v3.0) for 5 min at room temperature.
   CAUTION: *HCR™ Probe Hybridization Buffer (v3.0) contains formamide, a hazardous material.*
2. Centrifuge at 200 × g for 2 min to remove solution.
3. Pre-hybridize larvae in 300 μL of HCR™ Probe Hybridization Buffer (v3.0) at 37 °C for 1 h.
4. Prepare probe solution by adding 2 pmol of each HCR™ Probe (v3.0) (e.g. 2 μL of 1 μM stock) to 200 μL 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 subcellular quantitative RNA imaging.*
5. Add the probe solution to reach a final hybridization volume of 500 μL.
6. Incubate larvae overnight (>12 h) at 37 °C.
7. Remove excess probes by washing larvae 4 × 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.*
   NOTE: *bring larvae to the bottom of the tube with centrifugation at 500 × g for 2 min for each wash.*
8. Wash larvae 2 × 5 min with 1 mL of 5× SSCT at room temperature.

Amplification stage

1. Pre-amplify larvae with 300 μL of HCR™ Amplifier Buffer (v3.0) for 30 min at room temperature.
   NOTE: *equilibrate HCR™ Amplifier Buffer (v3.0) to room temperature before use.*
2. Separately prepare 30 pmol of hairpin h1 and 30 pmol of hairpin h2 by snap cooling 10 μL of 3 μM 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 200 μL of HCR™ Amplifier Buffer (v3.0) at room temperature.
4. Add the hairpin solution to reach a final amplification volume of 500 μL.
5. Incubate the larvae 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× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 30 min
   (c) 1 × 5 min

   NOTE: *bring larvae to the bottom of the tube with centrifugation at 500 × g for 2 min for each wash.*

7. Samples can be stored at 4 °C protected from light before microscopy.

Buffer recipes

M9 buffer
22 mM KH_2PO_4
42 mM Na_2HPO_4
20.5 mM NaCl
1 mM $MgSO_4$

For 1 L of solution
3 g of KH_2PO_4
6 g of Na_2HPO_4
5 g of NaCl
1 mL of 1 M $MgSO_4$
Fill up to 1 L with ultrapure H_2O
Sterilize by autoclaving
Store buffer at 4 °C before use

4% Paraformaldehyde (PFA)
4% PFA
1× PBS

For 40 mL of solution
10 mL of 16% PFA solution
4 mL of 10× PBS
Fill up to 40 mL with ultrapure H_2O

PBST
1× PBS
0.1% Tween 20

For 50 mL of solution
5 mL of 10× PBS
500 μL of 10% Tween 20
Fill up to 50 mL with ultrapure H_2O

Proteinase K solution
100 μg/mL proteinase K

For 1 mL of solution
5 μL of 20 mg/mL proteinase K
Fill up to 1 mL with PBST

Glycine solution
2 mg/mL glycine
PBST

For 50 mL of solution
100 mg of glycine
Fill up to 50 mL with PBST

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

For 40 mL of solution
10 mL of 20× SSC
400 μL of 10% Tween 20
Fill up to 40 mL with ultrapure H_2O

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

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).