HCR™ RNA-FISH (v3.0) protocol for whole-mount sea urchin 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 sea urchin embryos

1. Incubate fertilized embryos with fresh filtered seawater in a 24-well plate at 16 °C until 45 hpf.
2. Transfer embryos from the 24-well plate into a 50 mL conical tube.
3. Centrifuge at 100 × g for 2 min to bring embryos to the bottom of the tube.
4. Remove seawater to reach 20 mL volume and add 2.4 mL of 4.45 M NaCl to deciliate embryos.
5. Incubate on ice until embryos settle to the bottom of the tube.
6. Remove seawater (with NaCl) and add 20 mL of filtered sea water.
7. Incubate on ice until embryos settle to the bottom of the tube.
8. Repeat steps 6 and 7.
9. Aspirate as much sea water as possible without removing embryos.
10. Fix embryos in 40 mL of 4% paraformaldehyde (PFA) overnight at 4 °C on a nutator.
    CAUTION: *use PFA with extreme care as it is a hazardous material.*
    NOTE: *use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.*
11. Remove fixative to reach a volume of ≈ 6 mL.
12. Transfer embryos to six 1.5 mL tubes (1 mL each).
13. Centrifuge at 100 × g for 2 min.
14. Wash embryos 5 times with 1 mL of PBST each. Centrifuge at 100 × g for 2 min to bring embryos to the bottom of a tube in between washes.
15. Wash embryos 3 times with 1 mL of 70% ethanol each. Centrifuge at 100 × g for 2 min to bring embryos to the bottom of a tube in between ethanol washes.
16. Store embryos in 1 mL of 70% ethanol at -20 °C before use.

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

Detection stage

1. Transfer ≈200 embryos to each well of a 96 well plate.
   NOTE: *Adding and removing solution should be performed under a dissecting scope to avoid losing a large number of embryos.*
2. Rehydrate embryos 3 × 5 min with 250 μL of 5× SSCT.
3. Aspirate with care to reach ≈10 μL of volume.
4. Add 50 μL of HCR™ Probe Hybridization Buffer (v3.0) and pre-hybridize for 30 min at 37 °C.
   CAUTION: *HCR™ Probe Hybridization Buffer (v3.0) contains formamide, a hazardous material.*
5. Prepare probe solution by adding 0.4 pmol of each HCR™ Probe (v3.0) (e.g. 0.4 μL of 1 μM stock) to 50 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.*
6. Add the probe solution to reach a final hybridization volume of 100 μL.
7. Gently stir the solution with a 10 μL pipette tip.
8. Cover plate with Bio-Rad Microseal 'A' film and incubate embryos overnight (>12 h) at 37 °C.
9. Add 150 μL of HCR™ Probe Wash Buffer (v3.0) to each well of embryos.
   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.*
10. Incubate at 37 °C for 5 min.
11. Remove excess probes by washing with ≈200 μL of HCR™ Probe Wash Buffer (v3.0) at 37 °C:
    (a) 2 × 5 min
    (b) 2 × 30 min

    NOTE: *fill HCR™ Probe Wash Buffer (v3.0) to top of each well but do not overfill.*
    NOTE: *It is important to maintain plate temperature at 37 °C during probe washing steps. This can be achieved by placing the 96-well plate containing the embryos on a heat plate while removing solution from each well.*
12. Wash embryos 2 × 5 min with ≈200 μL of 5× SSCT.

Amplification stage

1. Separately prepare 6 pmol of hairpin h1 and 6 pmol of hairpin h2 by snap cooling 2 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.*
2. Equilibrate HCR™ Amplifier Buffer (v3.0) to room temperature.
3. Prepare hairpin solution by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 100 μL of HCR™ Amplifier Buffer (v3.0) at room temperature.
4. Aspirate as much 5× SSCT as possible without removing embryos.
5. Add the hairpin solution and incubate 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. Add 150 μL of 5× SSCT and incubate for 5 min at room temperature.
7. Remove excess hairpins by washing with ≈200 μL of 5× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 30 min
8. Samples can be stored at 4 °C protected from light before microscopy.

Buffer recipes

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

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: *16% PFA solution is filter sterilized using a 25 mm syringe filter with 0.2 μm membrane before use.*
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).