HCR RNA-FISH protocol for whole-mount zebrafish embryos and larvae (*Danio rerio*)

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)
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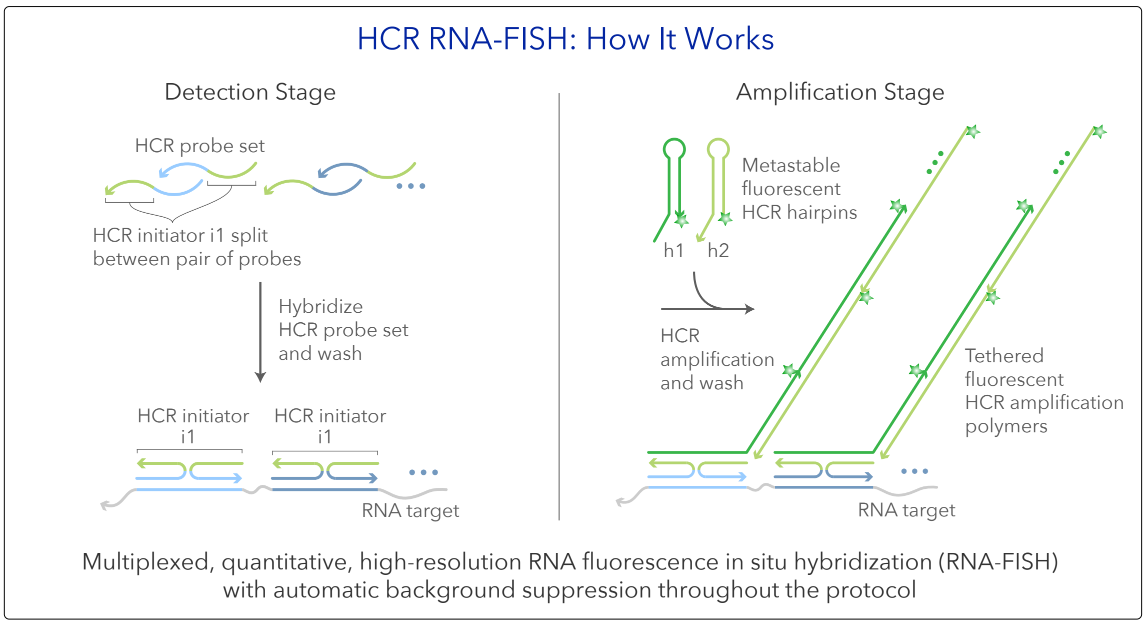

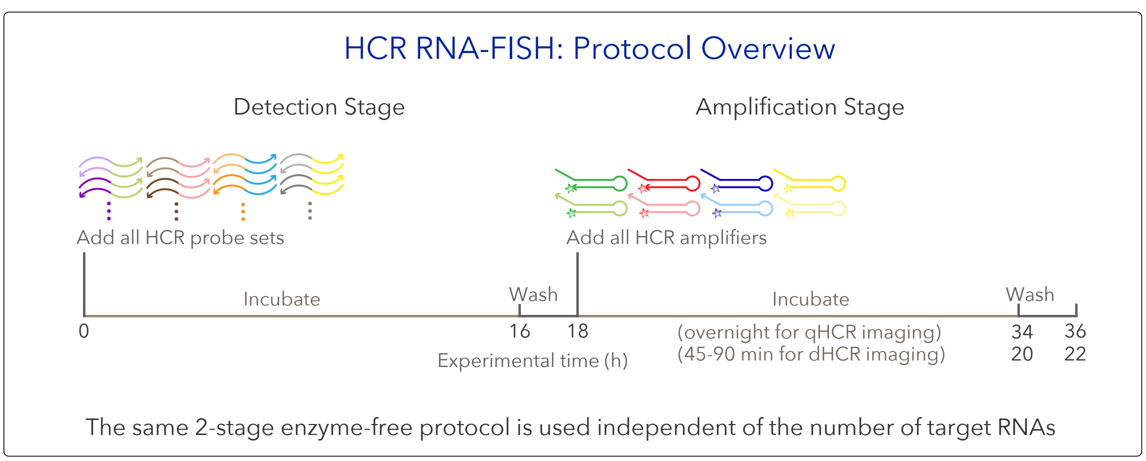

HCR RNA-FISH

Multiplexed, quantitative, high-resolution RNA imaging

Multiplexed Experiment

- Order one HCR RNA-FISH bundle per target RNA

Example 2-Plex Experiment

- HCR bundle for target mRNA1
  - HCR split-initiator probe set: target mRNA1 for use with amplifier B1
  - HCR amplifier: B1-647
  - HCR RNA-FISH buffers: probe hybridization buffer, probe wash buffer, amplification buffer (for use with all bundles)
- HCR bundle for target mRNA2
  - HCR split-initiator probe set: target mRNA2 for use with amplifier B2
  - HCR amplifier: B2-488

Storage conditions

- Store HCR probe sets, HCR amplifiers, HCR probe hybridization buffer, and HCR probe wash buffer at -20 °C.
- Store HCR amplification buffer 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 zebrafish embryos and larvae

1. Collect zebrafish embryos and incubate at 28 °C in a petri dish with egg H_2O.
2. Exchange egg H_2O with egg H_2O containing 0.003% of 1-phenyl 2-thiourea (PTU) when embryos reach 12 hpf.
   NOTE: *PTU treatment is not necessary if working with embryos younger than 30 hpf.*
   NOTE: *PTU inhibits melanogenesis but can be toxic at high concentrations. PTU treatment must start before the initial pigmentation occurs as PTU does not remove pigment that has already formed. PTU treatment is not necessary for nacre embryos used in this paper.*
3. Replace with fresh egg H_2O containing 0.003% of PTU everyday until the larvae reach 5 dpf (days post-fertilization).
4. Transfer ∼40 embryos/larvae (5 dpf) to a 2 mL eppendorf tube and remove excess egg H_2O.
5. Fix embryos/larvae in 2 mL of 4% paraformaldehyde (PFA) for 24 h at 4 °C.
   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.*
6. Wash embryos/larvae 3 × 5 min with 1 mL of 1× phosphate-buffered saline (PBS) to stop the fixation.
   NOTE: *avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the embryos/larvae.*
7. Dehydrate and permeabilize with a series of methanol (MeOH) washes (1 mL each):
   (a) 100% MeOH for 4 × 10 min
   (b) 100% MeOH for 1 × 50 min.
8. Store embryos/larvae at -20 °C overnight before use.
   NOTE: *Embryos/larvae can be stored for six months at -20 °C.*
9. Transfer the required number of embryos/larvae for an experiment to a 2 mL eppendorf tube.
10. Rehydrate with a series of graded 1 mL MeOH/PBST washes for 5 min each at room temperature:
    (a) 75% MeOH / 25% PBST
    (b) 50% MeOH / 50% PBST
    (c) 25% MeOH / 75% PBST
    (d) 5 × 100% PBST.
11. Treat 5 dpf embryos/larvae with 1 mL of proteinase K (30 μg/mL) for 45 min at room temperature.
    NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage. Skip proteinase K treatment and postfixation (steps 11–14) for embryos 30 hpf and younger.*

12. Wash embryos/larvae two times with PBST (1 mL each) without incubation.

13. Postfix with 1 mL of 4% PFA for 20 min at room temperature.

14. Wash embryos/larvae 5 × 5 min with 1 mL of PBST.

Multiplexed HCR RNA-FISH protocol

Detection stage

1. For each sample, transfer 8 embryos/larvae to a 1.5 mL tube.
2. Pre-hybridize with 500 μL of probe hybridization buffer for 30 min at 37 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
3. Prepare probe solution by adding 2 pmol of each probe set (e.g. 2 μL of 1 μM stock) to 500 μL of probe hybridization buffer at 37 °C.
   NOTE: *For dHCR 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.*
4. Remove the pre-hybridization solution and add the probe solution.
5. Incubate embryos/larvae overnight (>12 h) at 37 °C.
6. Remove excess probes by washing embryos/larvae 4 × 15 min with 500 μL of probe wash buffer at 37 °C.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *pre-heat probe wash buffer to 37 °C before use.*
7. Wash embryos/larvae 2 × 5 min with 5× SSCT at room temperature.

Amplification stage

1. Pre-amplify embryos/larvae with 500 μL of amplification buffer for 30 min at room temperature.
   NOTE: *equilibrate amplification buffer 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: *HCR 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 500 μL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate the embryos/larvae overnight (>12 h) in the dark at room temperature.
   NOTE: *For dHCR imaging, amplify for a shorter period of time to ensure single-molecule dots are diffraction-limited.*
6. Remove excess hairpins by washing with 500 μL of 5× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 30 min
   (c) 1 × 5 min
7. Samples can be stored at 4 °C protected from light before microscopy.

Buffer recipes

6% PTU stock solution	For 100 mL of solution
6% PTU	6 g of 1-phenyl 2-thiourea powder Fill up to 100 mL with egg H_2O Heat solution at 50–60 °C overnight to dissolve powder
0.3% PTU in egg H_2O	For 50 mL of solution
0.3% PTU	2.5 mL of 6% PTU Fill up to 50 mL with with egg H_2O
4% paraformaldehyde (PFA)	For 25 mL of solution
4% PFA 1× PBS	1 g of PFA powder 25 mL of 1× PBS Heat solution at 50–60 °C to dissolve powder
PBST	For 50 mL of solution
1× PBS 0.1% Tween 20	5 mL of 10× PBS 500 μL of 10% Tween 20 Fill up to 50 mL with ultrapure H_2O
Proteinase K solution	For 1 mL of solution
30 μg/mL proteinase K	1.5 μL of 20 mg/mL proteinase K Fill up to 1 mL with PBST
5× SSCT	For 40 mL of solution
5× sodium chloride sodium citrate (SSC) 0.1% Tween 20	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.*

HCR Technology Citation Notes

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

- **HCR IHC + HCR RNA-FISH**
  HCR IHC + HCR RNA-FISH enables a unified approach to multiplexed, quantitative, high-resolution protein immunohistochemistry (IHC) and RNA fluorescence in situ hybridization (RNA-FISH), with quantitative 1-step enzyme-free HCR signal amplification performed for all protein and RNA targets simultaneously (Schwarzkopf et al., 2021).

- **HCR IHC**
  HCR IHC enables multiplexed, quantitative, high-resolution protein immunohistochemistry (IHC) in highly autofluorescent samples (e.g., FFPE brain tissue sections) (Schwarzkopf et al., 2021).

- **HCR RNA-FISH (v3.0)**
  Third-generation HCR RNA-FISH (v3.0) enables multiplexed, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) with automatic background suppression *throughout the protocol* for dramatically enhanced performance (signal-to-background, qHCR precision, dHCR fidelity) and ease-of-use (no probe set optimization for new targets and organisms) (Choi et al., 2018). Quantitative analysis modes:

  - qHCR RNA imaging: analog mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples.
  - dHCR RNA imaging: digital mRNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples.
  - qHCR RNA flow cytometry: analog mRNA relative quantitation for high-throughput expression profiling of mammalian cells and bacteria.

  Protocols for HCR RNA-FISH (v3.0) in diverse organisms are adapted from the Zoo paper.

- **qHCR RNA imaging**
  qHCR RNA imaging enables mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples (e.g., whole-mount vertebrate embryos). The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (Trivedi et al., 2018).

- **Zoo paper**
  Protocols for multiplexed mRNA imaging in diverse sample types (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

- **dHCR imaging**
  dHCR RNA imaging enables 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).

- **qHCR northern blots**
  qHCR northern blots enable multiplexed quantification of RNA target size and abundance for up to 5 target RNAs (Schwarzkopf & Pierce, 2016).

- **HCR RNA-FISH (v2.0)**
  Second-generation in situ HCR RNA-FISH technology (v2.0) using DNA HCR probes and DNA HCR probes and DNA HCR amplifiers: 10$\times$ increase in signal, 10$\times$ reduction in cost, dramatic increase in reagent durability (Choi et al., 2014).

- **HCR RNA-FISH (v1.0)**
  First-generation HCR RNA-FISH technology (v1.0) using RNA HCR probes and RNA HCR amplifiers: multiplexed mRNA imaging in whole-mount vertebrate embryos with simultaneous signal amplification for up to 5 target mRNAs (Choi et al., 2010).

- **HCR mechanism**
  The hybridization chain reaction (HCR) mechanism enables multiplexed, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings (Dirks & Pierce, 2004).

HCR Technology References

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