HCR™ RNA-FISH protocol for mammalian cells on a chambered slide

This protocol has not been validated for all mammalian cell types and should only be used as a template.

Technical support
support@molecularinstruments.com

Safety data sheets (SDS)
www.molecularinstruments.com/safety

Patents
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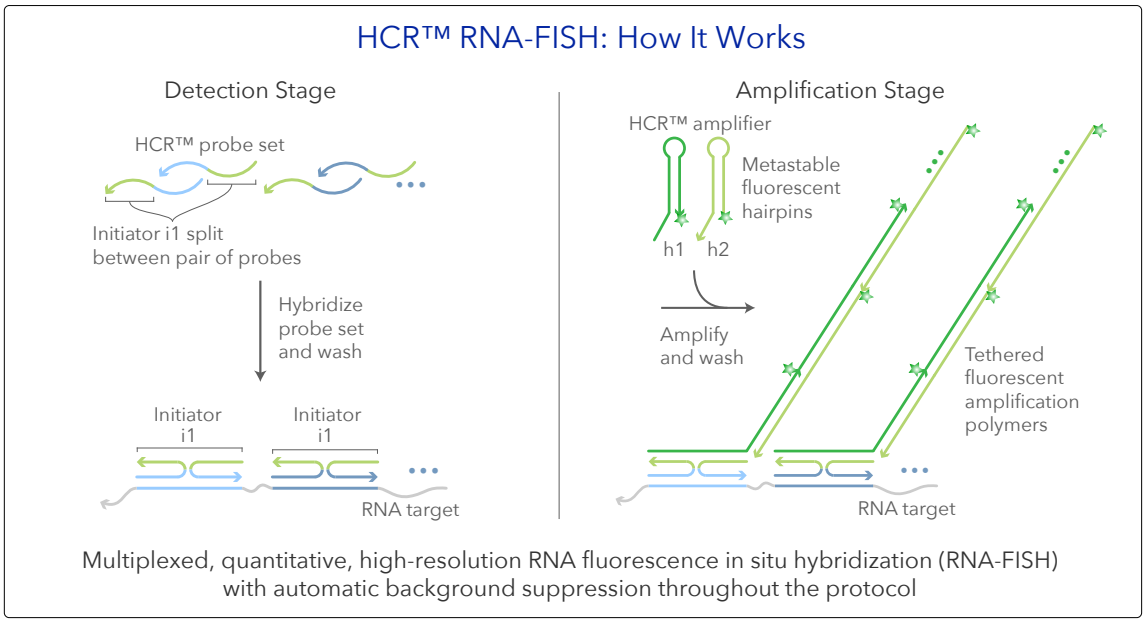

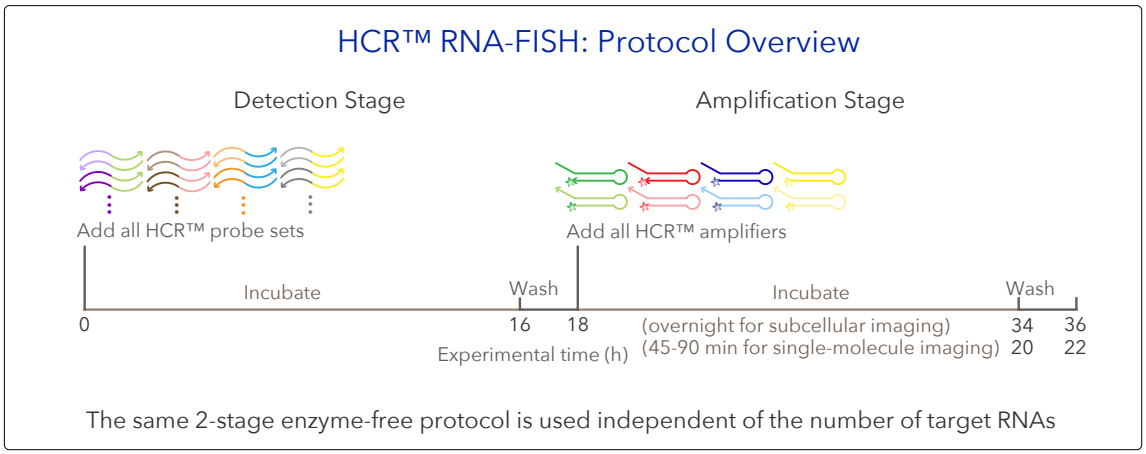

HCR™ RNA-FISH

Multiplexed, quantitative, high-resolution RNA imaging

Multiplexed Experiment

- Order one HCR™ RNA-FISH bundle per target RNA

Example 2-Plex Experiment

- HCR™ RNA-FISH bundle for target mRNA1
  - HCR™ 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™ RNA-FISH bundle for target mRNA2
  - HCR™ probe set: target mRNA2 for use with amplifier B2
  - HCR™ amplifier: B2-488

Storage conditions

- Store HCR™ probe sets, HCR™ amplifiers, HCR™ RNA-FISH probe hybridization buffer, and HCR™ RNA-FISH 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 mammalian cells on a chambered slide

1. Coat bottom of each chamber by applying 300 μL of 0.01% poly-D-lysine prepared in cell culture grade H_2O.
   NOTE: *A volume of 300 μL is sufficient per chamber on an 8-chamber slide.*
2. Incubate for at least 30 min at room temperature.
3. Aspirate the coating solution and wash each chamber twice with molecular biology grade H_2O.
4. Plate desired number of cells in each chamber.
5. Grow cells to desired confluency for 24–48 h.
6. Aspirate growth media and wash each chamber with 300 μL of DPBS.
   NOTE: *avoid using calcium chloride and magnesium chloride in DPBS as this leads to increased autofluorescence.*
7. Add 300 μL of 4% formaldehyde to each chamber.
   CAUTION: *use formaldehyde with extreme care as it is a hazardous material.*
8. Incubate for 10 min at room temperature.
9. Aspirate fixative and wash each chamber 2 $\times$ 300 μL of DPBS.
10. Aspirate DPBS and add 300 μL of ice-cold 70% ethanol.
11. Permeabilize cells overnight at -20 °C.
12. Cells can be stored at -20 °C or 4 °C until use.

Multiplexed HCR™ RNA-FISH protocol

Detection stage

1. Aspirate EtOH and wash samples 2 × 300 μL of 2× SSC.
2. Pre-hybridize samples in 300 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 1.2 pmol of each probe set (e.g. 1.2 μL of 1 μM stock) to 300 μL of probe hybridization buffer 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.*
4. Remove the pre-hybridization solution and add the probe solution.
5. Incubate samples overnight (>12 h) at 37 °C.
6. Remove excess probes by washing 4 × 5 min with 300 μL of probe wash buffer at 37 °C.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *Probe wash buffer should be pre-heated to 37 °C before use.*
7. Wash samples 2 × 5 min with 5× SSCT at room temperature.

Amplification stage

1. Pre-amplify samples in 300 μL of amplification buffer for 30 min at room temperature.
2. Separately prepare 18 pmol of hairpin h1 and 18 pmol of hairpin h2 by snap cooling 6 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 300 μL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate samples overnight (>12 h) in the dark at room temperature.
   NOTE: *For single-molecule detection, a 45 min amplification time was used to ensure single-molecule dots are diffraction-limited.*
6. Remove excess hairpins by washing 5 × 5 min with 300 μL of 5× SSCT at room temperature.
7. Aspirate 5× SSCT and add ≈100 μL of antifade mounting reagent.
8. Samples can be stored at 4 °C protected from light prior to imaging.

Buffer recipes

4% formaldehyde in PBS	For 10 mL of solution
4% formaldehyde	2.5 mL of 16% formaldehyde
1× PBS	1 mL of 10× PBS
	Fill up to 10 mL with molecular biology grade H_2O

5× SSCT	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	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™ IF + HCR™ RNA-FISH**
  HCR™ IF + HCR™ RNA-FISH enables a unified approach to multiplexed, quantitative, high-resolution protein immunofluorescence (IF) and RNA fluorescence in situ hybridization (RNA-FISH), with quantitative 1-step enzyme-free signal amplification performed for all protein and RNA targets simultaneously (Schwarzkopf et al., 2021).

- **HCR™ IF**
  HCR™ IF enables multiplexed, quantitative, high-resolution protein immunofluorescence (IF) 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, subcellular quantitative RNA imaging precision, single-molecule quantitative RNA imaging fidelity) and ease-of-use (no probe set optimization for new targets and organisms) (Choi et al., 2018). Quantitative analysis modes:

  - Subcellular quantitative RNA imaging: analog mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples.
  - Single-molecule quantitative RNA imaging: digital mRNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples.
  - Quantitative 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.

- **Subcellular quantitative RNA imaging**
  Subcellular quantitative 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

- **Single-molecule quantitative RNA imaging**
  Single-molecule quantitative 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).

- **HCR™ northern blots**
  HCR™ 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™ amplifiers: 10× increase in signal, 10× 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™ technology**
  HCR™ amplifiers enable multiplexed, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings (Dirks & Pierce, 2004).