

HCR RNA-FISH v3.0 protocol for samples in solution

This protocol has not been validated for all sample types yet, and should only be used as a template.

Technical support: support@moleculartechnologies.org

Sample pre-treatment

Samples should be prepared in the same manner as for a traditional in situ hybridization, up to the probe hybridization step. This may include permeabilization and protease digestion. Then proceed with the protocol described below.

Multiplexed HCR RNA-FISH v3.0 protocol

Detection stage

1. Pre-hybridize samples in 500 μ L of 30% probe hybridization buffer for 30 min at 37 °C.
CAUTION: probe hybridization buffer contains formamide, a hazardous material.
2. Prepare probe solution by adding 2 pmol of each probe mixture (e.g. 2 μ L of 1 μ M stock) to 500 μ L of 30% 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.
3. Remove the pre-hybridization solution and add the probe solution.
4. Incubate samples overnight (12–16 h) at 37 °C.
5. Remove excess probes by washing 4 \times 15 min with 500 μ L of 30% probe wash buffer at 37 °C.
CAUTION: probe wash buffer contains formamide, a hazardous material.
NOTE: Wash solutions should be pre-heated to 37 °C before use.
6. Wash samples 3 \times 5 min with 5 \times SSCT at room temperature.

Amplification stage

1. Pre-amplify samples in 500 μ L of amplification buffer for 30 min at room temperature.
2. Separately prepare 30 pmol of hairpin H1 and 30 pmol of hairpin H2 by snap cooling 10 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: 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 all snap-cooled hairpins to 500 μ L of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate samples overnight (12–16 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 μ L of 5 \times SSCT at room temperature:
 - (a) 2 \times 5 min
 - (b) 2 \times 30 min
 - (c) 1 \times 5 min

Sample mounting for microscopy

Samples can be mounted in SlowFade Gold antifade mountant inside an imaging chamber tailored for the sample of interest. For whole-mount embryos or larvae, a Zeiss 710 NLO inverted confocal microscope equipped with either an LD LCI Plan-Apochromat 25×/0.8 Imm Corr DIC objective or an LD C-Apochromat 40×/1.1 W Korr M27 objective was used in our lab to acquire images.

HCR reagents storage conditions

Probes, amplifiers, probe hybridization buffer, and probe wash buffer should be stored at -20 °C. Amplification buffer should be stored at 4 °C. Keep these reagents on ice at all times during probe and amplifier preparation. Make sure all solutions are well mixed before use.

General buffer recipes for HCR RNA-FISH

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 H₂O

Reagents and supplies

20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
50% Tween 20 (Life Technologies Cat. # 00-3005)
SlowFade Gold antifade mountant (Life Technologies Cat. # S36936)

Citation Notes

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- **10-plex HCR spectral imaging**
HCR spectral imaging of any combination of 10 RNA and/or protein targets with 1-step quantitative HCR signal amplification for all targets simultaneously (Schulte *et al.*, 2024a).
Software: [HCR Imaging Python module including Dot Detection 2.0 and Unmix 1.0 packages](#).
- **HCR protein:protein complex imaging**
Multiplexed, quantitative, high-resolution imaging of protein:protein complexes with/without HCR IF and/or HCR RNA-FISH with simultaneous HCR signal amplification for all protein:protein, protein, and RNA targets (Schulte *et al.*, 2024b).
- **HCR RNA-FISH/IF**
A unified approach to multiplexed, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) and protein immunofluorescence (IF), with quantitative 1-step enzyme-free HCR signal amplification performed for all RNA and protein targets simultaneously (Schwarzkopf *et al.*, 2021).
- **HCR IF**
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)**
Automatic background suppression for dramatically enhanced performance (signal-to-background and quantitative precision) and ease-of-use (no probe set optimization for new targets) (Choi *et al.*, 2018). v3.0 supports three quantitative modes:
 - qHCR imaging:** analog mRNA relative quantitation with subcellular resolution in an anatomical context;
 - qHCR flow cytometry:** analog mRNA relative quantitation for high-throughput single-cell analysis;
 - dHCR imaging:** digital mRNA absolute quantitation with single-molecule resolution in an anatomical context.
 Protocols for v3.0 in diverse organisms are adapted from the zoo paper.
Software: [Dot Analysis 1.0 package](#).
- **Quantitative HCR (qHCR) imaging with subcellular resolution**
Analog mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos. The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (Trivedi *et al.*, 2018).
Software: [Read-out/Read-in 1.0 package](#).
- **Zoo paper**
Protocols for multiplexed mRNA imaging in diverse sample types (Choi *et al.*, 2016):

bacteria in suspension	whole-mount zebrafish embryos and larvae
whole-mount fruit fly embryos	whole-mount chicken embryos
whole-mount worm larvae	whole-mount mouse embryos
whole-mount sea urchin embryos	FFPE human tissue sections
- **Digital HCR (dHCR) imaging with single-molecule resolution**
Digital mRNA absolute quantitation with single-molecule resolution in thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah *et al.*, 2016).

- **Multiplexed quantitative HCR (qHCR) northern blots**

Simultaneous quantification of RNA target size and abundance with signal amplification for up to 5 target RNAs (Schwarzkopf & Pierce, 2016).

- **HCR RNA-FISH (v2.0)**

2nd generation HCR RNA-FISH technology (v2.0) using DNA 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)**

1st generation HCR RNA-FISH technology (v1.0) using RNA 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 isothermal enzyme-free molecular signal amplification (Dirks & Pierce, 2004).

We are happy to provide advice and technical support: support@moleculartechnologies.org.

– The Molecular Technologies Team

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