HCR™ RNA flow cytometry (v3.0) protocol for bacteria in suspension

This protocol has been optimized for *Escherichia coli* and should only be used as a template for other types of bacteria.

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.

Sample preparation

1. Grow *E. coli* from streaked plate or frozen glycerol stocks in 2–3 mL of LB media overnight in a 37 °C shaker.
2. Dilute to make a 5 mL liquid culture with OD_{600} = 0.05.
3. Incubate in a 37 °C shaker until $OD_{600} \approx 0.5$ (exponential phase).
4. Aliquot 1 mL of cells and centrifuge for 10 min.
   NOTE: *Centrifugation should be as gentle as possible to pellet cells. For E. coli, all centrifugation steps are done at 4000 × g.*
5. Remove supernatant and re-suspend cells in 750 μL of 1× phosphate-buffered saline (PBS).
   NOTE: *remove all solutions via pipetting throughout the protocol.*
6. Add 250 μL of 4% formaldehyde and incubate overnight at 4 °C.
   CAUTION: *use formaldehyde with extreme care as it is a hazardous material.*
7. Centrifuge for 10 min and remove supernatant.
8. Re-suspend cells in 150 μL of 1× PBS.
9. Add 850 μL of 100% MeOH and store cells at -20 °C before use.

Multiplexed HCR™ RNA flow cytometry (v3.0) protocol

Detection stage

1. Transfer 150 μL of cells into a 1.5 mL eppendorf tube.
2. Centrifuge for 5 min and remove supernatant.
3. Wash cells with 500 μL of 1$\times$ PBST. Centrifuge for 5 min and remove the supernatant.
4. Re-suspend the pellet with 400 μL of HCR™ Probe Hybridization Buffer (v3.0) and pre-hybridize for 1 h at 37 °C.
   CAUTION: *HCR™ Probe Hybridization Buffer (v3.0) contains formamide, a hazardous material.*
   NOTE: *HCR™ Probe Hybridization Buffer (v3.0) for cells in suspension will be used in this protocol.*
5. In the meantime, prepare probe solution by adding 2 pmol of each HCR™ Probe (v3.0) (e.g. 2 μL of 1 μM stock) to 100 μL of HCR™ Probe Hybridization Buffer (v3.0) pre-heated to 37 °C.
6. Add the probe solution directly to the sample to reach a final probe concentration of 4 nM.
   NOTE: *use higher probe concentration (e.g., 16 nM) to improve signal for quantitative RNA flow cytometry.*
7. Incubate the sample overnight (>12 h) at 37 °C.
8. Add 1 mL of HCR™ Probe Wash Buffer (v3.0) to the sample to dilute the probes.
   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.*
9. Centrifuge for 5 min and remove the wash solution.
10. Re-suspend the cell pellet with 500 μL of HCR™ Probe Wash Buffer (v3.0) (pre-heated to 37 °C).
11. Incubate for 5 min at 37 °C and remove the wash solution by centrifugation for 5 min.
12. Repeat steps 10 and 11 for two additional times but with 10 min incubation.
13. Proceed to hairpin amplification.

Amplification stage

1. Re-suspend the cell pellet with 150 μL of HCR™ Amplifier Buffer (v3.0) and pre-amplify for 30 min at room temperature.
   NOTE: *HCR™ Amplifier Buffer (v3.0) for cells in suspension will be used in this protocol. Equilibrate HCR™ Amplifier Buffer (v3.0) to room temperature before use.*
2. Separately prepare 15 pmol of hairpin h1 and 15 pmol of hairpin h2 by snap cooling 5 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 100 μL of HCR™ Amplifier Buffer (v3.0) at room temperature.

4. Add the hairpin solution directly to the sample to reach a final hairpin concentration of 60 nM.
5. Incubate the sample overnight (>12 h) in the dark at room temperature.
6. Add 1 mL of 5× SSCT at room temperature to the sample to dilute the hairpins.
7. Centrifuge for 5 min and remove the wash solution.
8. Re-suspend the cell pellet with 500 μL of 5× SSCT.
9. Incubate for 5 min at room temperature and remove the wash solution by centrifugation for 5 min.
10. Repeat steps 8 and 9 for two additional times but with 10 min incubation.
11. Re-suspend the cell pellet in desired buffer (e.g. 5× SSCT) and volume.
12. Samples can be stored at 4 °C protected from light before microscopy or flow cytometry.
    NOTE: *filter cells before flow cytometry.*

Buffer recipes

LB media
5 g of Novagen LB Broth Miller powder
Fill up to 200 mL with ultrapure H_2O
Autoclave at 121 °C for 20 min

4% formaldehyde
4% formaldehyde
1× PBS

For 4 mL of solution
1 mL of 16% formaldehyde
0.4 mL of 10× PBS
Fill up to 4 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: *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:

- **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).

- **HCR™ RNA-FISH**
  - Third-generation HCR™ RNA-FISH (v3.0) enables multiplex, 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).
  - Second-generation HCR™ RNA-FISH (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).
  - First-generation HCR™ RNA-FISH (v1.0) using RNA HCR™ Probes and RNA HCR™ Amplifiers: multiplex mRNA imaging in whole-mount vertebrate embryos with simultaneous signal amplification for up to 5 target mRNAs (Choi et al., 2010).

- **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).