HCR RNA-FISH v3.0 protocol for bacteria in suspension (*Escherichia coli*)

This protocol has not been validated for all types of bacteria and should only be used as a template.
Technical support: support@moleculartechnologies.org

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.

Buffer recipes for sample preparation

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 in PBS
4% formaldehyde
1× PBS

For 10 mL of solution
2.5 mL of 16% formaldehyde
1 mL of 10× PBS
Fill up to 10 mL with ultrapure H_2O

NOTE: *Avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the samples.*

Multiplexed HCR RNA-FISH 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 and remove the solution by centrifugation.
4. Re-suspend the pellet with 400 μL of 30% LMW probe hybridization buffer and pre-hybridize for 1 hr at 37 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
   NOTE: *Pre-heat probe hybridization buffer to 37 °C before use.*
5. In the meantime, prepare probe solution by adding 2 pmol of each probe mixture (e.g. 2 μL of 1 μM stock) to 100 μL of 30% LMW probe hybridization buffer at 37 °C.
6. Add the probe mixture directly to the sample to reach a final probe concentration of 4 nM.
   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.*
7. Incubate the sample overnight at 37 °C.
8. Add 1mL of probe wash buffer to the sample.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *Pre-heat probe wash buffer 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 probe wash buffer (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 LMW amplification buffer and pre-amplify for 30 min at room temperature.
   NOTE: *Equilibrate amplification buffer 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: *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 mixture by adding all snap-cooled hairpins to 100 μL of amplification buffer at room temperature.
4. Add the hairpin mixture 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.
   NOTE: *For dHCR imaging, amplify for a shorter period of time to ensure single-molecule dots are diffraction-limited.*
6. Add 1 mL of 5× SSCT at room temperature to the sample to dilute the solution.
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 500 μL of 5× SSCT.
12. Store the sample at 4 °C before imaging.

Sample mounting for microscopy

1. Add 50 μL of SlowFade Gold antifade mountant to each sample.
2. Pipette 50 μL of 0.1% (w/v) poly-L-lysine onto a 22 mm × 22 mm coverslip.
3. Allow to sit for 10 min and tap off extra solution. Allow it to air dry at room temperature.
4. Add 10 μL of sample to the middle of the coverslip.
5. Place the sample side of the coverslip on top of a 25 mm × 75 mm glass slide. Lower the coverslip carefully to avoid air bubbles.
6. Sample slide could be imaged on a wide-field fluorescent microscope. A Zeiss Axio Observer Z1 fluorescent inverted microscope equipped with an Plan-Apochromat 100×/1.4 Oil DIC objective was used in our lab to acquire bacterial images. Samples were illuminated with X-Cite Series 120 (EXFO Photonics) and images were collected with an ImagEM-1K EM-CCD digital camera (Hamamatsu).

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	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

1× PBST	For 40 mL of solution
1× PBS	10 mL of 10× PBST (0.5% Tween 20)
0.1% Tween 20	200 μL of 10% Tween 20
	Fill up to 40 mL with ultrapure H_2O

Reagents and supplies

LB Broth Miller (Novagen Cat. # 71753-5)
10× PBS (Life Technologies Cat.# AM9625)
10× PBST (Rockland Cat. # MB-075-1000)
Methanol (Mallinckrodt Chemicals Cat. # 3016-16)
16% Formaldehyde (w/v), methanol-free (Life Technologies Cat. # 28908)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
10% Tween 20 solution (Bio-Rad Cat. # 161-0781)
25 mm × 75 mm glass slide (VWR Cat. # 48300-025)
22 mm × 22 mm No. 1 coverslip (VWR Cat. # 48366-067)
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

- **Digitial 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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