

HCR RNA flow cytometry 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

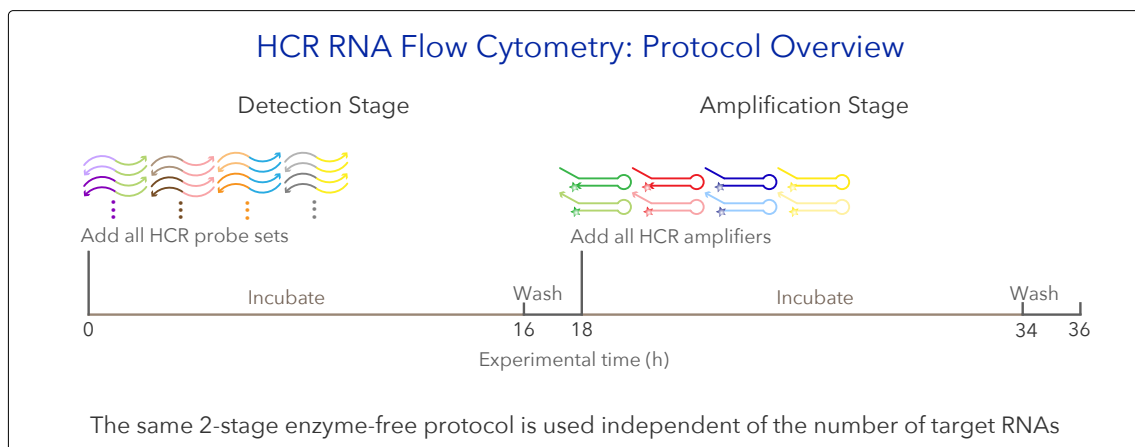
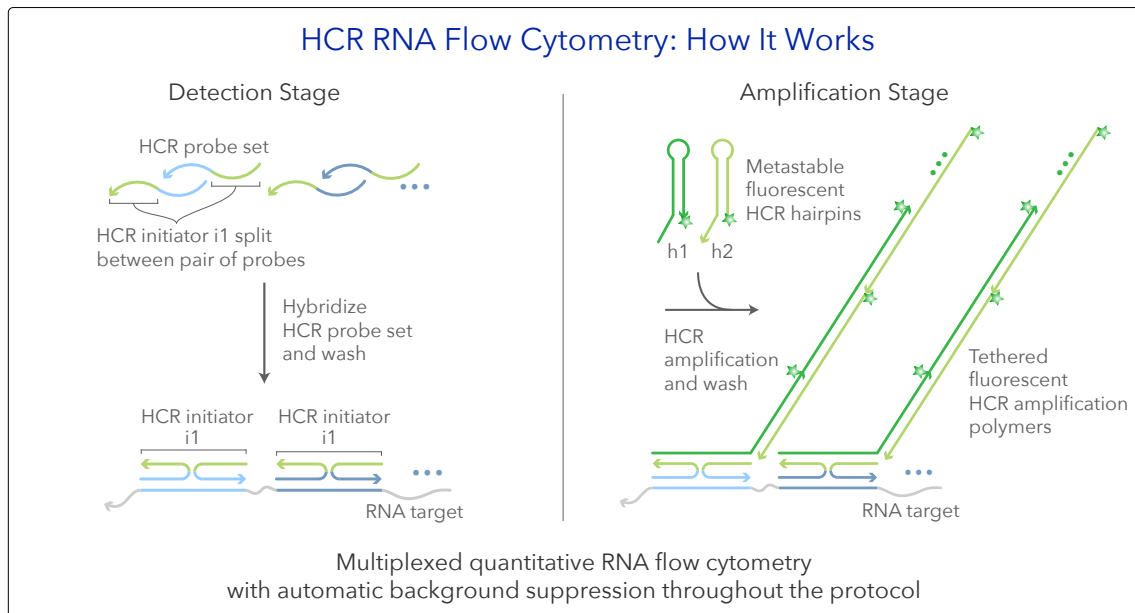
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HCR RNA Flow Cytometry

Multiplexed quantitative RNA flow cytometry

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.

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 $4000 \times g$.
5. Remove supernatant and re-suspend cells in 750 μL of $1 \times$ 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 $1 \times$ PBS.
9. Add 850 μL of 100% MeOH and store cells at -20 °C before use.

Multiplexed HCR RNA flow cytometry 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 probe hybridization buffer and pre-hybridize for 1 h at 37 $^{\circ}\text{C}$.
CAUTION: probe hybridization buffer contains formamide, a hazardous material.
NOTE: Probe hybridization buffer for cells in suspension will be used in this protocol.
5. In the meantime, prepare probe solution by adding 2 pmol of each probe set (e.g. 2 μL of 1 μM stock) to 100 μL of probe hybridization buffer pre-heated to 37 $^{\circ}\text{C}$.
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 qHCR flow cytometry.
7. Incubate the sample overnight at 37 $^{\circ}\text{C}$.
8. Add 1 mL of probe wash buffer to the sample to dilute the probes.
CAUTION: probe wash buffer contains formamide, a hazardous material.
NOTE: pre-heat probe wash buffer to 37 $^{\circ}\text{C}$ 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 $^{\circ}\text{C}$).
11. Incubate for 5 min at 37 $^{\circ}\text{C}$ 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 amplification buffer and pre-amplify for 30 min at room temperature.
NOTE: Amplification buffer for cells in suspension will be used in this protocol. 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 $^{\circ}\text{C}$ 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 100 μL of amplification buffer 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 μ L 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 H₂O

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 H₂O

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

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× 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 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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