HCR 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
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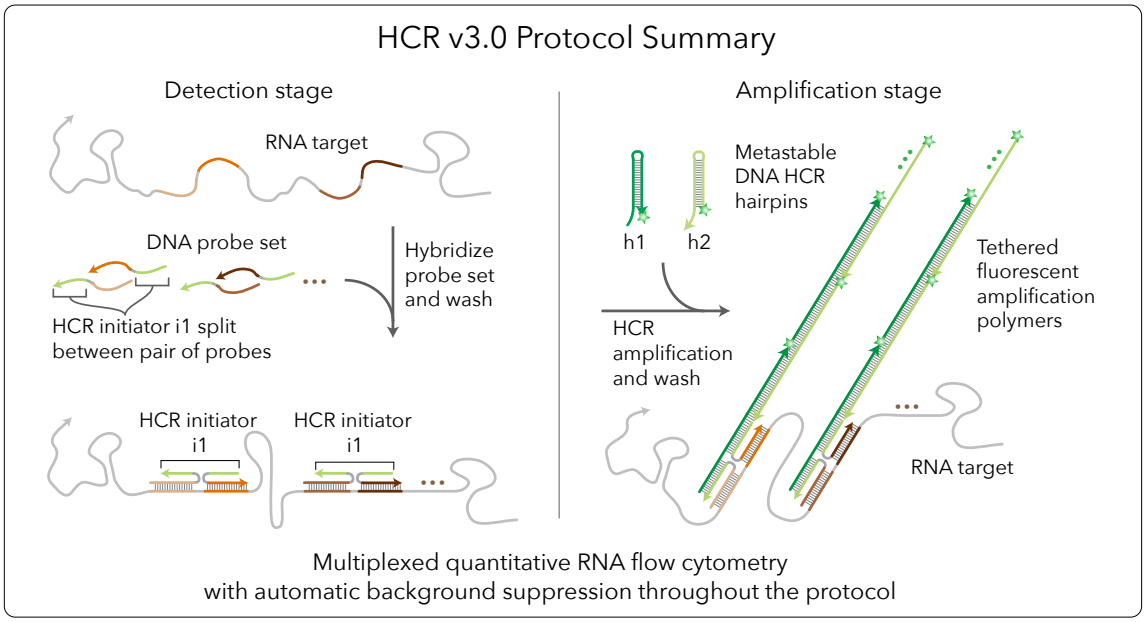
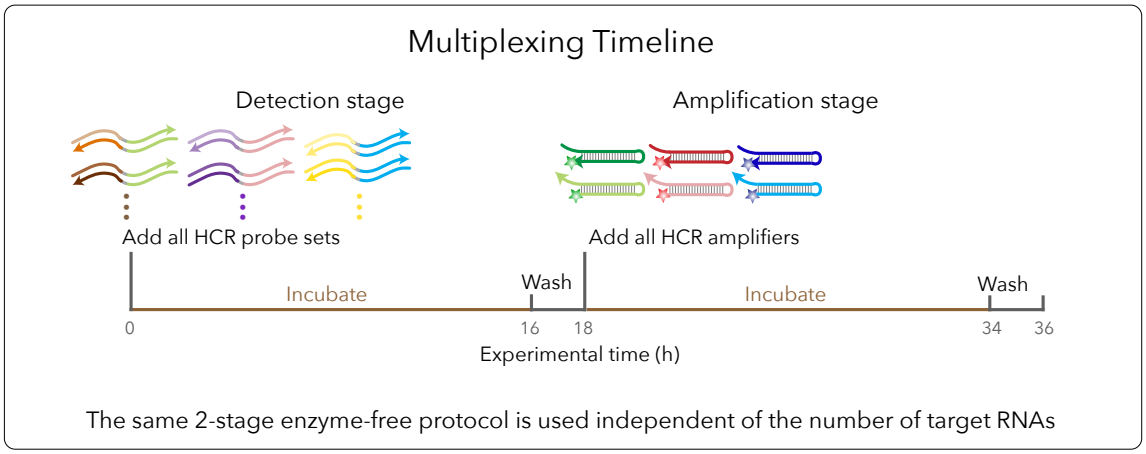
Storage conditions

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 × 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
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

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

Multiplexed HCR 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 probe hybridization buffer (with low M.W. dextran sulfate) and pre-hybridize for 1 h at 37 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
   NOTE: *Probe hybridization buffer with low M.W. dextran sulfate will be used in this protocol.*
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 probe hybridization buffer pre-heated to 37 °C.
6. Add the probe mixture directly to the sample to reach a final probe concentration of 4 nM.
7. Incubate the sample overnight at 37 °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 °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 amplification buffer (with low M.W. dextran sulfate) and pre-amplify for 30 min at room temperature.
   NOTE: *Amplification buffer with low M.W. dextran sulfate 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 °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 snap-cooled h1 hairpins and snap-cooled h2 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.
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.*

Citation Notes

For citation, please select from the list below as appropriate for your application:

- **HCR v3.0**
  Automatic background suppression 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 imaging: analog mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples.

  qHCR flow cytometry: analog mRNA relative quantitation for high-throughput expression profiling of mammalian and bacterial cells.

  dHCR imaging: digital mRNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples.

  Protocols for v3.0 in diverse organisms are adapted from the Zoo paper.

- **qHCR imaging**
  mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos; read-out/read-in analysis framwork (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 suspension	generic sample on slide
whole-mount chicken embryos	whole-mount fruit fly embryos
whole-mount mouse embryos	whole-mount sea urchin embryos
whole-mount worm larvae	whole-mount zebrafish embryos and larvae

- **dHCR imaging**
  Single-molecule mRNA imaging in thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah *et al.*, 2016).

- **qHCR northern blot**
  Simultaneous quantification of RNA target size and abundance for up to 5 target RNAs (Schwarzkopf & Pierce, 2016).

- **HCR v2.0**
  2nd generation in situ HCR 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 v1.0**
  1st generation in situ HCR 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**
  Isothermal enzyme-free molecular signal amplification (Dirks & Pierce, 2004).

References

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Choi, H. M. T., Beck, V. A., & Pierce, N. A. (2014). Next-generation in situ hybridization chain reaction: higher gain, lower cost, greater durability. *ACS Nano*, **8**(5), 4284–4294.

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