

HCR v3.0 protocol for mammalian cells in suspension

This protocol has not been validated for all mammalian cell types and should only be used as a template.

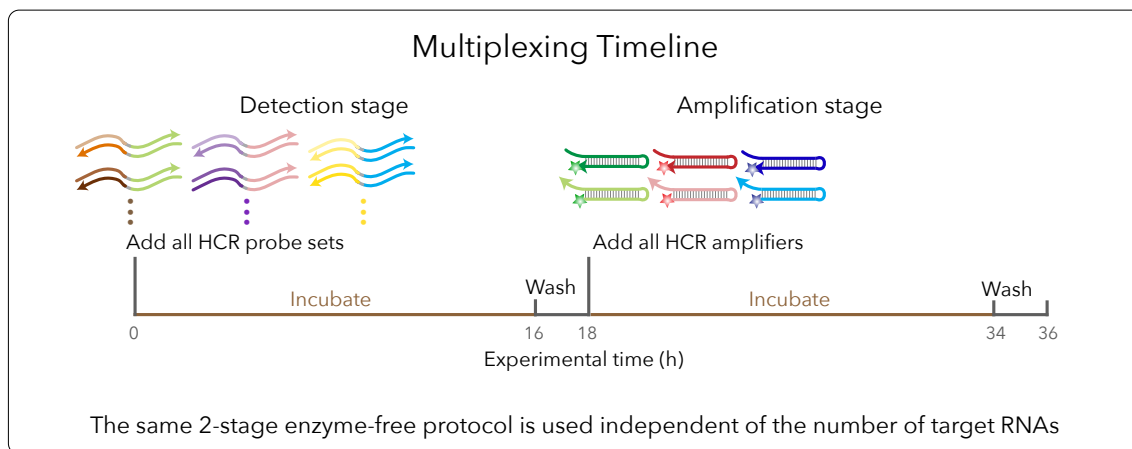
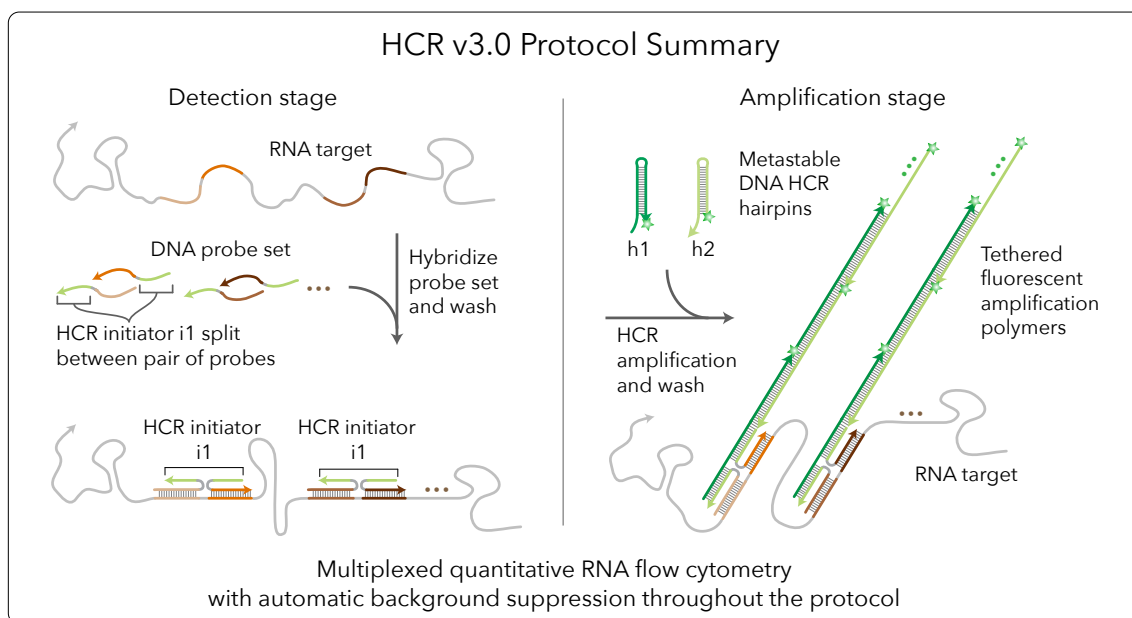
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Storage conditions

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 protocol

1. Aspirate growth media from culture plate and wash cells with DPBS.
NOTE: avoid using calcium chloride and magnesium chloride in DPBS as this leads to increased autofluorescence.
2. Add 3 mL of trypsin per 10 cm plate and incubate in a 5% CO₂ incubator at 37 °C for 5 min.
3. Quench trypsin by adding 3 mL of growth media.
4. Transfer cells to a 15 mL conical tube and centrifuge for 5 min at 180 × g.
5. Aspirate supernatant and re-suspend cells in 4% formaldehyde to reach approximately 10⁶ cells/mL.
CAUTION: use formaldehyde with extreme care as it is a hazardous material.
6. Fix cells for at least 1 hr at room temperature.
7. Centrifuge for 5 min at 180 × g and aspirate supernatant.
8. Wash cells 4 times with PBST (use the same volume as formaldehyde solution).
9. Re-suspend cells in cold 70% ethanol (use the same volume as formaldehyde and PBST solutions).
10. Store cells at 4 °C overnight before use.

Buffer recipes for sample preparation

4% formaldehyde in PBST

4% formaldehyde
1 × PBS
0.1% Tween 20

For 10 mL of solution

2.5 mL of 16% formaldehyde
1 mL of 10× PBS
100 μL of 10% Tween 20
Fill up to 10 mL with ultrapure H₂O

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 desired amount ($0.5-1 \times 10^6$) of fixed cells into a 1.5 mL tube.
2. Centrifuge for 5 min and remove supernatant.
NOTE: Centrifugation should be as gentle as possible to pellet cells. All centrifugation steps are done at $180 \times g$.
3. Wash cells twice with 500 μ L of PBST. Centrifuge for 5 min to remove supernatant.
4. Re-suspend the pellet with 400 μ L of probe hybridization buffer and pre-hybridize for 30 min at 37 °C.
CAUTION: probe hybridization buffer contains formamide, a hazardous material.
5. In the meantime, prepare probe solution by adding 2 pmol of each probe mixture (e.g. 2 μ L of 1 μ M stock) to 100 μ L of probe hybridization buffer pre-heated to 37 °C.
6. Add the probe solution directly to the sample to reach a final probe concentration of 4 nM.
7. Incubate the sample overnight at 37 °C.
8. Centrifuge for 5 min to remove probe solution.
9. Re-suspend the cell pellet with 500 μ L of probe wash buffer.
CAUTION: probe wash buffer contains formamide, a hazardous material.
NOTE: Probe wash buffer should be pre-heated to 37° C before use.
10. Incubate for 10 min at 37 °C and remove the wash solution by centrifugation for 5 min.
11. Repeat steps 9 and 10 for three additional times.
12. Re-suspend the cell pellet with 500 μ L of 5 \times SSCT.
13. Incubate for 5 min at room temperature.
14. Proceed to hairpin amplification.

Amplification stage

1. Centrifuge for 5 min to pellet the cells.
2. Re-suspend the cell pellet with 150 μ L of amplification buffer and pre-amplify for 30 min at room temperature.
3. Separately prepare 15 pmol of hairpin h1 and 15 pmol of hairpin h2 by snap cooling 5 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.
4. Prepare hairpin mixture by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 100 μ L of amplification buffer at room temperature.
5. Add the hairpin mixture directly to the sample to reach a final hairpin concentration of 60 nM.
6. Incubate the sample overnight (>12 h) in the dark at room temperature.

7. Centrifuge for 5 min and remove the hairpin solution.
8. Re-suspend the cell pellet with 500 μ L of 5 \times SSCT.
9. Without incubation, remove the wash solution by centrifugation for 5 min.
10. Repeat steps 8 and 9 for five additional times.
11. Re-suspend the cell pellet in desired buffer and volume.
12. 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 framework (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).

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