

## 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.

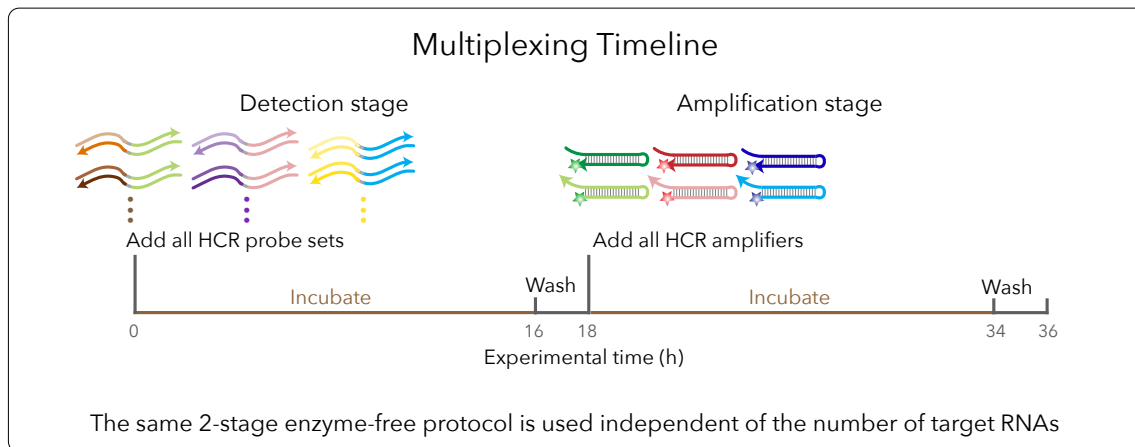
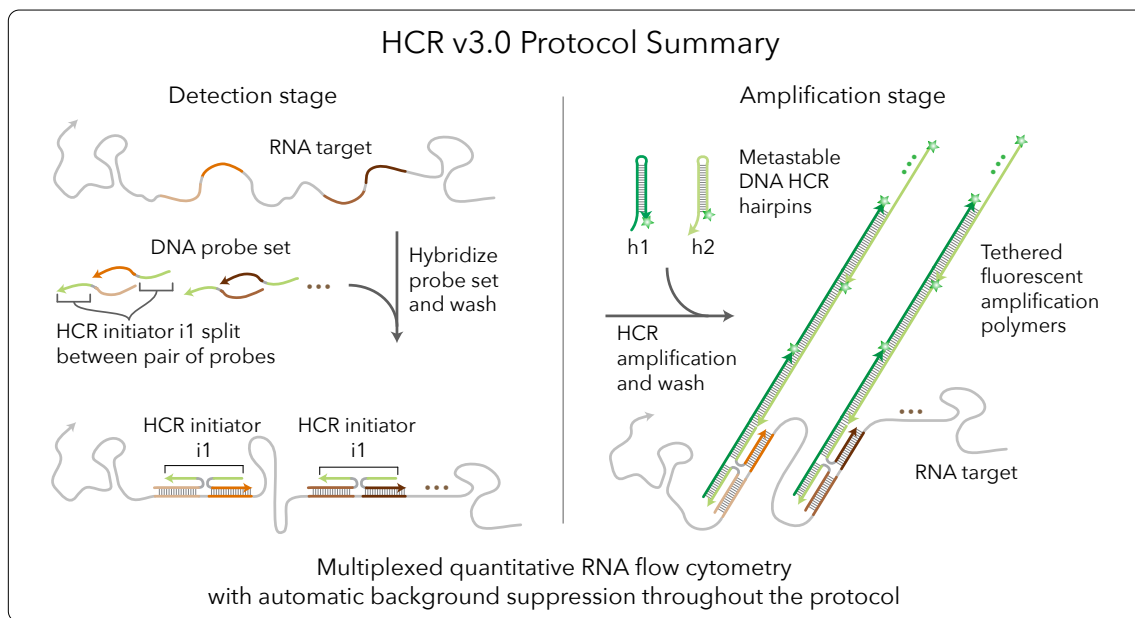
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### Storage conditions

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% CO<sub>2</sub> 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 10<sup>6</sup> 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 H<sub>2</sub>O

*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  $\mu\text{L}$  of PBST. Centrifuge for 5 min to remove supernatant.
4. Re-suspend the pellet with 400  $\mu\text{L}$  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 set (e.g. 2  $\mu\text{L}$  of 1  $\mu\text{M}$  stock) to 100  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{L}$  of 3  $\mu\text{M}$  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 solution by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 100  $\mu\text{L}$  of amplification buffer at room temperature.
5. Add the hairpin solution 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  $\mu$ 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).

## References

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