HCR v3.0 protocol for mammalian cells on a chambered slide

This protocol has not been validated for all mammalian cell types and should only be used as a template.
Technical support: support@molecularinstruments.com
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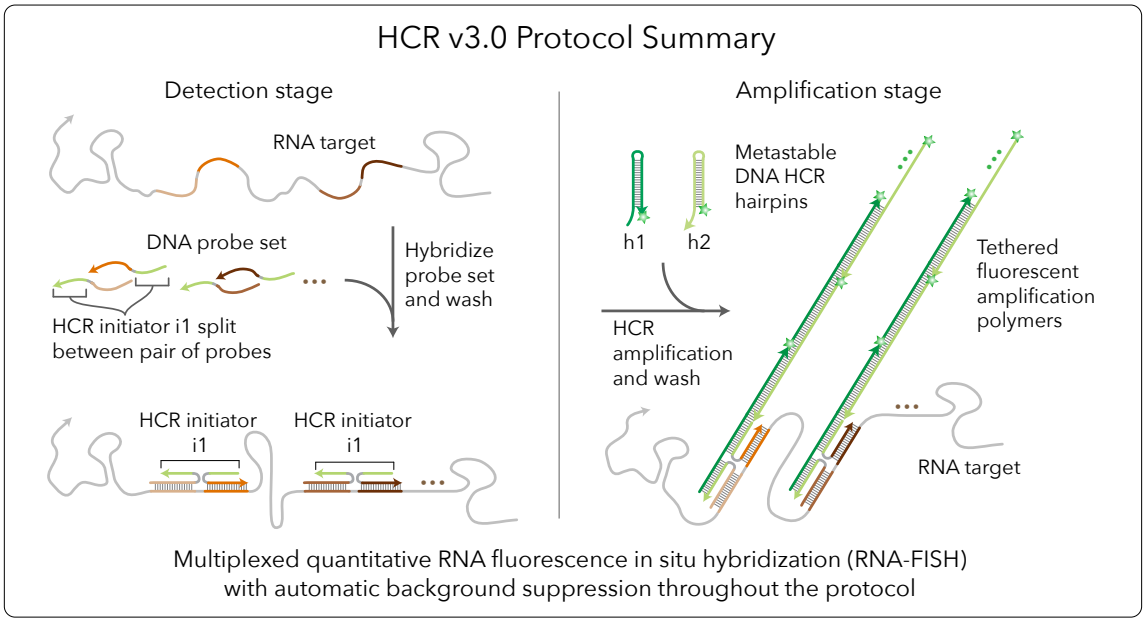
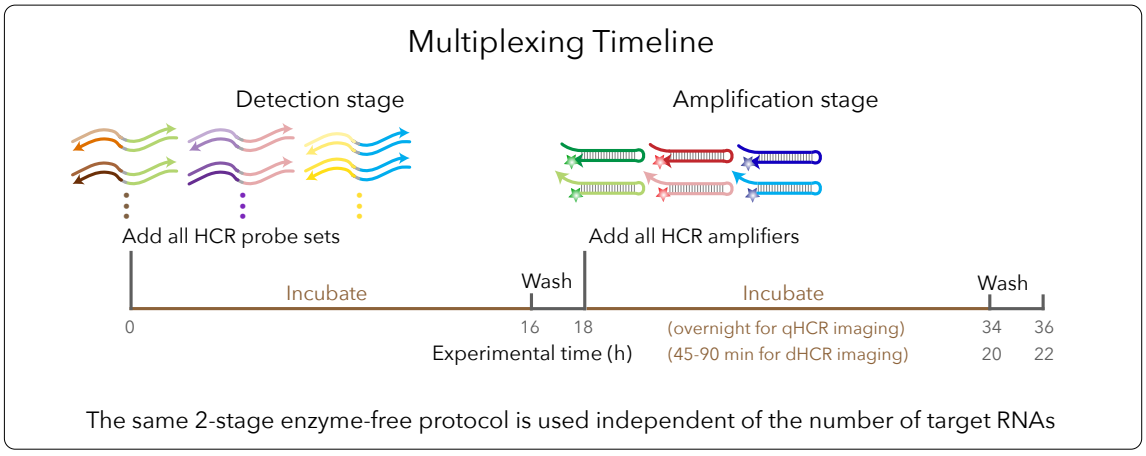
Storage conditions

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.





Preparation of fixed mammalian cells on a chambered slide

1. Coat bottom of each chamber by applying 300 μL of 0.01% poly-D-lysine prepared in cell culture grade H_2O.
   NOTE: *A volume of 300 μL is sufficient per chamber on an 8-chamber slide.*
2. Incubate for at least 30 min at room temperature.
3. Aspirate the coating solution and wash each chamber twice with molecular biology grade H_2O.
4. Plate desired number of cells in each chamber.
5. Grow cells to desired confluency for 24–48 h.
6. Aspirate growth media and wash each chamber with 300 μL of DPBS.
   NOTE: *avoid using calcium chloride and magnesium chloride in DPBS as this leads to increased autofluorescence.*
7. Add 300 μL of 4% formaldehyde to each chamber.
   CAUTION: *use formaldehyde with extreme care as it is a hazardous material.*
8. Incubate for 10 min at room temperature.
9. Aspirate fixative and wash each chamber 2 $\times$ 300 μL of DPBS.
10. Aspirate DPBS and add 300 μL of ice-cold 70% ethanol.
11. Permeabilize cells overnight at -20 °C.
12. Cells can be stored at -20 °C or 4 °C until use.

Buffer recipes for sample preparation

4% formaldehyde in PBS	For 10 mL of solution
4% formaldehyde	2.5 mL of 16% formaldehyde
1$\times$ PBS	1 mL of 10$\times$ PBS
	Fill up to 10 mL with molecular biology grade 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. Aspirate EtOH and air dry samples at room temperature.
2. Wash samples 2 × 300 μL of 2× SSC.
3. Pre-hybridize samples in 300 μL of probe hybridization buffer for 30 min at 37 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
4. Prepare probe solution by adding 1.2 pmol of each probe set (e.g. 1.2 μL of 1 μM stock) to 300 μL of probe hybridization buffer at 37 °C.
   NOTE: *For single-molecule detection, 3 pmol of each probe was used to improve probe hybridization efficiency.*
5. Remove the pre-hybridization solution and add the probe solution.
6. Incubate samples overnight (12–16 h) at 37 °C.
7. Remove excess probes by washing 4 × 5 min with 300 μL of probe wash buffer at 37 °C.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *Probe wash buffer should be pre-heated to 37 °C before use.*
8. Wash samples 2 × 5 min with 5× SSCT at room temperature.

Amplification stage

1. Pre-amplify samples in 300 μL of amplification buffer for 30 min at room temperature.
2. Separately prepare 18 pmol of hairpin h1 and 18 pmol of hairpin h2 by snap cooling 6 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 solution by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 300 μL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate samples overnight (12–16 h) in the dark at room temperature.
   NOTE: *For single-molecule detection, a 45 min amplification time was used to ensure single-molecule dots are diffraction-limited.*
6. Remove excess hairpins by washing 5 × 5 min with 300 μL of 5× SSCT at room temperature.
7. Aspirate 5× SSCT and add ≈100 μL of antifade mounting reagent.
8. Samples can be stored at 4 °C protected from light prior to imaging.

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).

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