HCR v3.0 protocol for sample in solution

This protocol has not been validated for all sample types yet, and should only be used as a template.
Technical support: support@molecularinstruments.com
Safety data sheets (SDS): www.molecularinstruments.com/safety
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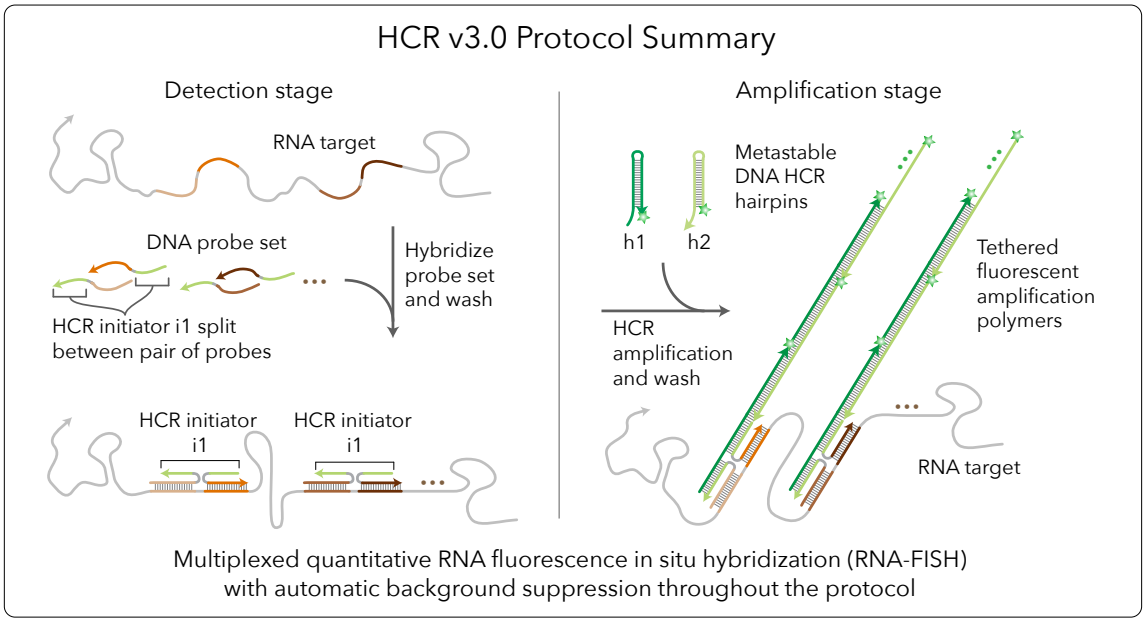
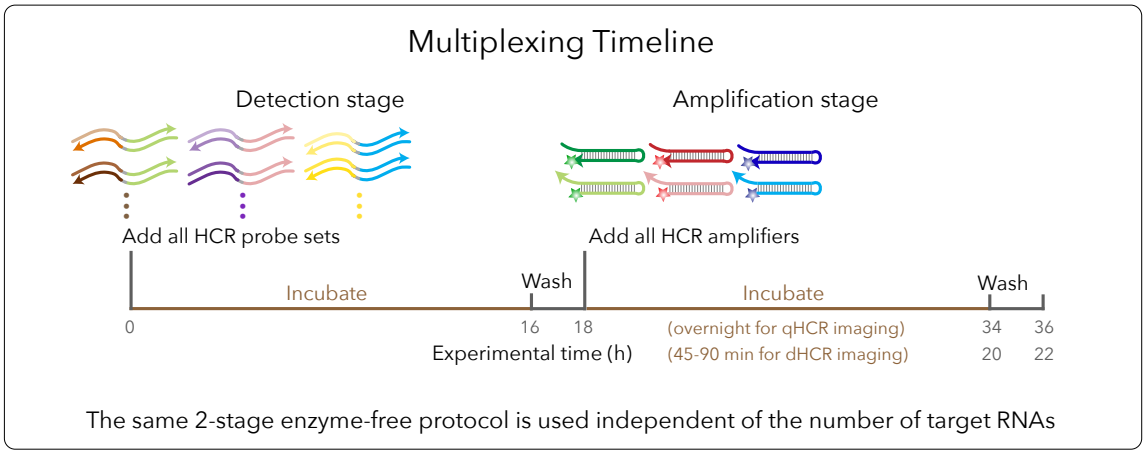
Storage conditions

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

Samples should be prepared in the same manner as for a traditional in situ hybridization, up to the probe hybridization step. This may include permeabilization and protease digestion. Then proceed with the protocol described below.

Multiplexed HCR v3.0 protocol

Detection stage

1. Pre-hybridize samples in 500 μL of probe hybridization buffer for 30 min at 37 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
2. Prepare probe solution by adding 2 pmol of each probe mixture (e.g. 2 μL of 1 μM stock) to 500 μL of probe hybridization buffer at 37 °C.
   NOTE: *For dHCR imaging, use higher concentration of probe to improve probe hybridization efficiency.*
3. Remove the pre-hybridization solution and add the probe solution.
4. Incubate samples overnight (12–16 h) at 37 °C.
5. Remove excess probes by washing 4 × 15 min with 500 μL of probe wash buffer at 37 °C.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *Wash solutions should be pre-heated to 37 °C before use.*
6. Wash samples 3 × 5 min with 500 μL of 5× SSCT at room temperature.

Amplification stage

1. Pre-amplify samples in 500 μL of amplification buffer for 30 min at room temperature.
2. Separately prepare 30 pmol of hairpin h1 and 30 pmol of hairpin h2 by snap cooling 10 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 500 μL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin mixture.
5. Incubate samples overnight (12–16 h) in the dark at room temperature.
   NOTE: *For dHCR imaging, amplify for a shorter period of time to ensure single-molecule dots are diffraction-limited.*

6. Remove excess hairpins by washing with 500 μL of 5$\times$ SSCT at room temperature:
   (a) 2 $\times$ 5 min
   (b) 2 $\times$ 30 min
   (c) 1 $\times$ 5 min
7. Samples can be stored at 4 °C protected from light before microscopy.

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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