HCR v3.0 protocol for FFPE human tissue sections (*Homo sapiens sapiens*)

This protocol has not been optimized for all tissue types 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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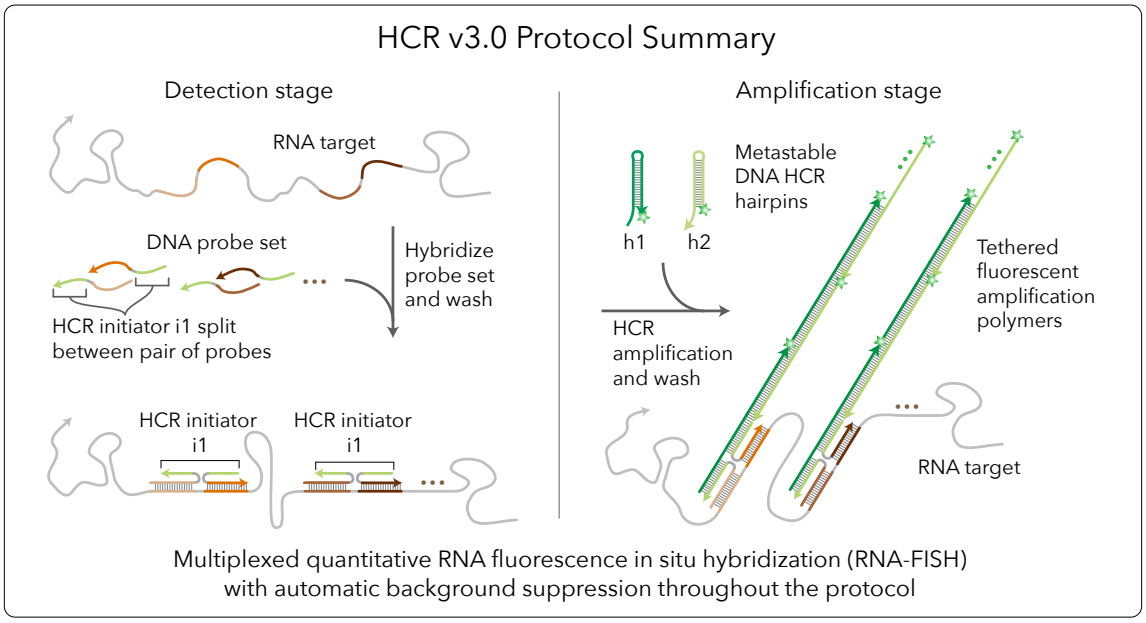
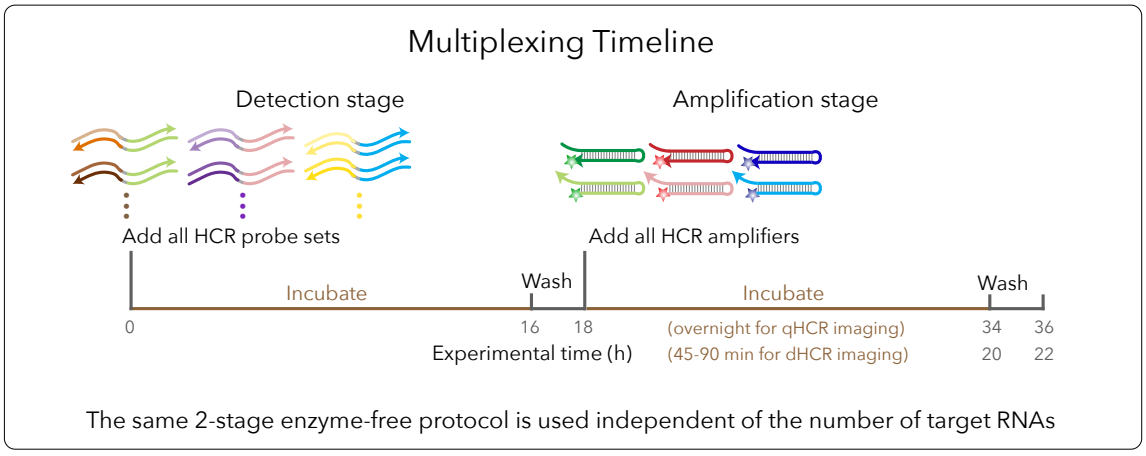
Storage conditions

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 protocol

1. Bake slides in a dry oven for 1 h at 60 °C to improve sample adhesion to the slide.
2. In a fume hood, deparaffinize FFPE tissue by immersing slide in xylene for 2 × 5 min. Move slides up and down occasionally.
   CAUTION: *use xylene with care as it is a hazardous material.*
   NOTE: *Each 50 mL tube can fit two outward-facing slides. A volume of 30 mL is sufficient to immerse sections in the tube. If desired, a larger number of slides can be processed together using a Coplin jar.*
3. Incubate slides in 100% ethanol for 2 × 2 min at room temperature. Move slides up and down occasionally.
4. Dry slide by blotting edges on a Kimwipe.
5. Allow slides to air dry at room temperature.
6. Introduce 200 μL of 10 μg/mL of proteinase K solution on top of sample and incubate slides in a humidified chamber for 10 min at 37 °C.
   NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K.*
7. Immerse slides in nanopure water. Move slides up and down occasionally.
8. Dry slide by blotting edges on a Kimwipe.
9. Proceed immediately to HCR assay.
   NOTE: *do not let tissues dry out.*

Multiplexed HCR v3.0 protocol

Detection stage

1. Add 200 μL of probe hybridization buffer on top of the tissue sample.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
2. Pre-hybridize for 10 min inside the 37 °C humidified chamber.
3. Prepare probe solution by adding 0.4 pmol of each probe set (e.g. 0.4 μL of 1 μM stock) to 100 μL of probe hybridization buffer at 37 °C.
   NOTE: *For dHCR imaging, use higher concentration of probe to improve probe hybridization efficiency.*
4. Remove the pre-hybridization solution and drain excess buffer on slide by blotting edges on a Kimwipe.
5. Add 50–100 μL of the probe solution on top of the tissue sample.
   NOTE: *Amount of probe solution depends on the size of the tissue.*
6. Place a coverslip on the sample and incubate overnight (12–16 h) in the 37 °C humidified chamber.
7. Immerse slide in probe wash buffer at 37 °C to float off coverslip.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
8. Remove excess probes by incubating slide at 37 °C in:
   (a) 75% of probe wash buffer / 25% 5× SSCT for 15 min
   (b) 50% of probe wash buffer / 50% 5× SSCT for 15 min
   (c) 25% of probe wash buffer / 75% 5× SSCT for 15 min
   (d) 100% 5× SSCT for 15 min

   NOTE: *Wash solutions should be pre-heated to 37 °C before use.*
9. Immerse slide in 5× SSCT for 5 min at room temperature.

Amplification stage

1. Dry slide by blotting edges on a Kimwipe.
2. Add 200 μL of amplification buffer on top of the tissue sample and pre-amplify in a humidified chamber for 30 min at room temperature.
3. Separately prepare 6 pmol of hairpin h1 and 6 pmol of hairpin h2 by snap cooling 2 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.*
4. Prepare hairpin solution by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 100 μL of amplification buffer at room temperature.
5. Remove the pre-amplification solution and drain excess buffer on slide by blotting edges on a Kimwipe.

6. Add 50–100 μL of the hairpin solution on top of the tissue sample.
   NOTE: *Amount of hairpin solution depends on the size of the tissue.*
7. Incubate overnight (12–16 h) in a dark humidified chamber at room temperature.
   NOTE: *For dHCR imaging, amplify for a shorter period of time to ensure single-molecule dots are diffraction-limited.*
8. Remove excess hairpins by incubating slide in 5 $\times$ SSCT at room temperature for:
   (a) 1 $\times$ 5 min
   (b) 2 $\times$ 15 min
   (c) 1 $\times$ 5 min
9. Dry slide by blotting edges on a Kimwipe.
10. Add 50–100 μL of antifade mounting reagent on top of the tissue.
11. Place a coverslip on top for 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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