HCR v3.0 protocol for fresh frozen or fixed frozen tissue sections

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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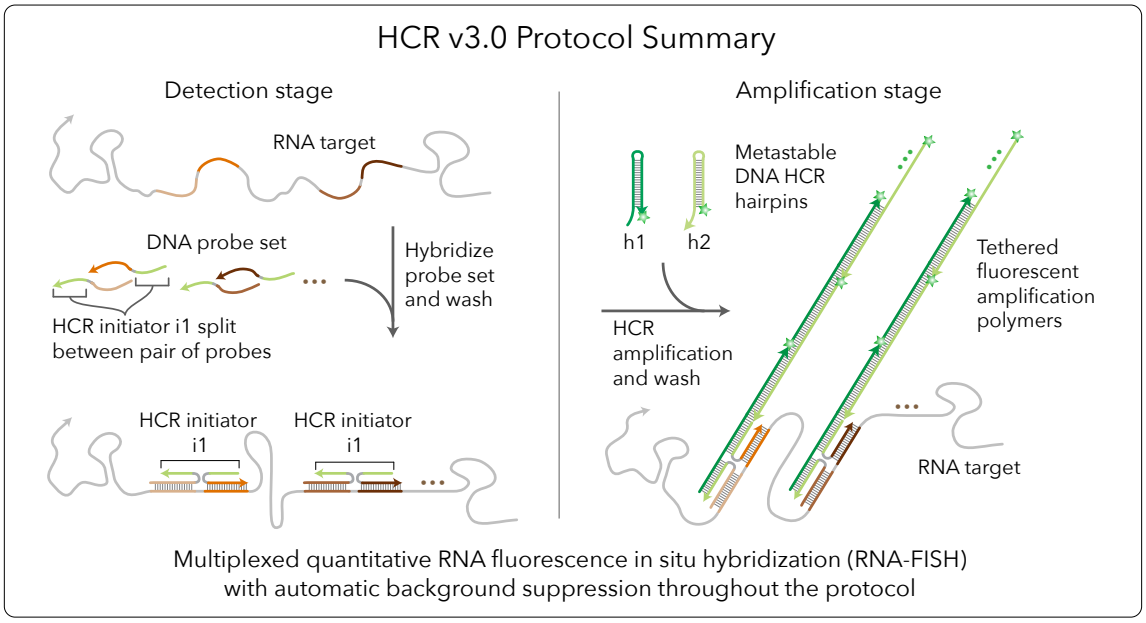
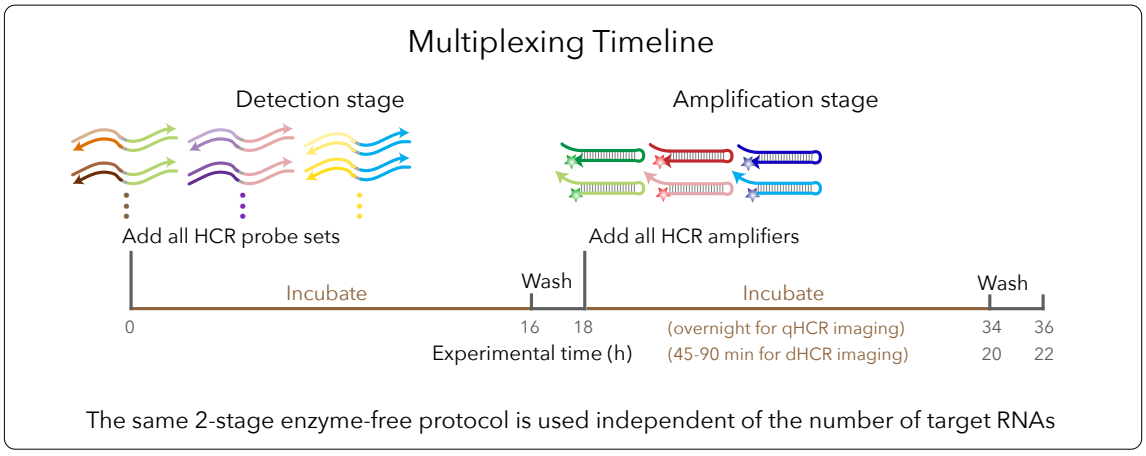
Storage conditions

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 protocol

1. Remove frozen sections on slide from -80 °C.
2. Fix tissues by immersing slides in ice-cold 4% paraformaldehye (PFA) for 15 min at 4 °C.
   CAUTION: *use PFA with extreme care as it is a hazardous material.*
   NOTE: *use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.*
   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. Immerse slides in 50% EtOH for 5 min at room temperature.
4. Immerse slides in 70% EtOH for 5 min at room temperature.
5. Immerse slides in 100% EtOH for 5 min at room temperature.
6. Immerse slides in another fresh 100% EtOH for 5 min at room temperature.
7. Remove slides and allow them to air day for 5 min at room temperature.
8. 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.*
9. Wash slides by immersing in 1$\times$ PBS.
   NOTE: *avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the tissue.*
10. Repeat with fresh 1$\times$ PBS.
11. Proceed to HCR assay.

Buffer recipes for sample preparation

4% paraformaldehyde (PFA)	For 30 mL of solution
4% PFA	7.5 mL of 16% PFA solution
1× PBS	3 mL of 10× PBS
	Fill up to 30 mL with water

Proteinase K solution	For 1 mL of solution
10 μg/mL proteinase K	0.5 μL of 20 mg/mL proteinase K
	Fill up to 1 mL with 1× PBS

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. Pre-warm a humidified chamber to 37 °C.
2. Dry slide by blotting edges on a Kimwipe.
3. Add 200 μL of probe hybridization buffer on top of the sample.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
4. Pre-hybridize for 10 min inside the humidified chamber.
5. 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.*
6. Remove the pre-hybridization solution and drain excess buffer on slide by blotting edges on a Kimwipe.
7. Add 50–100 μL of the probe solution on top of the sample.
   NOTE: *Amount of probe solution depends on the size of the coverslip.*
8. Place a coverslip on the sample and incubate overnight (12–16 h) in the 37 °C humidified chamber.
9. Immerse slide in probe wash buffer at 37 °C to float off coverslip.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
10. 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.*
11. 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 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 sample.
   NOTE: *Amount of hairpin solution depends on the size of the coverslip.*
7. Place a coverslip on the sample and 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. Immerse slide in 5 × SSCT at room temperature to float off coverslip.
9. Remove excess hairpins by incubating slide in 5 × SSCT at room temperature for:
   (a) 2 × 30 min
   (b) 1 × 5 min
10. Dry slide by blotting edges on a Kimwipe.
11. Add 50–100 μL of antifade mounting reagent on top of the sample.
12. 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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Choi, H. M. T., Beck, V. A., & Pierce, N. A. (2014). Next-generation in situ hybridization chain reaction: higher gain, lower cost, greater durability. *ACS Nano*, **8**(5), 4284–4294.

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