HCR RNA-FISH protocol for fresh frozen or fixed frozen tissue sections

This protocol has not been validated for all tissue types and should only be used as a template.

Technical support
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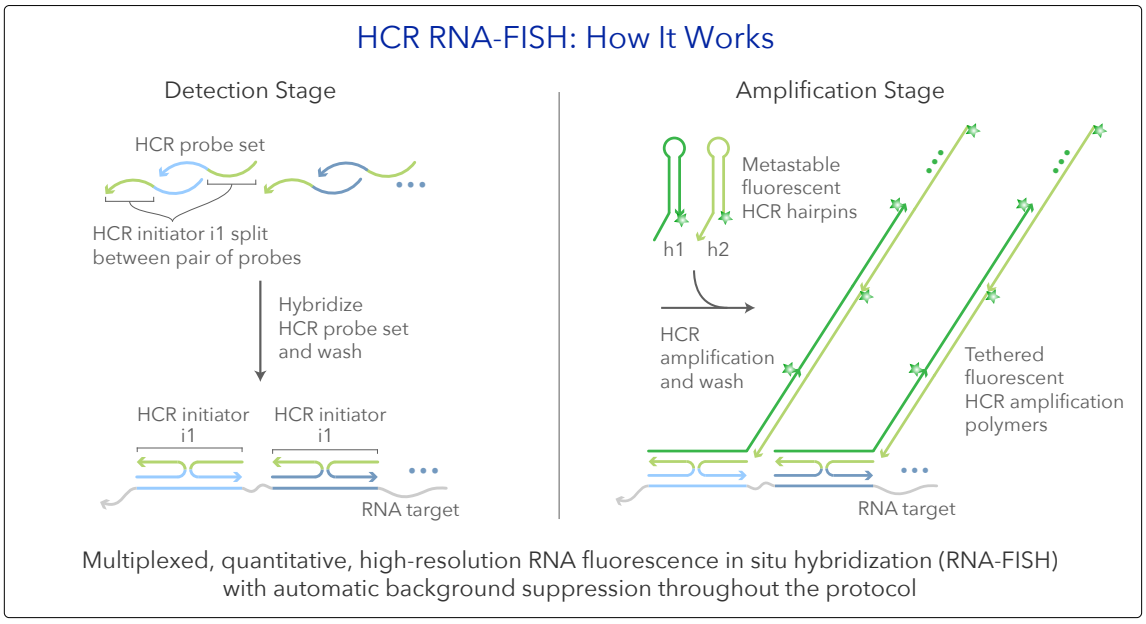

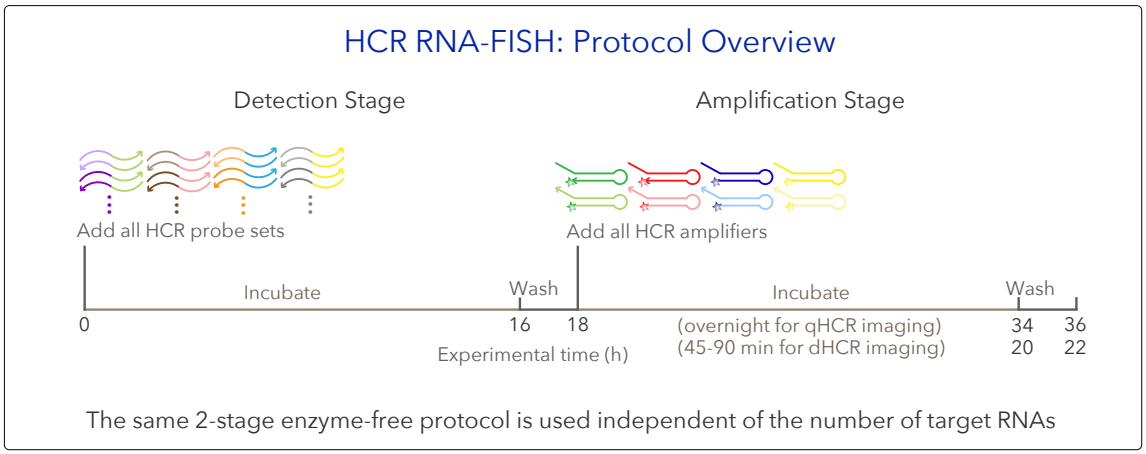

HCR RNA-FISH

Multiplexed, quantitative, high-resolution RNA imaging

Multiplexed Experiment

- Order one HCR RNA-FISH bundle per target RNA

Example 2-Plex Experiment

- HCR bundle for target mRNA1
  - HCR split-initiator probe set: target mRNA1 for use with amplifier B1
  - HCR amplifier: B1-647
  - HCR RNA-FISH buffers: probe hybridization buffer, probe wash buffer, amplification buffer (for use with all bundles)
- HCR bundle for target mRNA2
  - HCR split-initiator probe set: target mRNA2 for use with amplifier B2
  - HCR amplifier: B2-488

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. Immerse slides in PBS.
8. Dry slide using a Kimwipe. Avoid touching the tissue.
9. Draw a barrier around the tissue using a hydrophobic pen.
10. Optional: 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.*
11. Wash slides by immersing in 1× PBS.
    NOTE: *avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the tissue.*
12. Repeat with fresh 1× PBS.
13. Proceed to HCR assay.

Multiplexed HCR RNA-FISH 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 probe concentration (e.g., 16 nM) to increase probe hybridization yield. If desired, this approach can also be used to increase signal for qHCR imaging.*
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 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 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.

Buffer recipes

4% paraformaldehyde (PFA)
4% PFA
1× PBS

For 30 mL of solution
7.5 mL of 16% PFA solution
3 mL of 10× PBS
Fill up to 30 mL with water

Proteinase K solution
10 μg/mL proteinase K

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

5× SSCT
5× sodium chloride sodium citrate (SSC)
0.1% Tween 20

For 40 mL of solution
10 mL of 20× SSC
400 μL of 10% Tween 20
Fill up to 40 mL with ultrapure H_2O

NOTE: *avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the samples.*

HCR Technology Citation Notes

For citation, please select from the list below as appropriate for your application:

- **HCR IHC + HCR RNA-FISH**
  HCR IHC + HCR RNA-FISH enables a unified approach to multiplexed, quantitative, high-resolution protein immunohistochemistry (IHC) and RNA fluorescence in situ hybridization (RNA-FISH), with quantitative 1-step enzyme-free HCR signal amplification performed for all protein and RNA targets simultaneously (Schwarzkopf et al., 2021).

- **HCR IHC**
  HCR IHC enables multiplexed, quantitative, high-resolution protein immunohistochemistry (IHC) in highly autofluorescent samples (e.g., FFPE brain tissue sections) (Schwarzkopf et al., 2021).

- **HCR RNA-FISH (v3.0)**
  Third-generation HCR RNA-FISH (v3.0) enables multiplexed, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) with automatic background suppression *throughout the protocol* 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 RNA imaging: analog mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples.
  - dHCR RNA imaging: digital mRNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples.
  - qHCR RNA flow cytometry: analog mRNA relative quantitation for high-throughput expression profiling of mammalian cells and bacteria.

  Protocols for HCR RNA-FISH (v3.0) in diverse organisms are adapted from the Zoo paper.

- **qHCR RNA imaging**
  qHCR RNA imaging enables mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples (e.g., whole-mount vertebrate embryos). The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (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 solution
  - generic sample on a slide
  - mammalian cells on a slide
  - mammalian cells in suspension
  - whole-mount chicken embryos
  - whole-mount fruit fly embryos
  - whole-mount mouse embryos
  - whole-mount nematode larvae
  - whole-mount sea urchin embryos
  - whole-mount zebrafish embryos and larvae

- **dHCR imaging**
  dHCR RNA imaging enables RNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah et al., 2016).

- **qHCR northern blots**
  qHCR northern blots enable multiplexed quantification of RNA target size and abundance for up to 5 target RNAs (Schwarzkopf & Pierce, 2016).

- **HCR RNA-FISH (v2.0)**
  Second-generation in situ HCR RNA-FISH technology (v2.0) using DNA HCR probes and 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 RNA-FISH (v1.0)**
  First-generation HCR RNA-FISH 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**
  The hybridization chain reaction (HCR) mechanism enables multiplexed, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings (Dirks & Pierce, 2004).

HCR Technology References

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