

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

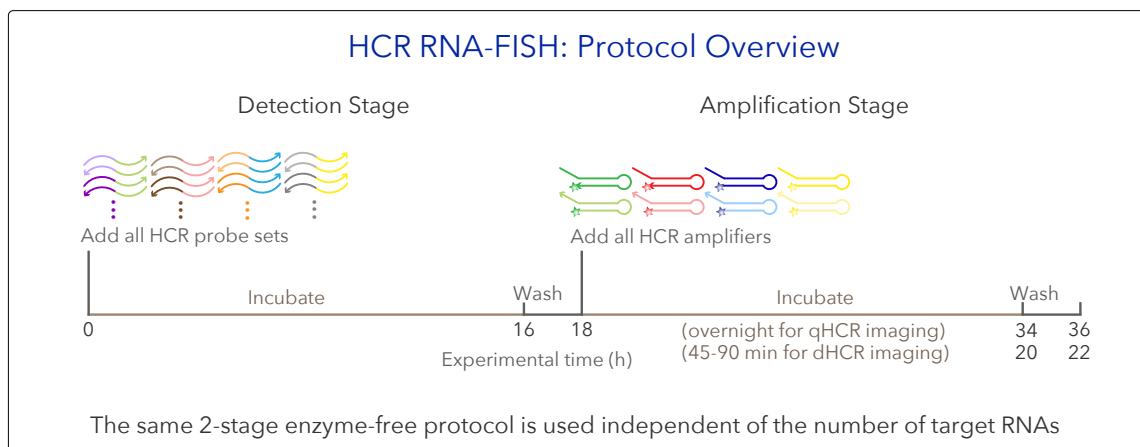
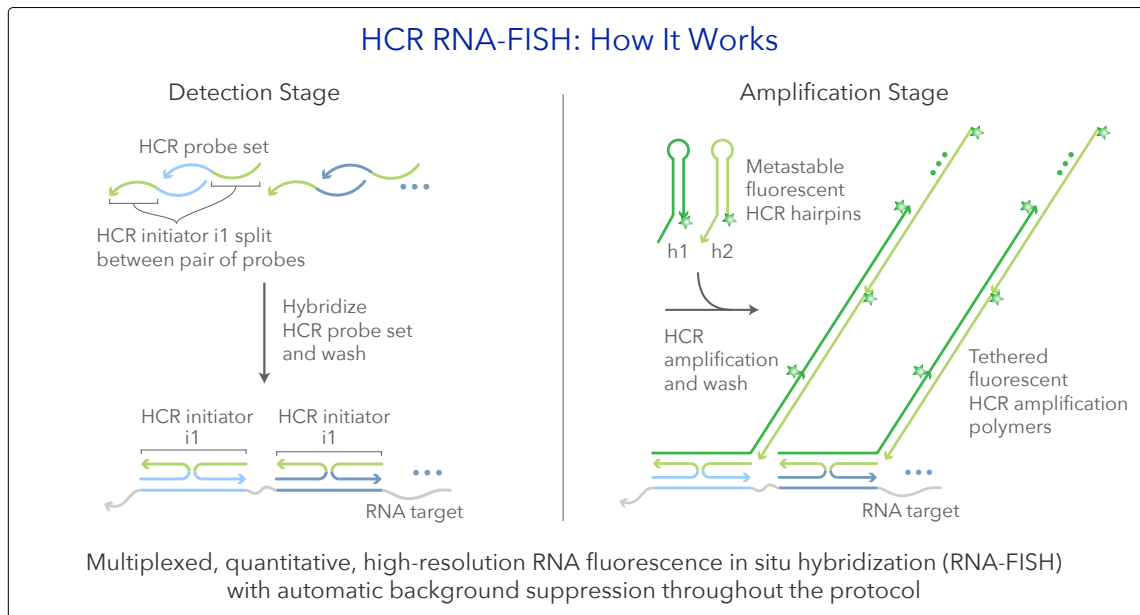
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HCR RNA-FISH

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% paraformaldehyde (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 dry for 5 min at room temperature.
8. Introduce 200 μ L of 10 μ g/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.

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 (12–16 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 \times SSCT for 15 min
 - (b) 50% of probe wash buffer / 50% 5 \times SSCT for 15 min
 - (c) 25% of probe wash buffer / 75% 5 \times SSCT for 15 min
 - (d) 100% 5 \times SSCT for 15 min
NOTE: Wash solutions should be pre-heated to 37 °C before use.
11. Immerse slide in 5 \times 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 \times SSCT at room temperature to float off coverslip.
9. Remove excess hairpins by incubating slide in 5 \times SSCT at room temperature for:
 - (a) 2 \times 30 min
 - (b) 1 \times 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 $\mu\text{g}/\text{mL}$ 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 H₂O

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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