

## HCR RNA-FISH protocol for sample on slide

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

### Technical support

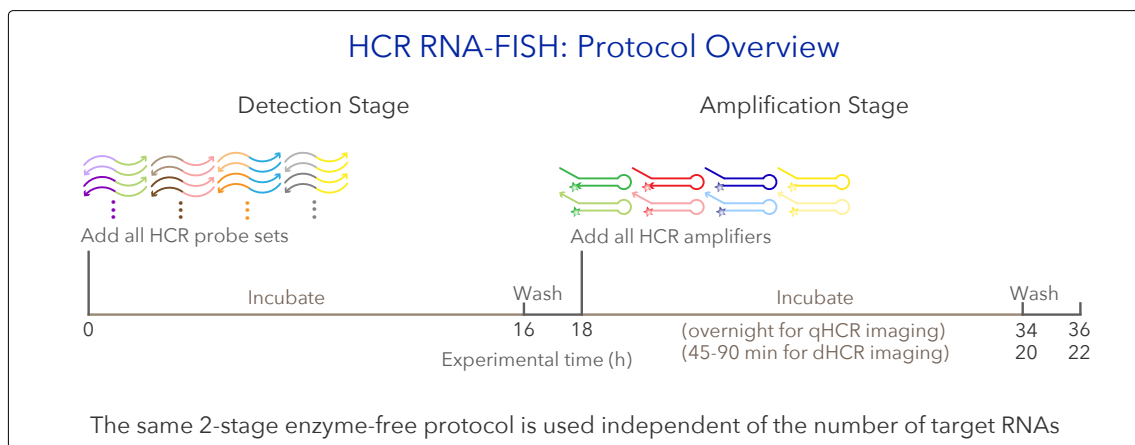
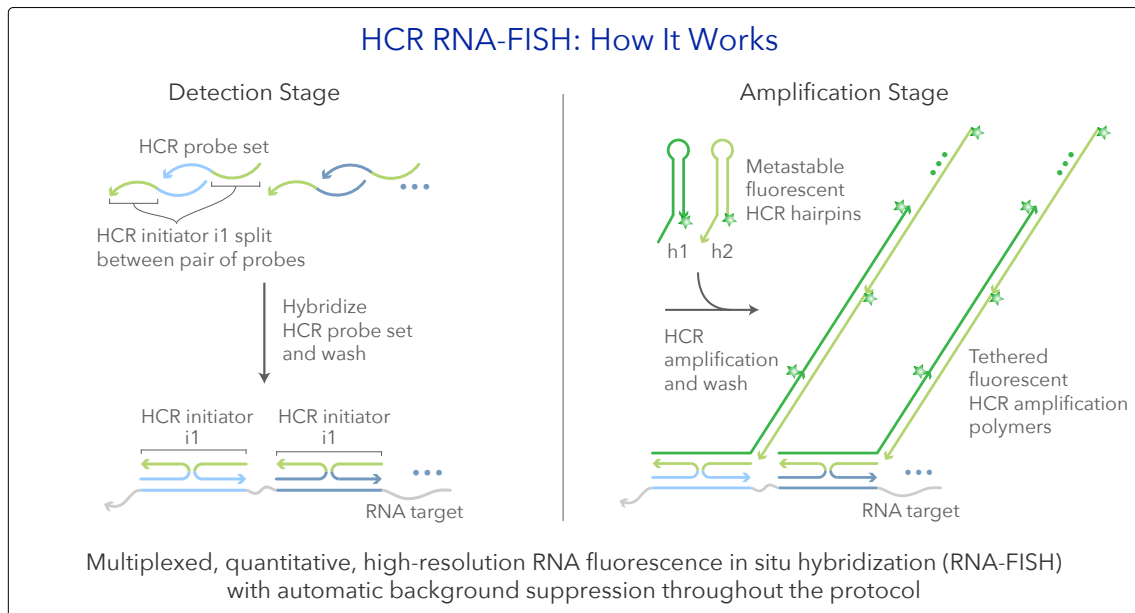
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## **HCR RNA-FISH**

*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

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 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  $\mu$ L 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  $\mu$ L of 1  $\mu$ M stock) to 100  $\mu$ L 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  $\mu$ L 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  $\mu\text{L}$  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  $\mu\text{L}$  of 3  $\mu\text{M}$  stock (heat at 95  $^{\circ}\text{C}$  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  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{L}$  of antifade mounting reagent on top of the sample.
12. Place a coverslip on top for microscopy.

## Buffer recipe

### 5× SSCT

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

### For 40 mL of solution

10 mL of 20× SSC  
400  $\mu$ L of 10% Tween 20  
Fill up to 40 mL with ultrapure H<sub>2</sub>O

## 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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