

## 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

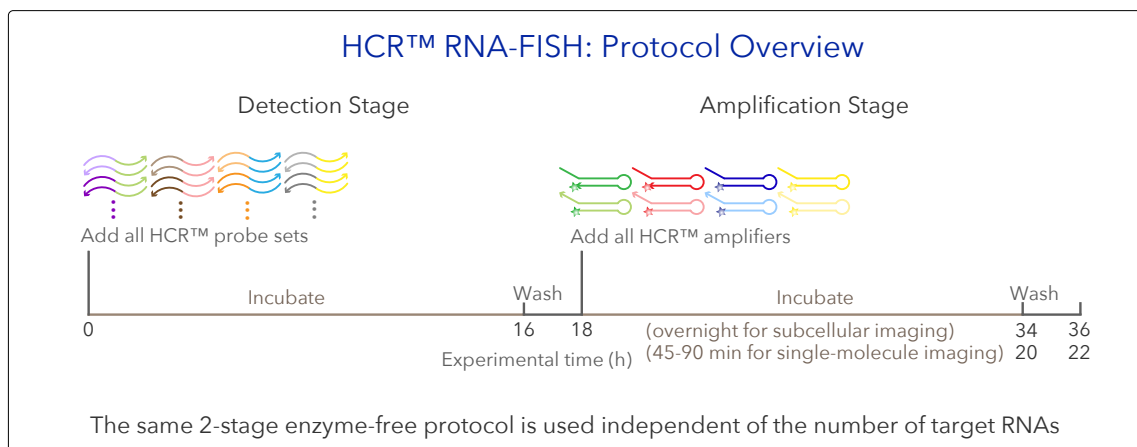
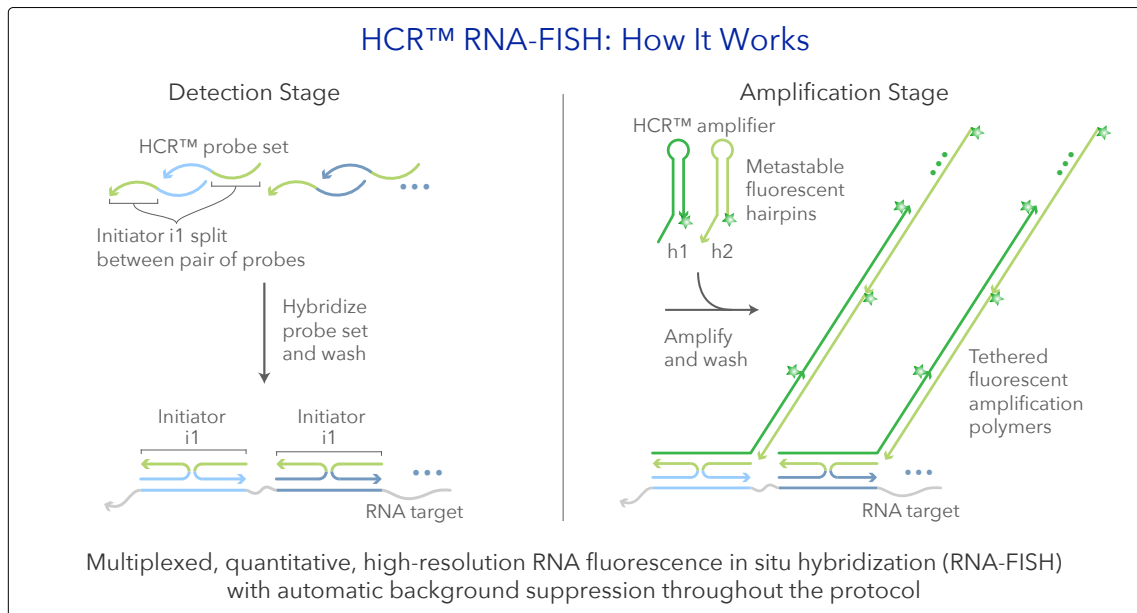
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### Safety data sheets (SDS)

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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™ RNA-FISH bundle for target mRNA1
  - HCR™ 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™ RNA-FISH bundle for target mRNA2
  - HCR™ probe set: target mRNA2 for use with amplifier B2
  - HCR™ amplifier: B2-488

### **Storage conditions**

- Store HCR™ probe sets, HCR™ amplifiers, HCR™ RNA-FISH probe hybridization buffer, and HCR™ RNA-FISH 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 single-molecule RNA 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 subcellular quantitative RNA 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 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 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).  
*NOTE: 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 h) in a dark humidified chamber at room temperature.  
*NOTE: For single-molecule RNA 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™ IF + HCR™ RNA-FISH**

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

- **HCR™ IF**

[HCR™ IF](#) enables multiplexed, quantitative, high-resolution protein immunofluorescence (IF) 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, subcellular quantitative RNA imaging precision, single-molecule quantitative RNA imaging fidelity) and ease-of-use (no probe set optimization for new targets and organisms) ([Choi et al., 2018](#)). Quantitative analysis modes:

- [Subcellular quantitative RNA imaging](#): analog mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples.
- [Single-molecule quantitative RNA imaging](#): digital mRNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples.
- [Quantitative 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.

- **Subcellular quantitative RNA imaging**

[Subcellular quantitative 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

- **Single-molecule quantitative RNA imaging**

[Single-molecule quantitative 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](#)).

- **HCR™ northern blots**

[HCR™ 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™ 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™ technology**

[HCR™ amplifiers](#) enable multiplexed, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings ([Dirks & Pierce, 2004](#)).