

## HCR RNA-FISH protocol for sample in solution

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

### Technical support

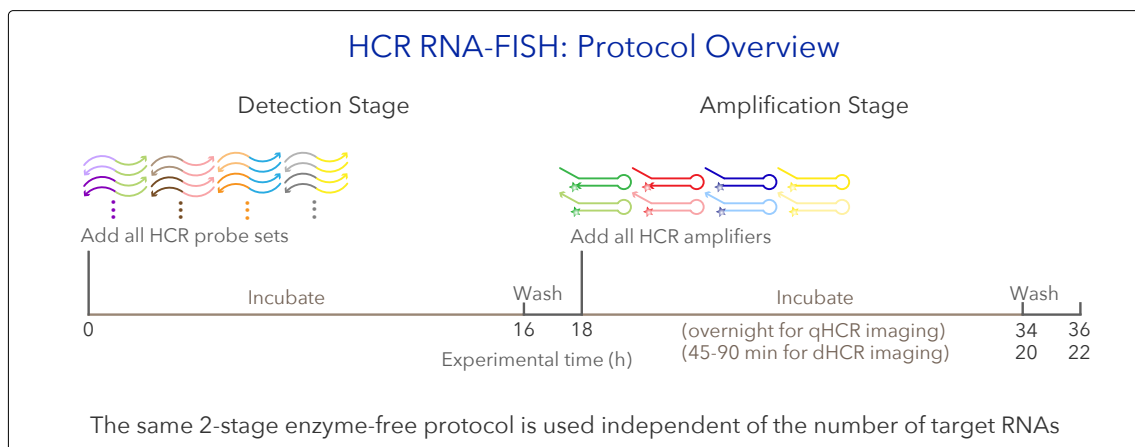
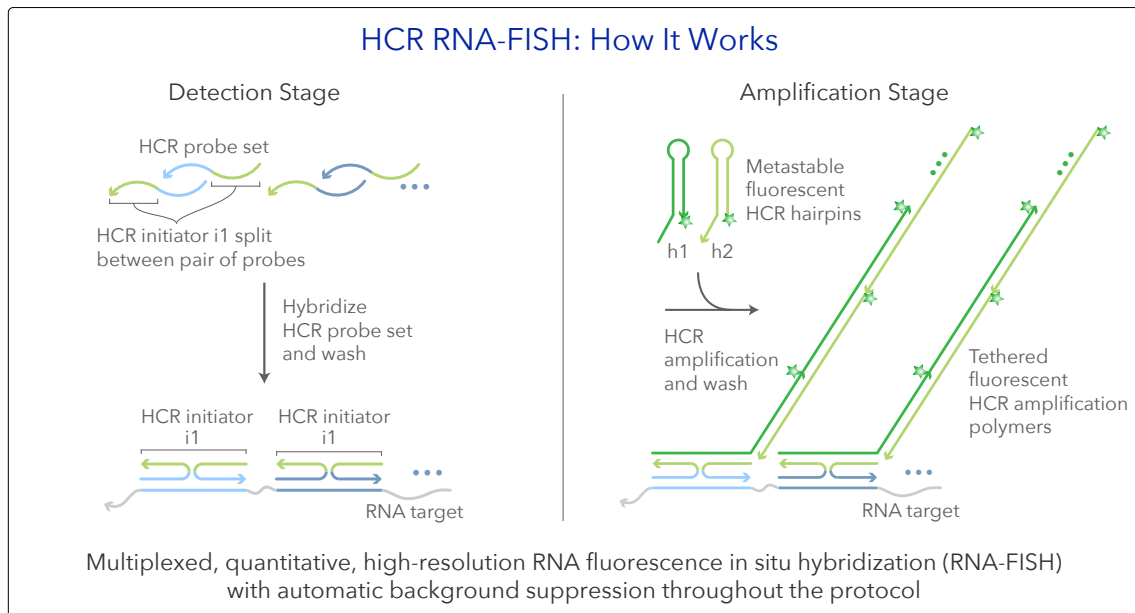
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## **HCR RNA-FISH**

*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-hybridize samples in 500  $\mu\text{L}$  of probe hybridization buffer for 30 min at 37  $^{\circ}\text{C}$ .  
*CAUTION: probe hybridization buffer contains formamide, a hazardous material.*
2. Prepare probe solution by adding 2 pmol of each probe set (e.g. 2  $\mu\text{L}$  of 1  $\mu\text{M}$  stock) to 500  $\mu\text{L}$  of probe hybridization buffer at 37  $^{\circ}\text{C}$ .  
*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.*
3. Remove the pre-hybridization solution and add the probe solution.
4. Incubate samples overnight (>12 h) at 37  $^{\circ}\text{C}$ .
5. Remove excess probes by washing 4  $\times$  15 min with 500  $\mu\text{L}$  of probe wash buffer at 37  $^{\circ}\text{C}$ .  
*CAUTION: probe wash buffer contains formamide, a hazardous material.*  
*NOTE: Wash solutions should be pre-heated to 37  $^{\circ}\text{C}$  before use.*
6. Wash samples 3  $\times$  5 min with 500  $\mu\text{L}$  of 5 $\times$  SSCT at room temperature.

### Amplification stage

1. Pre-amplify samples in 500  $\mu\text{L}$  of amplification buffer for 30 min at room temperature.
2. Separately prepare 30 pmol of hairpin h1 and 30 pmol of hairpin h2 by snap cooling 10  $\mu\text{L}$  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.*
3. Prepare hairpin solution by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 500  $\mu\text{L}$  of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate samples overnight (>12 h) in the dark at room temperature.  
*NOTE: For dHCR imaging, amplify for a shorter period of time to ensure single-molecule dots are diffraction-limited.*
6. Remove excess hairpins by washing with 500  $\mu\text{L}$  of 5 $\times$  SSCT at room temperature:
  - (a) 2  $\times$  5 min
  - (b) 2  $\times$  30 min
  - (c) 1  $\times$  5 min
7. Samples can be stored at 4  $^{\circ}\text{C}$  protected from light before 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

- Choi, H. M. T., Chang, J. Y., Trinh, L. A., Padilla, J. E., Fraser, S. E., & Pierce, N. A. (2010). Programmable in situ amplification for multiplexed imaging of mRNA expression. *Nat Biotechnol*, **28**(11), 1208–12.
- 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.
- Choi, H. M. T., Calvert, C. R., Husain, N., Huss, D., Barsi, J. C., Deverman, B. E., Hunter, R. C., Kato, M., Lee, S. M., Abelin, A. C. T., Rosenthal, A. Z., Akbari, O. S., Li, Y., Hay, B. A., Sternberg, P. W., Patterson, P. H., Davidson, E. H., Mazmanian, S. K., Prober, D. A., van de Rijn, M., Leadbetter, J. R., Newman, D. K., Readhead, C., Bronner, M. E., Wold, B., Lansford, R., Sauka-Spengler, T., Fraser, S. E., & Pierce, N. A. (2016). Mapping a multiplexed zoo of mRNA expression. *Development*, **143**, 3632–3637.
- Choi, H. M. T., Schwarzkopf, M., Fornace, M. E., Acharya, A., Artavanis, G., Stegmaier, J., Cunha, A., & Pierce, N. A. (2018). Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development*, **145**, dev165753.
- Dirks, R. M., & Pierce, N. A. (2004). Triggered amplification by hybridization chain reaction. *Proc Natl Acad Sci USA*, **101**(43), 15275–15278.
- Schwarzkopf, M., & Pierce, N. A. (2016). Multiplexed miRNA northern blots via hybridization chain reaction. *Nucleic Acids Res*, **44**(15), e129.
- Schwarzkopf, M., Liu, M.C., Schulte, S.J., Ives, R., Husain, N., Choi, H.M.T., & Pierce, N. A. (2021). Hybridization chain reaction enables a unified approach to multiplexed, quantitative, high-resolution immunohistochemistry and in situ hybridization. *Development*, **148**(22), dev199847.
- Shah, S., Lubeck, E., Schwarzkopf, M., He, T.-F., Greenbaum, A., Sohn, C. H., Lignell, A., Choi, H. M. T., Gradinaru, V., Pierce, N. A., & Cai, L. (2016). Single-molecule RNA detection at depth via hybridization chain reaction and tissue hydrogel embedding and clearing. *Development*, **143**, 2862–2867.
- Trivedi, V., Choi, H. M. T., Fraser, S. E., & Pierce, N. A. (2018). Multidimensional quantitative analysis of mRNA expression within intact vertebrate embryos. *Development*, **145**, dev.156869.