

## HCR RNA-FISH protocol for FFPE tissue sections

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

### Technical support

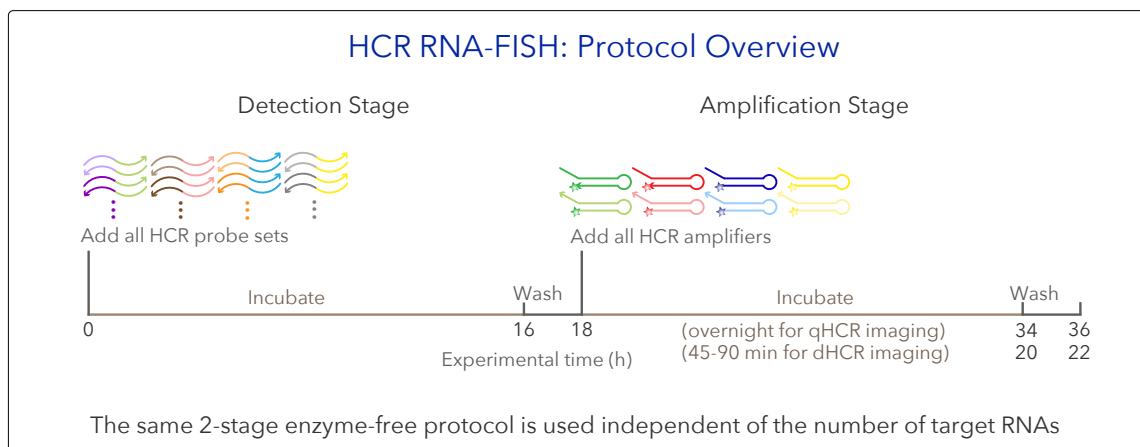
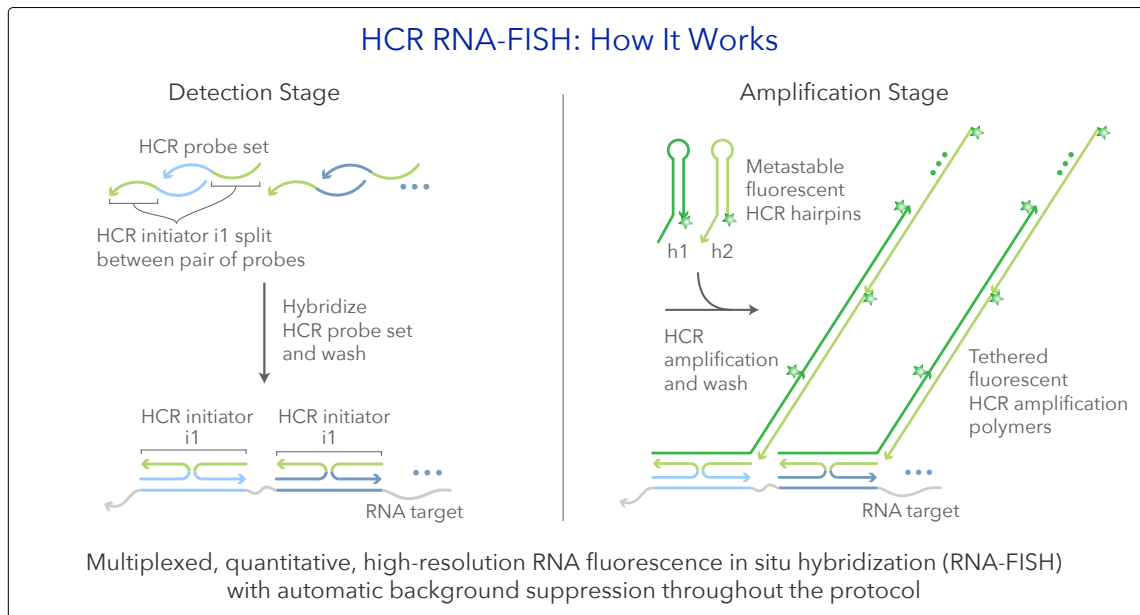
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## **HCR RNA-FISH**

*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. Bake slides in a dry oven for 1 h at 60 °C to improve sample adhesion to the slide.
2. In a fume hood, deparaffinize FFPE tissue by immersing slide in dewaxing solution (e.g., xylene, Pro-Par Clearant etc.) for 3 × 5 min. Move slides up and down occasionally.  
*CAUTION: dewaxing solution may contain hazardous material, use with care.*  
*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. Incubate slides in 100% ethanol (EtOH) for 2 × 3 min at room temperature. Move slides up and down occasionally.
4. Rehydrate with a series of graded EtOH washes at room temperature.
  - (a) 95% EtOH for 3 min
  - (b) 70% EtOH for 3 min
  - (c) 50% EtOH for 3 min
  - (d) Nanopure water for 3 min
5. Bring 500 mL of 1× Tris-EDTA buffer (pH 9.0) in a beaker to boil in a microwave.  
*NOTE: 1× citrate buffer (pH 6.0) can be used in place of Tris-EDTA buffer (pH 9.0).*
6. Maintain Tris-EDTA buffer temperature at 95 °C on a hot plate.
7. Immerse slides for 15 min.
8. Remove beaker from hot plate and add 100 mL of nanopure water every 5 min to allow temperature to decrease to 45 °C in 20 min.
9. Immerse slides in 400 mL of nanopure water in a separate container for 10 min at room temperature.
10. Immerse slides in 1× PBST for 2 × 2 min at room temperature.  
*NOTE: avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the tissue.*
11. Dry slide using a Kimwipe. Avoid touching the tissue.
12. Draw a barrier around the tissue using a hydrophobic pen.
13. 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.*
14. Wash slides by immersing in 1× PBST.
15. Repeat with fresh 1× PBST.
16. 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  $\mu$ L of probe hybridization buffer on top of the tissue 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 tissue sample.  
*NOTE: Amount of probe solution depends on the size of the tissue.*
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 tissue 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 tissue sample.  
*NOTE: Amount of hairpin solution depends on the size of the tissue.*
7. 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. Remove excess hairpins by incubating slide in 5  $\times$  SSCT at room temperature for:
  - (a) 1  $\times$  5 min
  - (b) 2  $\times$  15 min
  - (c) 1  $\times$  5 min
9. Dry slide by blotting edges on a Kimwipe.
10. Add 50–100  $\mu\text{L}$  of antifade mounting reagent on top of the tissue.
11. 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

### 1 × Tris-EDTA buffer

1 × Tris-EDTA buffer

### For 500 mL of solution

5 mL of 100 × Tris-EDTA buffer pH 9.0 (Abcam Cat. #ab93684)  
Fill up to 500 mL with water

### 1 × citrate buffer

1 × citrate buffer

### For 500 mL of solution

5 mL of 100 × citrate buffer pH 6.0 (Abcam Cat. #ab93678)  
Fill up to 500 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<sub>2</sub>O

*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

- 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., Gradi-naru, 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.