

HCR v3.0 protocol for sample on slide

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

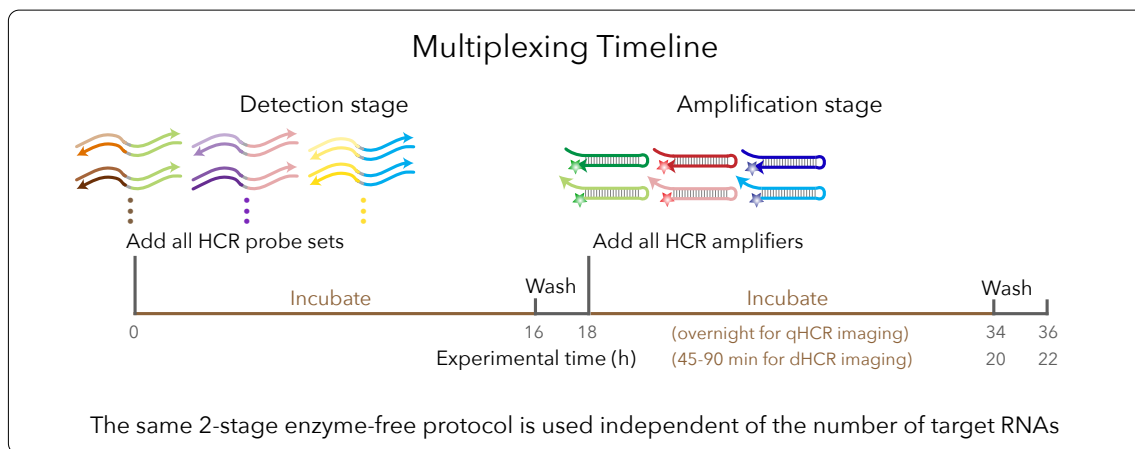
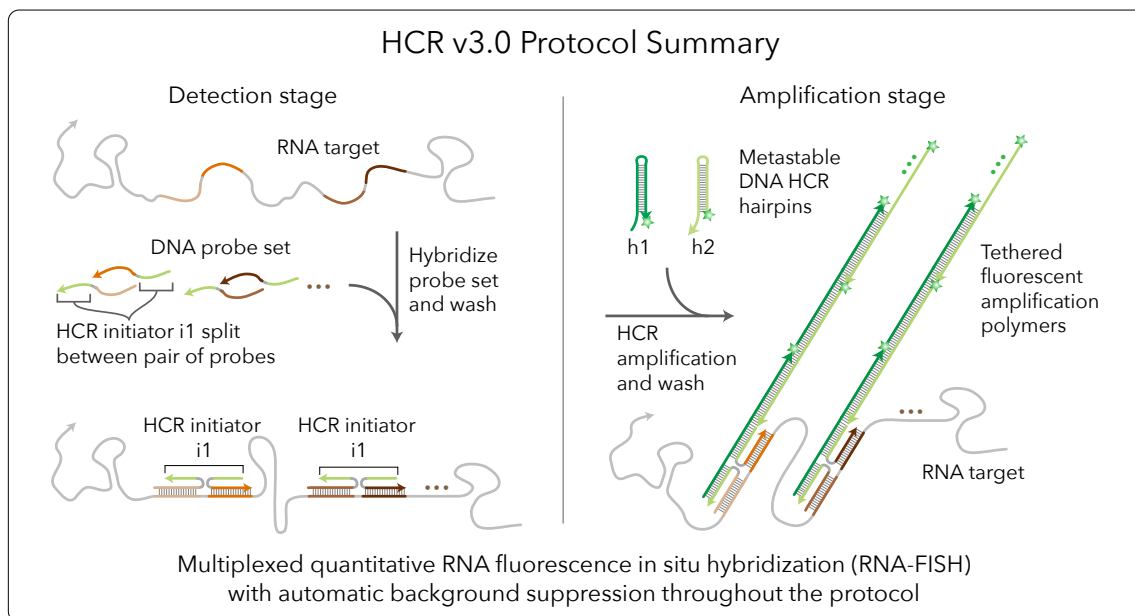
Technical support: support@molecularinstruments.com

Safety data sheets (SDS): www.molecularinstruments.com/safety

Molecular Instruments products are protected by and for use under patents: www.molecularinstruments.com/patents

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 v3.0 protocol

Detection stage

1. Pre-warm a humidified chamber to 37 °C.
2. Dry slide by blotting edges on a Kimwipe.
3. Add 200 μ 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 mixture (e.g. 0.4 μ L of 1 μ M stock) to 100 μ L of probe hybridization buffer at 37 °C.
NOTE: For dHCR imaging, use higher concentration of probe to improve probe hybridization efficiency.
6. Remove the pre-hybridization solution and drain excess buffer on slide by blotting edges on a Kimwipe.
7. Add 50–100 μ 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 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 $^{\circ}\text{C}$ 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 mixture 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 mixture on top of the sample.
NOTE: Amount of hairpin mixture 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.

Citation Notes

For citation, please select from the list below as appropriate for your application:

- **HCR v3.0**

Automatic background suppression 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 imaging: analog mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples.

qHCR flow cytometry: analog mRNA relative quantitation for high-throughput expression profiling of mammalian and bacterial cells.

dHCR imaging: digital mRNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples.

Protocols for v3.0 in diverse organisms are adapted from the Zoo paper.

- **qHCR imaging**

mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos; read-out/read-in analysis framework (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 suspension	generic sample on slide
whole-mount chicken embryos	whole-mount fruit fly embryos
whole-mount mouse embryos	whole-mount sea urchin embryos
whole-mount worm larvae	whole-mount zebrafish embryos and larvae

- **dHCR imaging**

Single-molecule mRNA imaging in thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah *et al.*, 2016).

- **qHCR northern blot**

Simultaneous quantification of RNA target size and abundance for up to 5 target RNAs (Schwarzkopf & Pierce, 2016).

- **HCR v2.0**

2nd generation in situ HCR 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 v1.0**

1st generation in situ HCR 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**

Isothermal enzyme-free molecular signal amplification (Dirks & Pierce, 2004).

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.
- 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.