

HCR v3.0 protocol for whole-mount mouse embryos (*Mus musculus*)

This protocol has been optimized for embryos at stage E9.5 and should only be used as a template for other stages.

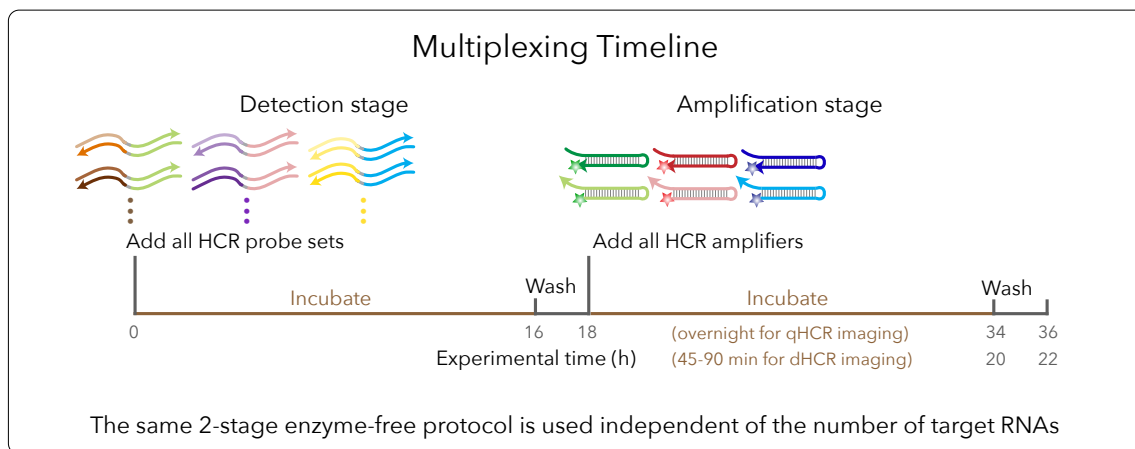
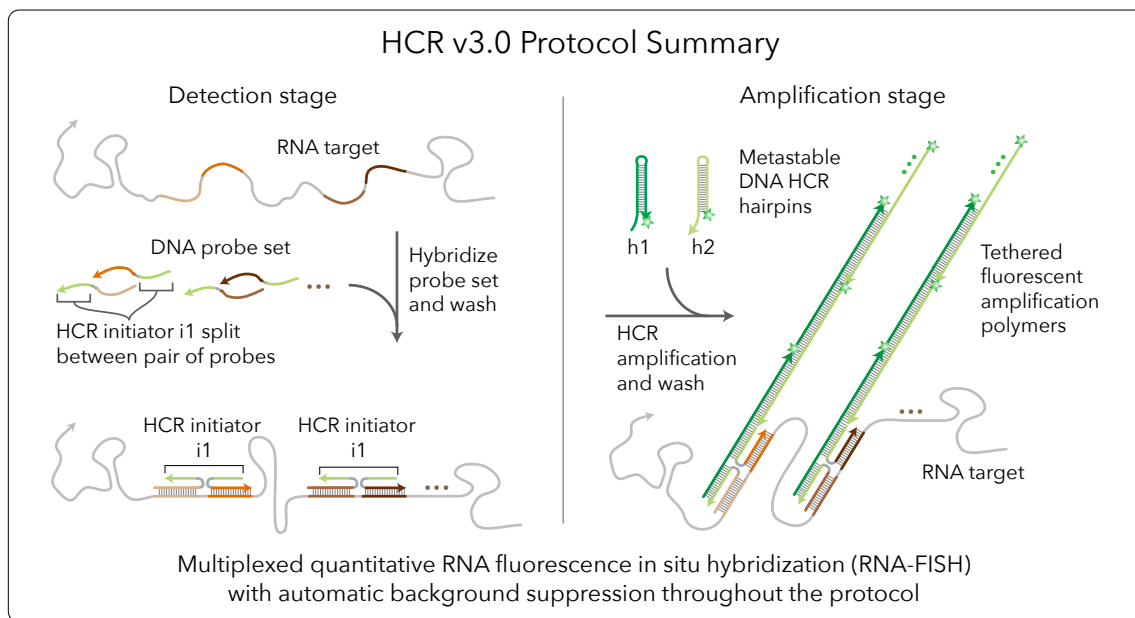
Technical support: support@molecularinstruments.com

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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.



Preparation of fixed whole-mount mouse embryos

1. Wipe all dissection equipment with RNaseZap.
2. Kill a pregnant female mouse using an IACUC-approved protocol.
3. Immediately remove the uterus and submerge it in 4% paraformaldehyde (PFA) in a fresh RNase-free petri dish.
NOTE: use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.
4. Dissect the mouse embryos from the uterus while it is submerged in 4% PFA.
CAUTION: use PFA with extreme care as it is a hazardous material.
NOTE: Each female mouse produces 6–9 embryos. We recommend using ≈2 mL of solution per group of 10 embryos.
5. Transfer the embryos to a clean vial containing fresh 4% PFA and fix them overnight or longer at 4 °C.
NOTE: make sure all embryos are submerged in PFA during fixation.
6. Wash 2 × 5 min with PBST on ice.
7. Dehydrate embryos into methanol (MeOH) with a series of graded MeOH/PBST washes for 10 min on ice:
 - (a) 25% MeOH / 75% PBST
 - (b) 50% MeOH / 50% PBST
 - (c) 75% MeOH / 25% PBST
 - (d) 100% MeOH
 - (e) 100% MeOH.
8. Incubate embryos at -20 °C overnight (> 16 h) or until use.
NOTE: Embryos could be stored for six months at -20 °C .
9. Transfer the required number of embryos for an experiment to a 2 mL tube.
NOTE: make sure embryos are submerged during washes.
10. Rehydrate with a series of graded MeOH/PBST washes for 10 min each on ice:
 - (a) 75% MeOH / 25% PBST
 - (b) 50% MeOH / 50% PBST
 - (c) 25% MeOH / 75% PBST
 - (d) 100% PBST
11. Wash embryos with PBST for 10 min at room temperature.

12. Immerse embryos in 10 $\mu\text{g}/\text{mL}$ proteinase K solution for 15 min at room temperature.
NOTE: Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage.
13. Wash embryos 2 \times 5 min with PBST.
14. Postfix with 4% PFA for 20 min at room temperature.
CAUTION: use PFA with extreme care as it is a hazardous material.
15. Wash embryos 3 \times 5 min with PBST.

Buffer recipes for sample preparation

4% paraformaldehyde (PFA)

4% PFA
1 \times PBS

For 25 mL of solution

1 g of PFA powder
25 mL of 1 \times PBS
Heat solution at 50–60 $^{\circ}\text{C}$ to dissolve powder

PBST

1 \times PBS
0.1% Tween 20

For 50 mL of solution

5 mL of 10 \times PBS
500 μL of 10% Tween 20
Fill up to 50 mL with ultrapure H_2O

Proteinase K solution

10 $\mu\text{g}/\text{mL}$ proteinase K

For 2 mL of solution

1 μL of 20 mg/mL proteinase K
Fill up to 2 mL with PBST

NOTE: avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the embryos.

Multiplexed HCR v3.0 protocol

Detection stage

1. For each sample, transfer 1-4 embryos to a 2 mL tube.
2. Incubate embryos in 1 mL of probe hybridization buffer for 5 min.
CAUTION: probe hybridization buffer contains formamide, a hazardous material.
3. Remove the buffer and pre-hybridize with 1 mL of probe hybridization buffer for 30 min at 37 °C.
4. Prepare probe solution by adding 2 pmol of each probe set (e.g. 2 μ L of 1 μ M stock) to 500 μ L of probe hybridization buffer at 37 °C.
NOTE: For dHCR imaging, use higher concentration of probe to improve probe hybridization efficiency.
5. Remove the pre-hybridization solution and add the probe solution.
6. Incubate embryos overnight (12–16 h) at 37 °C.
7. Remove excess probes by washing embryos 4 \times 15 min with 1 mL of probe wash buffer at 37 °C.
CAUTION: probe wash buffer contains formamide, a hazardous material.
NOTE: pre-heat probe wash buffer to 37 °C before use.
8. Wash samples 2 \times 5 min with 5 \times SSCT at room temperature.

Amplification stage

1. Pre-amplify embryos with 1 mL of amplification buffer for 5 min at room temperature.
NOTE: Equilibrate amplification buffer to room temperature before use.
2. Separately prepare 30 pmol of hairpin h1 and 30 pmol of hairpin h2 by snap cooling 10 μ L of 3 μ M stock (heat at 95 °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 μ L of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate the embryos overnight (12–16 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 1 mL 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 °C protected from light before 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.