

HCR v3.0 protocol for whole-mount chicken embryos (*Gallus gallus domesticus*)

This protocol has been optimized for embryos at stage HH 8–11 and should only be used as a template for other stages.

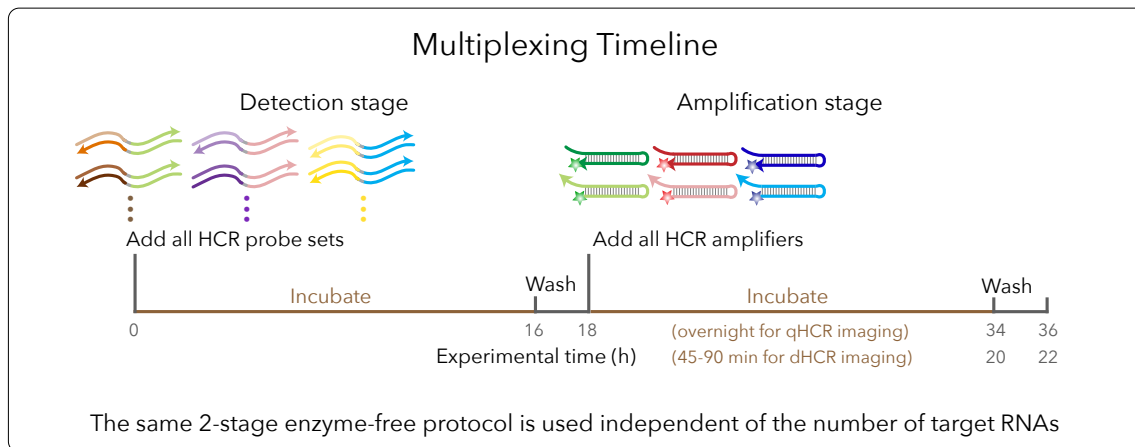
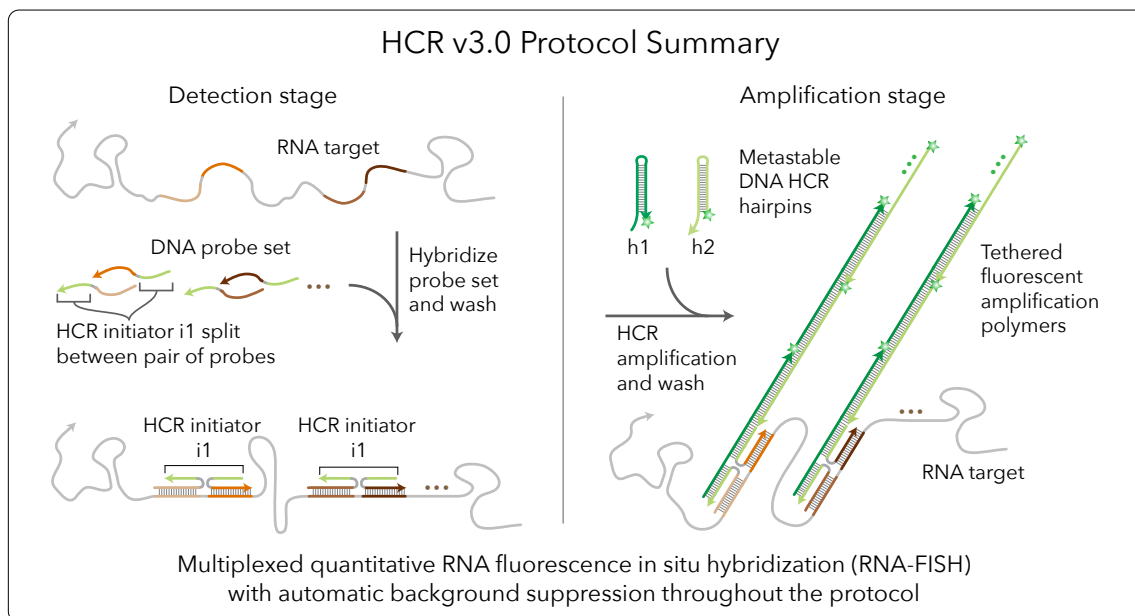
Technical support: support@molecularinstruments.com

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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 chicken embryos

1. Collect chick embryos on 3M paper circles and place in a petri dish containing Ringer's solution.
2. Transfer embryos into a new petri dish with fresh Ringer's solution.
NOTE: This is to rinse away egg yolk before fixation.
3. Transfer into a petri dish containing 4% paraformaldehyde (PFA).
CAUTION: use PFA with extreme care as it is a hazardous material.
NOTE: use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.
4. Fix the samples at room temperature for 1 h.
5. Transfer embryos into a petri dish containing PBST.
6. Dissect the embryos off the filter paper. Remove the fixed vitelline membrane (opaque) and cut the squares around area pellucida, without leaving excess of extra-embryonic tissue.
7. Transfer embryos into a 2 mL tube containing PBST.
8. Nutate for 5 min with the tube positioned horizontally in a small ice bucket.
9. Wash embryos two additional times with 2 mL of PBST, each time nutating for 5 min on ice.
10. Dehydrate embryos with 2 × 5 min washes of 2 mL methanol (MeOH) on ice.
11. Store embryos at -20 °C overnight before use.
NOTE: Embryos can be stored for six months at -20 °C.
12. Transfer the required number of embryos for an experiment to a 2 mL tube.
NOTE: do not place more than 4 embryos in each 2 mL tube.
13. Rehydrate with a series of graded 2 mL MeOH/PBST washes for 5 min each on ice:
 - (a) 75% MeOH / 25% PBST
 - (b) 50% MeOH / 50% PBST
 - (c) 25% MeOH / 75% PBST
 - (d) 100% PBST
 - (e) 100% PBST.
14. Treat embryos with 2 mL of 10 µg/mL proteinase K solution for 2 min (stage HH 8) or 2.5 min (stage HH 10–11) 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.
15. Postfix with 2 mL of 4% PFA for 20 min at room temperature.
CAUTION: use PFA with extreme care as it is a hazardous material.
16. Wash embryos 2 × 5 min with 2 mL of PBST on ice.
17. Wash embryos with 2 mL of 50% PBST / 50% 5× SSCT for 5 min on ice.
18. Wash embryos with 2 mL of 5× SSCT for 5 min on ice.

Buffer recipes for sample preparation

Ringer's solution

123 mM NaCl
1.53 mM CaCl₂
4.96 mM KCl₂
0.81 mM Na₂HPO₄
0.15 mM KH₂PO₄

For 2 L of solution

14.4 g of NaCl
340 mg of CaCl₂
740 mg of KCl
230 mg of Na₂HPO₄
40 mg of KH₂PO₄
Bring volume up to 1.5 L with ultrapure H₂O
Adjust pH to 7.4 and fill up to 2 L with ultrapure H₂O
Filter sterilize with 0.22 μm bottle top filter

4% paraformaldehyde (PFA)

4% PFA
1× PBS

For 25 mL of solution

1 g of PFA powder
25 mL of 1× PBS
Heat solution at 50–60 °C to dissolve powder

PBST

1× PBS
0.1% Tween 20

For 50 mL of solution

5 mL of 10× PBS
500 μL of 10% Tween 20
Fill up to 50 mL with ultrapure H₂O

Proteinase K solution

10 μg/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.
NOTE: do not place more than 4 embryos in each 2 mL tube.
2. Pre-hybridize embryos in 500 μL of probe hybridization buffer for 30 min at 37 °C.
CAUTION: probe hybridization buffer contains formamide, a hazardous material.
3. Prepare probe solution by adding 2 pmol of each probe mixture (e.g. 2 μL of 1 μM stock) to 500 μL of probe hybridization buffer at 37 °C.
NOTE: For dHCR imaging, use 5 pmol of each probe mixture to improve probe hybridization efficiency.
4. Remove the pre-hybridization solution and add the probe solution.
5. Incubate embryos overnight (12–16 h) at 37 °C.
6. 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.
7. Wash samples 2 \times 5 min with 5 \times SSCT at room temperature.

Amplification stage

1. Pre-amplify embryos with 500 μL 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 μL of 3 μM 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 mixture by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 500 μL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin mixture.
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 (e.g. 90 min for stage HH 8 embryos) 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).

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