HCR RNA-FISH 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

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

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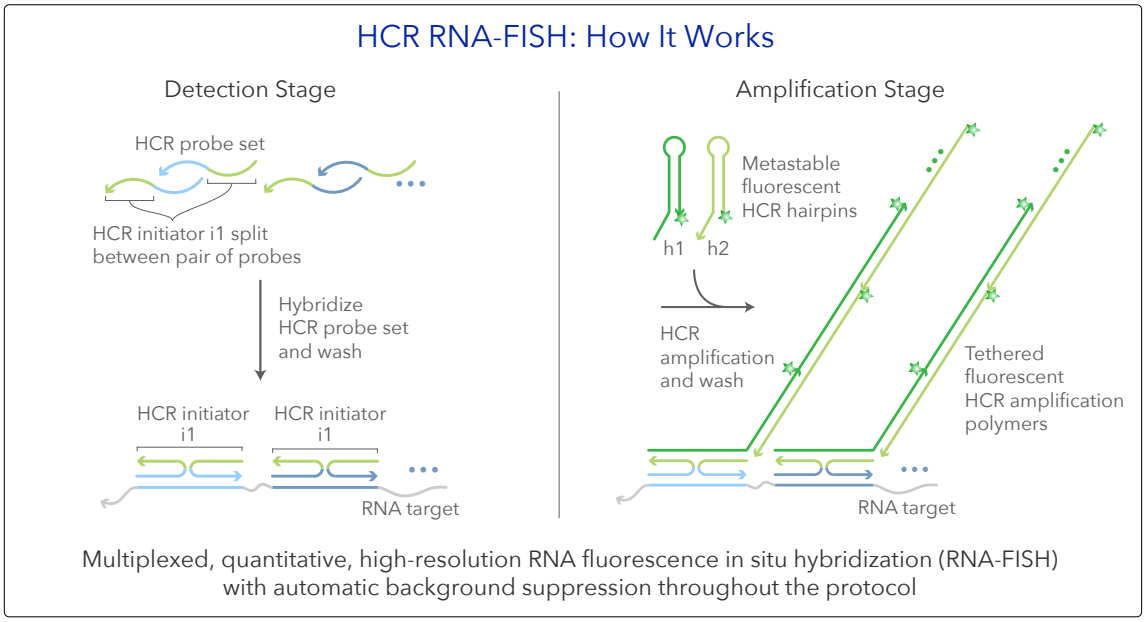

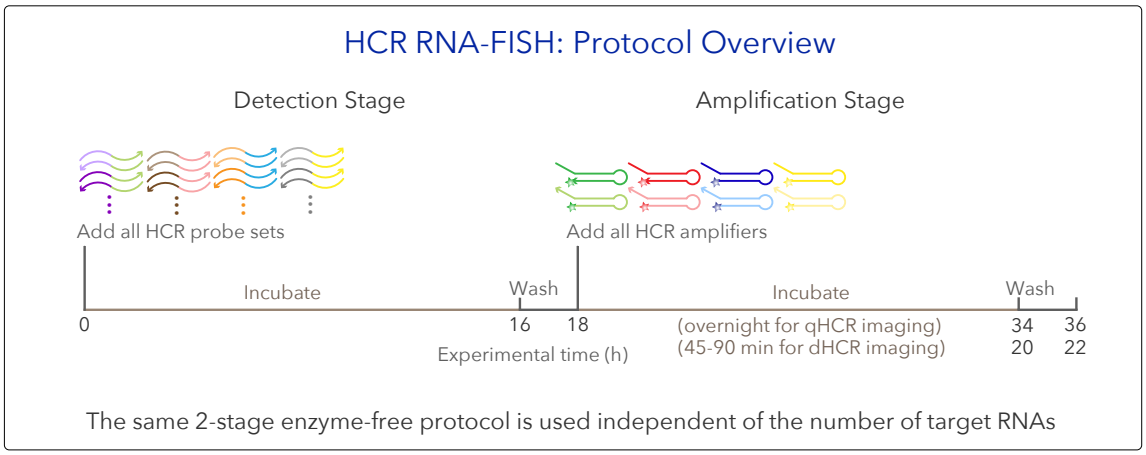

HCR RNA-FISH

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.

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.

Multiplexed HCR RNA-FISH 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 set (e.g. 2 μL of 1 μM stock) to 500 μL 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.*

4. Remove the pre-hybridization solution and add the probe solution.

5. Incubate embryos overnight (>12 h) at 37 °C.

6. Remove excess probes by washing embryos 4 × 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 × 5 min with 5× 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 solution 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 solution.

5. Incubate the embryos overnight (>12 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× SSCT at room temperature:

   (a) 2 × 5 min
   (b) 2 × 30 min
   (c) 1 × 5 min

7. Samples can be stored at 4 °C protected from light before microscopy.

Buffer recipes

Ringer's solution
123 mM NaCl
1.53 mM $CaCl_2$
4.96 mM KCl_2
0.81 mM Na_2HPO_4
0.15 mM KH_2PO_4

For 2 L of solution
14.4 g of NaCl
340 mg of $CaCl_2$
740 mg of KCl
230 mg of Na_2HPO_4
40 mg of KH_2PO_4
Bring volume up to 1.5 L with ultrapure H_2O
Adjust pH to 7.4 and fill up to 2 L with ultrapure H_2O
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 H_2O

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

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_2O

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

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

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