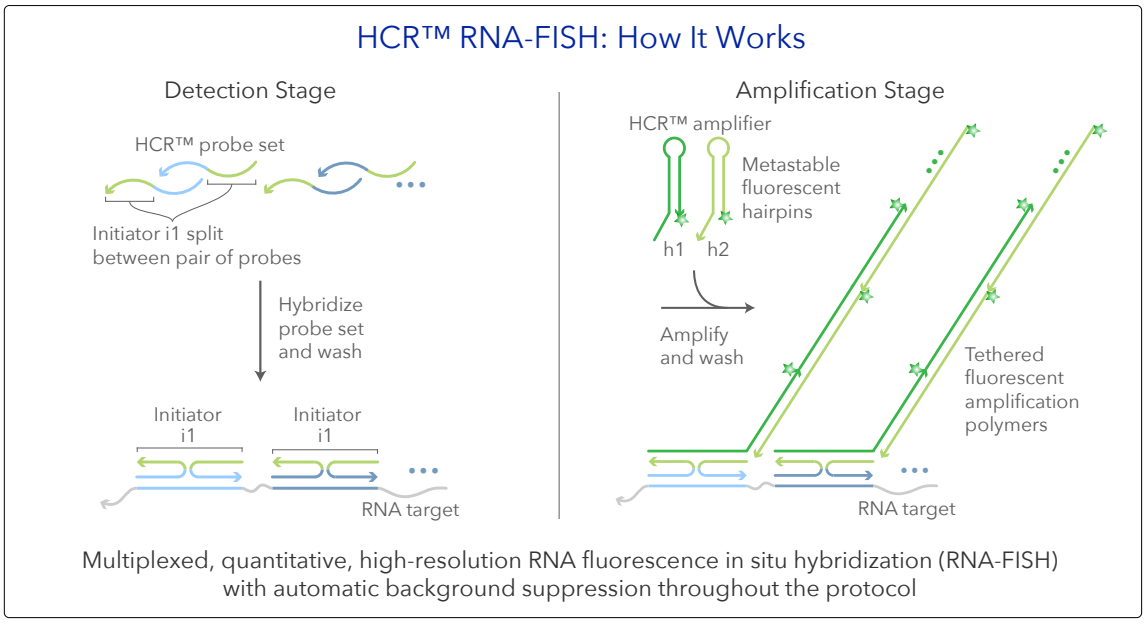
HCR™ RNA-FISH protocol for whole-mount mouse embryos (*Mus musculus*)

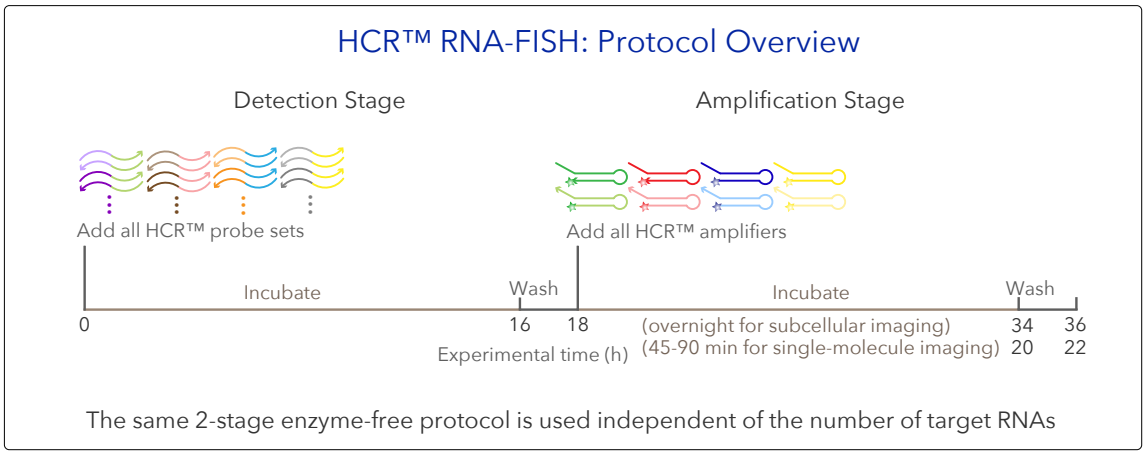
This protocol has been optimized for embryos at stage E9.5 and should only be used as a template for other stages.

Technical support
support@molecularinstruments.com

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

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





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™ RNA-FISH bundle for target mRNA1
  - HCR™ 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™ RNA-FISH bundle for target mRNA2
  - HCR™ probe set: target mRNA2 for use with amplifier B2
  - HCR™ amplifier: B2-488

Storage conditions

- Store HCR™ probe sets, HCR™ amplifiers, HCR™ RNA-FISH probe hybridization buffer, and HCR™ RNA-FISH 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 μg/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.

Multiplexed HCR™ RNA-FISH 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 μL of 1 μM stock) to 500 μL of probe hybridization buffer at 37 °C.
   NOTE: *For single-molecule RNA 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 subcellular quantitative RNA imaging.*
5. Remove the pre-hybridization solution and add the probe solution.
6. Incubate embryos overnight (>12 h) at 37 °C.
7. 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.*
8. Wash samples 2 × 5 min with 5× 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 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: *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 single-molecule RNA 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× 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

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™ IF + HCR™ RNA-FISH**
  HCR™ IF + HCR™ RNA-FISH enables a unified approach to multiplexed, quantitative, high-resolution protein immunofluorescence (IF) and RNA fluorescence in situ hybridization (RNA-FISH), with quantitative 1-step enzyme-free signal amplification performed for all protein and RNA targets simultaneously (Schwarzkopf et al., 2021).

- **HCR™ IF**
  HCR™ IF enables multiplexed, quantitative, high-resolution protein immunofluorescence (IF) 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, subcellular quantitative RNA imaging precision, single-molecule quantitative RNA imaging fidelity) and ease-of-use (no probe set optimization for new targets and organisms) (Choi et al., 2018). Quantitative analysis modes:

  - Subcellular quantitative RNA imaging: analog mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples.
  - Single-molecule quantitative RNA imaging: digital mRNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples.
  - Quantitative 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.

- **Subcellular quantitative RNA imaging**
  Subcellular quantitative RNA imaging enables mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples (e.g., whole-mount vertebrate embryos). The readout/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

- **Single-molecule quantitative RNA imaging**
  Single-molecule quantitative 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).

- **HCR™ northern blots**
  HCR™ 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™ 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™ technology**
  HCR™ amplifiers enable multiplexed, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings (Dirks & Pierce, 2004).