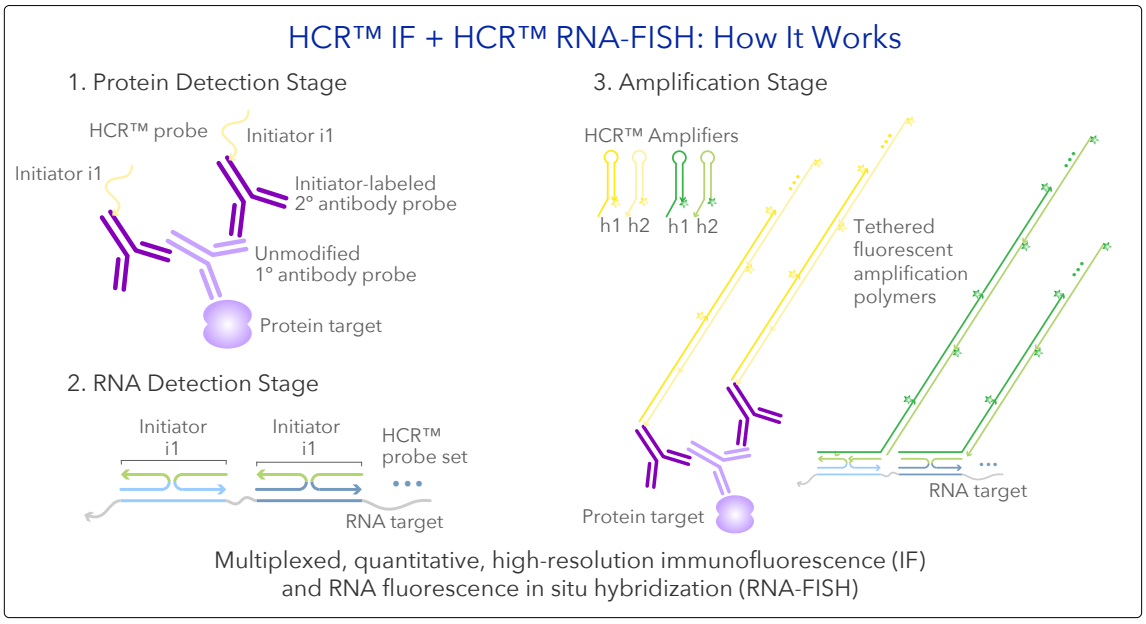
HCR™ IF + HCR™ RNA-FISH protocol for sample in solution

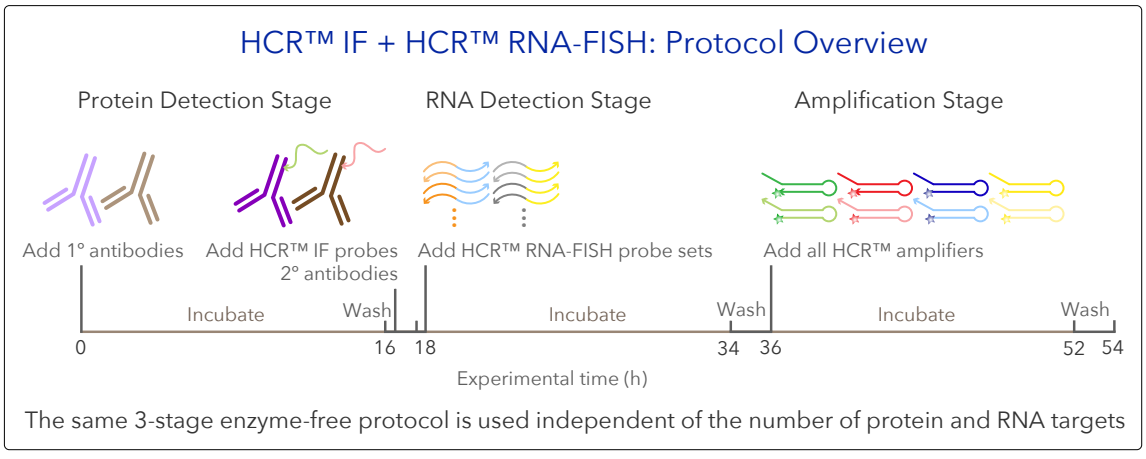
This protocol has not been validated for all sample types and should only be used as a template.

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™ IF + HCR™ RNA-FISH

Simultaneous multiplexed, quantitative, high-resolution protein and RNA imaging

Multiplexed Experiment

- Order one HCR™ IF bundle per target protein
- Order one HCR™ RNA-FISH bundle per target RNA

Example 2-Plex Experiment

- HCR™ RNA-FISH bundle for target mRNA1
  - HCR™ RNA-FISH 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
- HCR™ IF bundle for target Protein1
  - 1° Ab: Mouse Anti-Protein1 (your own 1° antibody)
  - HCR™ IF 2° Ab probe: Donkey Anti-Mouse for use with amplifier B2
  - HCR™ amplifier: B2-488
  - HCR™ IF buffers: antibody buffer, amplification buffer

Storage conditions

- Store HCR™ RNA-FISH probe sets, HCR™ IF antibody probes, HCR™ amplifiers, HCR™ RNA-FISH probe hybridization buffer, and HCR™ RNA-FISH probe wash buffer at -20 °C.
- Store HCR™ IF antibody buffer and 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 immunohistochemistry, up to the primary antibody application step. This may include permeabilization and antigen retrieval. Then proceed with the protocol described below.

Multiplexed HCR™ IF + HCR™ RNA-FISH protocol

Protein detection stage

1. Block samples with 250 μL of antibody buffer for 4 h at 4 °C.
2. Prepare working concentration of primary antibodies in antibody buffer. Prepare 250 μL per sample.
   NOTE: *follow manufacturer's guidelines for primary antibody working concentration.*
3. Remove antibody buffer and add primary antibody solution to samples.
4. Incubate samples overnight (>12 h) at 4 °C with gentle rotation.
   NOTE: *Incubation may be optimized (e.g., 3 h at room temperature) depending on sample type and thickness.*
5. Remove excess antibodies by washing 4 × 30 min with 500 μL of PBST at room temperature.
6. Prepare 1 μg/mL working concentration of initiator-labeled secondary antibodies in antibody buffer. Prepare 250 μL per sample.
   NOTE: *Concentration may be optimized depending on protein target and primary antibody.*
7. Remove PBST and add secondary antibody solution to embryos.
8. Incubate embryos for 3 h at room temperature with gentle rotation.
9. Remove excess antibodies by washing 5 × 5 min with 500 μL of PBST at room temperature.
10. Wash 1 × 5 min with 500 μL of 5× SSCT at room temperature.

RNA detection stage

1. Post-fix sample with 500 μL of 4% formaldehyde.
   CAUTION: *use formaldehyde with extreme care as it is a hazardous material.*
2. Incubate for 10 min at room temperature.
3. Remove fixative and wash each sample 2 $\times$ 500 μL of PBST.
4. Wash sample with 500 μL of 2$\times$ SSCT.
5. Pre-hybridize samples in 250 μL of probe hybridization buffer for 30 min at 37 °C.
   CAUTION: *Probe hybridization buffer contains formamide, a hazardous material.*
   NOTE: *pre-heat probe hybridization buffer to 37 °C before use.*
6. Prepare a 16 nM probe solution by adding 4 pmol of each probe mixture (e.g. 4 μL of 1 μM stock) to 250 μL of probe hybridization buffer at 37 °C.
   NOTE: *This is the amount of probe set needed for each target in a single sample using 250 μL of incubation volume.*
7. Remove the pre-hybridization solution and add the probe solution.
8. Incubate samples overnight (>12 h) at 37 °C.
9. Remove excess probes by washing 4 $\times$ 5 min with 500 μL 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.*
10. Wash with 500 μL 5$\times$ SSCT at room temperature for 5 min.
11. Proceed to **Amplification stage**.

Amplification stage

1. Pre-amplify each sample with 250 μL of amplification buffer for 30 min at room temperature.
   NOTE: *equilibrate amplification buffer to room temperature before use.*
2. Separately prepare 15 pmol of hairpin h1 and 15 pmol of hairpin h2 by snap cooling 5 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. This is the amount of hairpins needed for each target in a single sample using 250 μL of incubation volume.*
3. Prepare a 60 nM hairpin solution by adding all snap-cooled h1 hairpins and snap-cooled h2 hairpins to 250 μL of amplification buffer at room temperature per sample.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate the samples overnight (>12 h) in the dark at room temperature.
6. Remove excess hairpins by washing with 500 μL 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

1× PBST
1× phosphate buffered solution (PBS)
0.1% Tween 20

For 40 mL of solution
4 mL of 10× PBS
400 μL of 10% Tween 20
Fill up to 40 mL with ultrapure H_2O

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 PBT as this leads to increased autofluorescence in the samples.*

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).