HCR™ RNA-FISH (v3.0)/2°IF 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-v3

Patents
www.molecularinstruments.com/patents

Ordering for Multiplex Experiment
Order one HCR™ 2°IF kit per target protein
Order one HCR™ RNA-FISH (v3.0) kit per target RNA

Example 2-Plex Experiment

- HCR™ RNA-FISH (v3.0) kit for target mRNA1
  - HCR™ Probe (v3.0): target mRNA1 for use with amplifier B1
  - HCR™ Amplifier (v3.0): B1-647
  - HCR™ RNA-FISH Buffers (v3.0): HCR™ Probe Hybridization Buffer (v3.0), HCR™ Probe Wash Buffer (v3.0), HCR™ Amplifier Buffer (v3.0)
- HCR™ 2°IF kit for target Protein1
  - 1° Ab: Mouse Anti-Protein1 (your own 1° antibody)
  - HCR™ 2° Antibody Probe: Donkey Anti-Mouse for use with amplifier B2
  - HCR™ Amplifier (v3.0): B2-488
  - HCR™ 2°IF Buffers: HCR™ Antibody Buffer, HCR™ Amplifier Buffer (v3.0)

Storage conditions

- Store HCR™ Probes (v3.0), HCR™ 2° Antibody Probes, HCR™ Amplifiers (v3.0), HCR™ Probe Hybridization Buffer (v3.0), and HCR™ Probe Wash Buffer (v3.0) at -20 °C.
- Store HCR™ Antibody Buffer and HCR™ Amplifier Buffer (v3.0) 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™ 2°IF + HCR™ RNA-FISH (v3.0) protocol

Protein detection stage

1. Block samples with 250 μL of HCR™ Antibody Buffer for 4 h at 4 °C.
2. Prepare working concentration of primary antibodies in HCR™ Antibody Buffer. Prepare 250 μL per sample.
   NOTE: *follow manufacturer's guidelines for primary antibody working concentration.*
3. Remove HCR™ 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 1× PBST at room temperature.
6. Prepare 1 μg/mL working concentration of HCR™ 2° Antibody Probes in HCR™ Antibody Buffer. Prepare 250 μL per sample.
   NOTE: *Concentration may be optimized depending on protein target and primary antibody.*
7. Remove 1× 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 1× PBST at room temperature.
10. Wash 1 × 5 min with 500 μL of 5× SSCT at room temperature.
11. Proceed to **RNA detection stage** for co-detection of protein and RNA.

RNA detection stage

1. Post-fix sample with 500 μL of 4% paraformaldehyde (PFA).
   CAUTION: *use PFA with extreme care as it is a hazardous material.*
   NOTE: *use 4% formaldehyde for cells in suspension.*
2. Incubate for 10 min at room temperature.
3. Remove fixative and wash each sample 2 $\times$ 500 μL of 1$\times$ PBST.
4. Wash sample with 500 μL of 2$\times$ SSCT.
5. Pre-hybridize samples in 250 μL of HCR™ Probe Hybridization Buffer (v3.0) for 30 min at 37 °C.
   CAUTION: *HCR™ Probe Hybridization Buffer (v3.0) contains formamide, a hazardous material.*
   NOTE: *pre-heat HCR™ Probe Hybridization Buffer (v3.0) to 37 °C before use.*
6. Prepare a 16 nM probe solution by adding 4 pmol of each HCR™ Probe (v3.0) (e.g. 4 μL of 1 μM stock) to 250 μL of HCR™ Probe Hybridization Buffer (v3.0) at 37 °C.
   NOTE: *This is the amount of probe 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 HCR™ Probe Wash Buffer (v3.0) at 37 °C.
   CAUTION: *HCR™ Probe Wash Buffer (v3.0) contains formamide, a hazardous material.*
   NOTE: *pre-heat HCR™ Probe Wash Buffer (v3.0) 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 HCR™ Amplifier Buffer (v3.0) for 30 min at room temperature.
   NOTE: *equilibrate HCR™ Amplifier Buffer (v3.0) 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 HCR™ Amplifier Buffer (v3.0) 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

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

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

S1 HCR™ Technology Citation Notes

For citation, please select from the list below as appropriate for your application:

- **HCR™ RNA-ISH**
  HCR™ RNA in situ hybridization (RNA-ISH) offers unmatched performance, robustness, and versatility imaging RNA targets in diverse organisms and sample types (Choi et al., 2010, Choi et al., 2014, Choi et al., 2018):
  - **HCR™ RNA-FISH**
    HCR™ RNA-FISH enables 10-plex, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) with automatic background suppression throughout the protocol for dramatically enhanced performance (signal-to-background, quantitative precision, single-molecule fidelity) and ease-of-use (no probe set optimization for new targets and organisms).
  - **Enzymatic HCR™ RNA-CISH/RNA-FISH**
    Enzymatic HCR™ RNA-ISH integrates enzymatic signal amplification to enable extreme-sensitivity RNA imaging using either chromogenic or fluorescent staining (RNA-CISH/RNA-FISH). In tissue sections, entirely protease-free workflows preserve sample morphology and maintain protein target integrity, enabling seamless compatibility with existing immunohistochemistry (IHC)/immunofluorescence (IF) assays. HCR™ RNA-CISH offers the convenience of brightfield microscopy and the option of archival staining.
- **10-Plex HCR™ Spectral Imaging**
  HCR™ RNA-FISH/IF enables quantitative high-resolution imaging of 10 RNA and/or protein targets with 1-step HCR™ signal amplification for all targets simultaneously. The method is suitable even for whole-mounts and delicate samples as it requires no repeated staining, imaging, registration, or stripping (Schulte et al., 2024).
- **HCR™ RNA-FISH/IF**
  HCR™ RNA-FISH/IF enables a unified approach to multiplex, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) and protein immunofluorescence (IF), with quantitative 1-step enzyme-free signal amplification performed for all RNA and protein targets simultaneously (Schwarzkopf et al., 2021).
- **HCR™ IF**
  HCR™ IF enables multiplex, quantitative, high-resolution protein immunofluorescence (IF) in highly autofluorescent samples (e.g., FFPE brain tissue sections) (Schwarzkopf et al., 2021).
- **Subcellular Quantitative RNA and Protein Imaging**
  HCR™ RNA-FISH enables analog relative quantitation of RNA and/or protein targets with subcellular resolution in the anatomical context of thick autofluorescent samples (e.g., whole-mount vertebrate embryos) (Trivedi et al., 2018, Choi et al., 2018, Schwarzkopf et al., 2021).
- **Single-Molecule Quantitative RNA Imaging**
  HCR™ RNA-FISH enables digital 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, Choi et al., 2018).
- **Read-Out/Read-In Analysis Framework**
  The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (Trivedi et al., 2018).

- **Protocols in Diverse Sample Types**
  Protocols for HCR™ RNA-FISH and/or IF in diverse sample types are adapted from the zoo paper (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
- **HCR™ RNA Flow Cytometry**
  HCR™ RNA Flow Cytometry enables analog RNA relative quantitation for high-throughput expression profiling of mammalian cells and bacteria without the need to engineer reporter lines (Choi et al., 2018).
- **HCR™ Northern Blots**
  HCR™ Northern Blots enable simultaneous quantification of RNA target size and abundance with automatic background suppression throughout the protocol (Schwarzkopf & Pierce, 2016).
- **HCR™ Amplifiers**
  HCR™ Amplifiers enable multiplex, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings (Dirks & Pierce, 2004).