HCR™ RNA-FISH (v3.0)/IF protocol for mammalian cells on a chambered slide

This protocol has not been validated for all mammalian cell 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™ 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™ 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™ 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.

Preparation of fixed mammalian cells on a chambered slide

1. Coat bottom of each chamber by applying 300 μL of 0.01% poly-D-lysine prepared in cell culture grade H_2O.
   NOTE: *A volume of 300 μL is sufficient per chamber on an 8-chamber slide. Scale volume accordingly if using a different slide format.*
2. Incubate for at least 30 min at room temperature.
3. Aspirate the coating solution and wash each chamber twice with molecular biology grade H_2O.
4. Plate desired number of cells in each chamber.
5. Grow cells to desired confluency for 24–48 h.
6. Aspirate growth media and wash each chamber with 300 μL of DPBS.
   NOTE: *avoid using calcium chloride and magnesium chloride in DPBS as this leads to increased autofluorescence.*
7. Add 300 μL of 4% formaldehyde to each chamber.
   CAUTION: *use formaldehyde with extreme care as it is a hazardous material.*
8. Incubate for 10 min at room temperature.
9. Remove fixative and wash each chamber 2 $\times$ 300 μL of DPBS.
10. Aspirate DPBS and add 300 μL of ice-cold 70% ethanol (EtOH).
11. Permeabilize cells overnight (or longer) at -20 °C.
12. Proceed to HCR™ IF + HCR™ RNA-FISH assay.

Multiplexed HCR™ IF + HCR™ RNA-FISH (v3.0) protocol

Protein detection stage

1. Aspirate EtOH from sample and wash samples 2 × 5 min with 300 μL of 1× PBS.
2. Apply 300 μL HCR™ Antibody Buffer to each chamber. Incubate at room temperature for 1 hr with gentle agitation.
3. Prepare working concentration of primary antibodies in HCR™ Antibody Buffer. Prepare 300 μL per chamber.
   NOTE: *follow manufacturer's guidelines for primary antibody working concentration.*
4. Replace HCR™ Antibody Buffer with primary antibody solution and incubate overnight (>12 h) at 4 °C with gentle agitation.
   NOTE: *Incubation may be optimized (e.g., 1–2 h at room temperature) depending on sample type and thickness.*
5. Remove excess antibodies by washing 3 × 5 min with 1× PBST at room temperature with gentle agitation.
6. Prepare 1 μg/mL working concentration of HCR™ 2° Antibody Probes in HCR™ Antibody Buffer. Prepare 300 μL per chamber.
   NOTE: *Concentration may be optimized depending on protein target and primary antibody.*
7. Add secondary antibody solution to each chamber and incubate 1 h at room temperature with gentle agitation.
8. Remove excess antibodies by washing 3 × 5 min with 1× PBST at room temperature with gentle agitation.
9. Proceed to **RNA detection stage** for co-detection of protein and RNA.

RNA detection stage

1. Post-fix sample with 300 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 chamber 2 $\times$ 300 μL of PBS.
4. Wash sample with 300 μL of 2$\times$ SSC.
5. Pre-hybridize samples in 300 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 4.8 pmol of each HCR™ Probe (v3.0) (e.g. 4.8 μL of 1 μM stock) to 300 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 chamber of an 8-well chambered slide using 300 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 300 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 300 μL 5$\times$ SSCT at room temperature for 5 min.
11. Proceed to **Amplification stage**.

Amplification stage

1. Wash with 300 μL 5$\times$ SSCT at room temperature for 5 min.
2. Pre-amplify samples in 300 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.*
3. Separately prepare 18 pmol of hairpin h1 and 18 pmol of hairpin h2 by snap cooling 6 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 chamber of an 8-well chambered slide using 300 μL of incubation volume.*
4. Prepare a 60 nM hairpin solution by adding all snap-cooled h1 hairpins and snap-cooled h2 hairpins to 300 μL of HCR™ Amplifier Buffer (v3.0) at room temperature per sample.
5. Remove the pre-amplification solution and add the hairpin solution.
6. Incubate the slide overnight (>12 h) protected from light at room temperature.
7. Remove excess hairpins by washing 5 $\times$ 5 min with 300 μL of 5$\times$ SSCT at room temperature.
8. Remove final wash and add 150 μL of mounting medium.
9. Slides can be stored at 4 °C protected from light prior to imaging.

Buffer recipes

4% formaldehyde in PBS	For 10 mL of solution
4% formaldehyde	2.5 mL of 16% formaldehyde
1× PBS	1 mL of 10× PBS
	Fill up to 10 mL with molecular biology grade H_2O
1× PBST	For 40 mL of solution
1× phosphate buffered solution (PBS)	4 mL of 10× PBS
0.1% Tween 20	400 μL of 10% Tween 20
	Fill up to 40 mL with ultrapure H_2O
5× SSCT	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	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 samples.*

HCR™ Technology Citation Notes
For citation, please select from the list below as appropriate for your application:

- **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).

- **HCR™ RNA-FISH**
  - Third-generation HCR™ RNA-FISH (v3.0) enables multiplex, 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).
  - Second-generation HCR™ RNA-FISH (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).
  - First-generation HCR™ RNA-FISH (v1.0) using RNA HCR™ Probes and RNA HCR™ Amplifiers: multiplex mRNA imaging in whole-mount vertebrate embryos with simultaneous signal amplification for up to 5 target mRNAs (Choi et al., 2010).

- **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).