HCR™ 2°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™ 2°IF kit per target protein

Example 2-Plex Experiment

- HCR™ 2°IF kit for target Protein1
  - 1° Ab: Rabbit Anti-Protein1 (your own 1° antibody)
  - HCR™ 2° Antibody Probe: Donkey Anti-Rabbit for use with amplifier B1
  - HCR™ Amplifier (v3.0): B1-647
  - HCR™ 2°IF Buffers: HCR™ Antibody Buffer, HCR™ Amplifier Buffer (v3.0) (for use with all kits)

- HCR™ 2°IF kit for target Protein2
  - 1° Ab: Mouse Anti-Protein2 (your own 1° antibody)
  - HCR™ 2° Antibody Probe: Donkey Anti-Mouse for use with amplifier B2
  - HCR™ Amplifier (v3.0): B2-488

Storage conditions

- Store HCR™ 2° Antibody Probes and HCR™ Amplifiers (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™ 2°IF assay.

Multiplexed HCR™ 2°IF protocol

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 unlabeled 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.

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 sample 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.*

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 bright-field 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).