# HCR™ RNA-FISH (v3.0)/2°IF protocol for sample on slide

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 tissue by applying 200 $\mu$L of HCR™ Antibody Buffer on top of the sample. Incubate at room temperature for 1 h in a humidified chamber.
2. Prepare working concentration of primary antibodies in HCR™ Antibody Buffer. Prepare 100 $\mu$L per section.
   NOTE: *follow manufacturer's guidelines for primary antibody working concentration.*
3. Drain slide by blotting edges on a Kimwipe and wipe around the section with another Kimwipe.
4. Add primary antibody solution to each section and incubate overnight ($>$12 h) at 4 °C in a humidified chamber.
   NOTE: *Incubation may be optimized (e.g., 1–2 h at room temperature) depending on sample type and thickness.*
5. Remove excess antibodies by immersing slide in 1× PBST at room temperature for 3 × 5 min.
6. Prepare 1 $\mu$g/mL working concentration of HCR™ 2° Antibody Probes in HCR™ Antibody Buffer. Prepare 100 $\mu$L per section.
   NOTE: *Concentration may be optimized depending on protein target and primary antibody.*
7. Drain slide by blotting edges on a Kimwipe and wipe around the section with another Kimwipe.
8. Add secondary antibody solution to each section and incubate for 1 h at room temperature in a humidified chamber.
9. Remove excess antibodies by immersing slide in 1× PBST at room temperature for 3 × 5 min.
10. Proceed to **RNA detection stage** for co-detection of protein and RNA.

**RNA detection stage**

1. Drain slide by blotting edges on a Kimwipe and wipe around the section with another Kimwipe.

2. Post-fix sample by adding 200 $\mu$L of 4% formaldehyde on the tissue.
   CAUTION: *use formaldehyde with extreme care as it is a hazardous material.*

3. Incubate slides for 10 min at room temperature.

4. Immerse slides for 2 × 5 min in 1× PBST.

5. Immerse slides for 5 min in 5× SSCT.

6. Pre-warm a humidified chamber to 37 °C.

7. Drain slide by blotting edges on a Kimwipe and wipe around the section with another Kimwipe.

8. Add 200 $\mu$L of HCR™ Probe Hybridization Buffer (v3.0) on top of the tissue sample.
   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.*

9. Pre-hybridize for 10 min inside the humidified chamber.

10. Prepare a 16 nM probe solution by adding 1.6 pmol of each HCR™ Probe (v3.0) (e.g. 1.6 $\mu$L of 1 $\mu$M stock) to 100 $\mu$L of HCR™ Probe Hybridization Buffer (v3.0) at 37 °C.
    NOTE: *This is the amount of probe needed for each target on a single slide using 100 $\mu$L of incubation volume.*

11. Remove the pre-hybridization solution and drain excess buffer on slide by blotting edges on a Kimwipe.

12. Add 100 $\mu$L of the probe solution on top of the tissue sample.

13. Place a coverslip on the sample and incubate overnight (>12 h) in the 37 °C humidified chamber.

14. Immerse slide in HCR™ Probe Wash Buffer (v3.0) at 37 °C to float off coverslip.
    CAUTION: *HCR™ Probe Wash Buffer (v3.0) contains formamide, a hazardous material.*

15. Remove excess probes by incubating slide at 37 °C in:
    (a) 75% of HCR™ Probe Wash Buffer (v3.0) / 25% 5× SSCT for 15 min
    (b) 50% of HCR™ Probe Wash Buffer (v3.0) / 50% 5× SSCT for 15 min
    (c) 25% of HCR™ Probe Wash Buffer (v3.0) / 75% 5× SSCT for 15 min
    (d) 100% 5× SSCT for 15 min

    NOTE: *Wash solutions should be pre-heated to 37 °C before use.*

16. Proceed to **Amplification stage**.

**Amplification stage**

1. Immerse slide in 5× SSCT at room temperature for 5 min.

2. Drain slide by blotting edges on a Kimwipe and wipe around the section with another Kimwipe.

3. Add 200 $\mu$L of HCR™ Amplifier Buffer (v3.0) on top of the tissue sample and pre-amplify in a humidified chamber for 30 min at room temperature.
   NOTE: *equilibrate HCR™ Amplifier Buffer (v3.0) to room temperature before use.*

4. Separately prepare 6 pmol of hairpin h1 and 6 pmol of hairpin h2 by snap cooling 2 $\mu$L of 3 $\mu$M 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 on a single slide using 100 $\mu$L of incubation volume.*

5. Prepare a 60 nM hairpin solution by adding all snap-cooled h1 hairpins and snap-cooled h2 hairpins to 100 $\mu$L of HCR™ Amplifier Buffer (v3.0) at room temperature per section.

6. Remove the pre-amplification solution and drain excess buffer on slide by blotting edges on a Kimwipe.

7. Add 100 $\mu$L of the hairpin solution on top of the tissue sample.

8. Incubate overnight (>12 h) in a dark humidified chamber at room temperature.

9. Remove excess hairpins by immersing slide in 5× SSCT at room temperature for:
   (a) 2 × 5 min
   (b) 2 × 15 min
   (c) 1 × 5 min

10. Drain slide by blotting edges on a Kimwipe and dry around the section with another Kimwipe.

11. Add 50–100 $\mu$L of antifade mounting reagent on top of the sample.

12. Place a coverslip on top for microscopy.

## Buffer recipes

| **1× PBST** | For 50 mL of solution |
|---|---|
| 1× PBS | 5 mL of 10× PBS |
| 0.1% Tween 20 | 500 $\mu$L of 10% Tween 20 |
| | Fill up to 50 mL with ultrapure $H_2O$ |

| **4% Formaldehyde (FA)** | 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 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 $\mu$L 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 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).