HCR™ IF protocol for FFPE tissue sections

This protocol has not been validated for all tissue 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

Example 2-Plex Experiment

- HCR™ 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™ IF Buffers: HCR™ Antibody Buffer, HCR™ Amplifier Buffer (v3.0) (for use with all kits)

- HCR™ 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 formalin-fixed paraffin-embedded (FFPE) tissue sections

1. Bake slides in a dry oven for 1 h at 60 °C to improve sample adhesion to the slide.
2. In a fume hood, deparaffinize FFPE tissue by immersing in Pro-Par Clearant for 3 × 5 min. Move slides up and down occasionally.
   CAUTION: *use Pro-Par Clearant with care as it is a hazardous material.*
   NOTE: *Xylene can be used in place of Pro-Par Clearant.*
   NOTE: *Each 50 mL tube can fit two outward-facing slides. A volume of 30mL is sufficient to immerse sections in the tube. If desired, a larger number of slides can be processed together using a Coplin jar.*
3. Incubate slides in 100% ethanol (EtOH) for 2 × 3 min at room temperature. Move slides up and down occasionally.
4. Rehydrate with a series of graded EtOH washes at room temperature.
   (a) 95% EtOH for 3 min
   (b) 70% EtOH for 3 min
   (c) 50% EtOH for 3 min
   (d) Nanopure water for 3 min
5. Bring 500 mL of 1× citrate buffer (pH 6.0) in a beaker to boil in a microwave.
   NOTE: *1× Tris-EDTA buffer (pH 9.0) can be used in place of citrate buffer (pH 6.0). Optimal antigen retrieval method may differ depending on the antigen/antibody used.*
6. Maintain citrate buffer at 90–95 °C on a hot plate.
7. Immerse slides for 15 min.
   NOTE: *Alternatively, slides may be immersed at 95–99 °C for 15 min in a steamer.*
8. Remove beaker from hot plate and add 100 mL of nanopure water every 5 min to allow temperature to decrease to 45 °C in 20 min.
9. Immerse slides in 400 mL of nanopure water in a separate container for 10 min at room temperature.
10. Immerse slides in 1× PBST for 2 × 2 min at room temperature.
    NOTE: *avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the tissue.*
11. Drain slide by blotting edges on a Kimwipe.
12. Wipe around the section with a Kimwipe and circle tissue with a hydrophobic pen.
13. Proceed to HCR™ IF assay.

Multiplexed HCR™ IF protocol

Detection stage

1. Block tissue by applying 200 μL 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 μL 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$\times$ PBST at room temperature for 3 × 5 min.

6. Prepare 1 μg/mL working concentration of HCR™ 2° Antibody Probes in HCR™ Antibody Buffer. Prepare 100 μL 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$\times$ PBST at room temperature for 3 × 5 min.

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 μL 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 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 on a single slide using 100 μL of incubation volume.*
5. Prepare a 60 nM hairpin solution by adding all snap-cooled h1 hairpins and snap-cooled h2 hairpins to 100 μL 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 μL 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 μL of antifade mounting reagent on top of the sample.
12. Place a coverslip on top for microscopy.

Buffer recipes

1× citrate buffer 1× citrate buffer	For 500 mL of solution 5 mL of 100× citrate buffer (pH 6.0) Fill up to 500 mL with water
1× Tris-EDTA buffer 1× Tris-EDTA buffer	For 500 mL of solution 5 mL of 100× Tris-EDTA buffer (pH 9.0) Fill up to 500 mL with water
PBST 1× PBS 0.1% Tween 20	For 50 mL of solution 5 mL of 10× PBS 500 μL of 10% Tween 20 Fill up to 50 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 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).