HCR™ IF + HCR™ RNA-FISH 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

Patents
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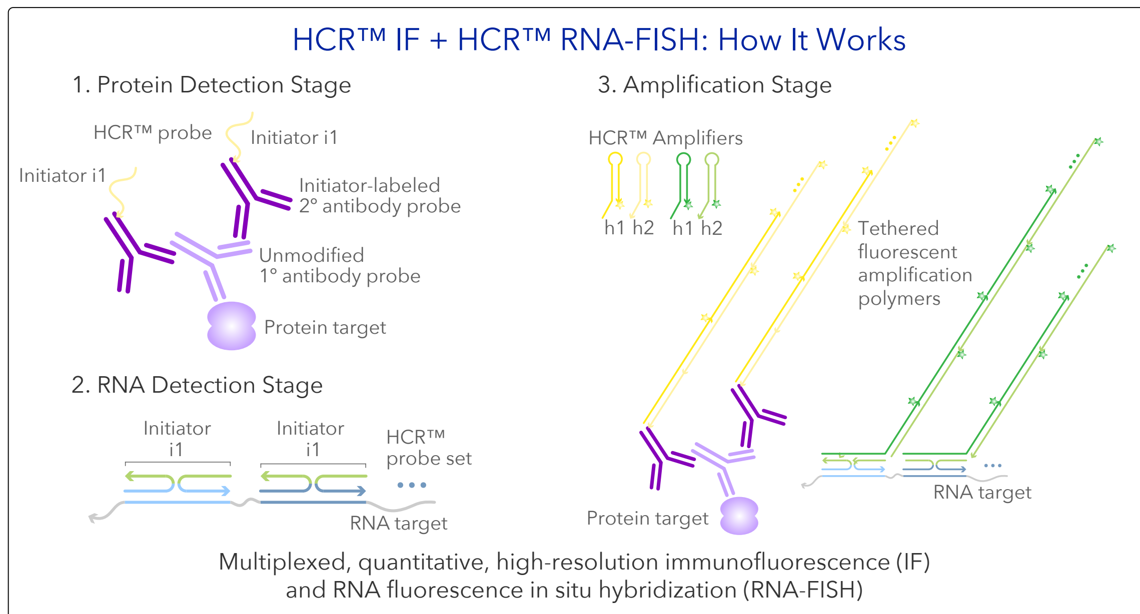

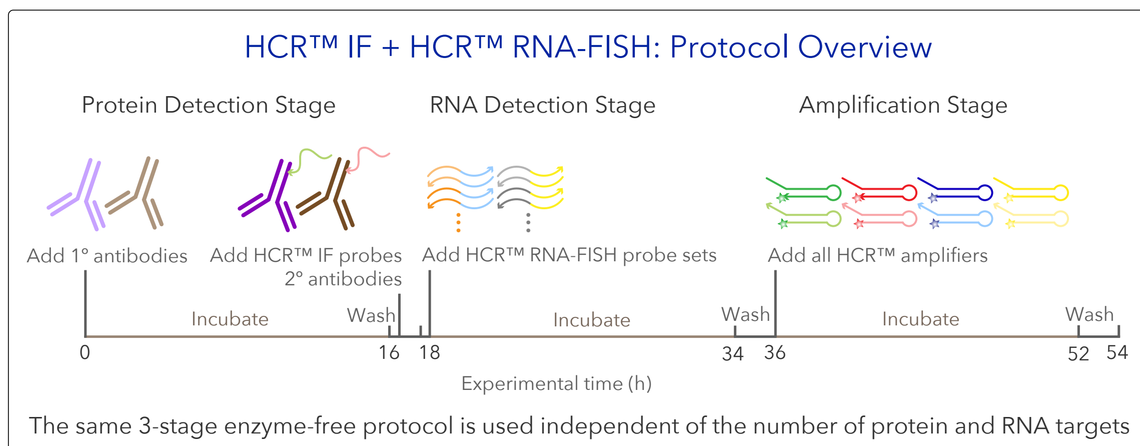

HCR™ IF + HCR™ RNA-FISH

Simultaneous multiplexed, quantitative, high-resolution protein and RNA imaging

Multiplexed Experiment

- Order one HCR™ IF bundle per target protein
- Order one HCR™ RNA-FISH bundle per target RNA

Example 2-Plex Experiment

- HCR™ RNA-FISH bundle for target mRNA1
  - HCR™ RNA-FISH probe set: target mRNA1 for use with amplifier B1
  - HCR™ amplifier: B1-647
  - HCR™ RNA-FISH buffers: probe hybridization buffer, probe wash buffer, amplification buffer
- HCR™ IF bundle for target Protein1
  - 1° Ab: Mouse Anti-Protein1 (your own 1° antibody)
  - HCR™ IF 2° Ab probe: Donkey Anti-Mouse for use with amplifier B2
  - HCR™ amplifier: B2-488
  - HCR™ IF buffers: antibody buffer, amplification buffer

Storage conditions

- Store HCR™ RNA-FISH probe sets, HCR™ IF antibody probes, HCR™ amplifiers, HCR™ RNA-FISH probe hybridization buffer, and HCR™ RNA-FISH probe wash buffer at -20 °C.
- Store HCR™ IF antibody buffer and HCR™ amplification buffer 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 + HCR™ RNA-FISH assay.

Multiplexed HCR™ IF + HCR™ RNA-FISH protocol

Protein detection stage

1. Block tissue by applying 200 μL of 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 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× PBST at room temperature for 3 × 5 min.
6. Prepare 1 μg/mL working concentration of initiator-labeled secondary antibodies in 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× 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 μL 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 $\times$ 5 min in PBST.
5. Immerse slides for 5 min in 5$\times$ 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 μL of probe hybridization buffer on top of the tissue sample.
   Caution: *Probe hybridization buffer contains formamide, a hazardous material.*
   Note: *pre-heat probe hybridization buffer 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 probe set (e.g. 1.6 μL of 1 μM stock) to 100 μL of probe hybridization buffer at 37 °C.
    Note: *This is the amount of probe set needed for each target on a single slide using 100 μL of incubation volume.*
11. Remove the pre-hybridization solution and drain excess buffer on slide by blotting edges on a Kimwipe.
12. Add 100 μL of the probe solution on top of the tissue sample.
13. Place a coverslip on the sample and incubate overnight ($>$12 h) in the 37 °C humidified chamber.
14. Immerse slide in probe wash buffer at 37 °C to float off coverslip.
    Caution: *Probe wash buffer contains formamide, a hazardous material.*
15. Remove excess probes by incubating slide at 37 °C in:
    (a) 75% of probe wash buffer / 25% 5$\times$ SSCT for 15 min
    (b) 50% of probe wash buffer / 50% 5$\times$ SSCT for 15 min
    (c) 25% of probe wash buffer / 75% 5$\times$ SSCT for 15 min
    (d) 100% 5$\times$ 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 μL of amplification buffer on top of the tissue sample and pre-amplify in a humidified chamber for 30 min at room temperature.
   NOTE: *equilibrate amplification buffer 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 amplification buffer 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	For 500 mL of solution
1× citrate buffer	5 mL of 100× citrate buffer (pH 6.0)
	Fill up to 500 mL with water
1× Tris-EDTA buffer	For 500 mL of solution
1× Tris-EDTA buffer	5 mL of 100× Tris-EDTA buffer (pH 9.0)
	Fill up to 500 mL with water
PBST	For 50 mL of solution
1× PBS	5 mL of 10× PBS
0.1% Tween 20	500 μL of 10% Tween 20
	Fill up to 50 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:

- **HCR™ IF + HCR™ RNA-FISH**
  HCR™ IF + HCR™ RNA-FISH enables a unified approach to multiplexed, quantitative, high-resolution protein immunofluorescence (IF) and RNA fluorescence in situ hybridization (RNA-FISH), with quantitative 1-step enzyme-free signal amplification performed for all protein and RNA targets simultaneously (Schwarzkopf et al., 2021).

- **HCR™ IF**
  HCR™ IF enables multiplexed, quantitative, high-resolution protein immunofluorescence (IF) in highly autofluorescent samples (e.g., FFPE brain tissue sections) (Schwarzkopf et al., 2021).

- **HCR™ RNA-FISH (v3.0)**
  Third-generation HCR™ RNA-FISH (v3.0) enables multiplexed, 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). Quantitative analysis modes:

  - Subcellular quantitative RNA imaging: analog mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples.
  - Single-molecule quantitative RNA imaging: digital mRNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples.
  - Quantitative RNA flow cytometry: analog mRNA relative quantitation for high-throughput expression profiling of mammalian cells and bacteria.

  Protocols for HCR™ RNA-FISH (v3.0) in diverse organisms are adapted from the Zoo paper.

- **Subcellular quantitative RNA imaging**
  Subcellular quantitative RNA imaging enables mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples (e.g., whole-mount vertebrate embryos). The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (Trivedi et al., 2018).

- **Zoo paper**
  Protocols for multiplexed mRNA imaging in diverse sample types (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

- **Single-molecule quantitative RNA imaging**
  Single-molecule quantitative RNA imaging enables 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).

- **HCR™ northern blots**
  HCR™ northern blots enable multiplexed quantification of RNA target size and abundance for up to 5 target RNAs (Schwarzkopf & Pierce, 2016).

- **HCR™ RNA-FISH (v2.0)**
  Second-generation in situ HCR™ RNA-FISH technology (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).

- **HCR™ RNA-FISH (v1.0)**
  First-generation HCR™ RNA-FISH technology (v1.0) using RNA HCR™ probes and RNA HCR™ amplifiers: multiplexed mRNA imaging in whole-mount vertebrate embryos with simultaneous signal amplification for up to 5 target mRNAs (Choi et al., 2010).

- **HCR™ technology**
  HCR™ amplifiers enable multiplexed, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings (Dirks & Pierce, 2004).