HCR RNA-FISH v3.0 protocol for FFPE human tissue sections (*Homo sapiens sapiens*)

This protocol has been optimized for 5-μm FFPE sections.
Technical support: support@moleculartechnologies.org

Sample preparation protocol

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 slide in xylene for 2 × 5 min. Move slides up and down occasionally.
   CAUTION: *Use xylene with care as it is a hazardous material.*
   NOTE: *Each 50 mL falcon tube can fit two outward-facing slides. A volume of 30 mL 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 for 2 × 2 min at room temperature. Move slides up and down occasionally.
4. Dry slide by blotting edges on a Kimwipe.
5. Allow slides to air dry at room temperature.
6. Introduce 200 μL of 10 μg/mL of proteinase K solution on top of sample and incubate slides in a humidified chamber for 10 min at 37 °C.
7. Immerse slides in nanopure water. Move slides up and down occasionally.
8. Dry slide by blotting edges on a Kimwipe.
9. Proceed immediately to HCR RNA-FISH assay.
   NOTE: *Do not let tissues dry out.*

Multiplexed HCR RNA-FISH v3.0 protocol

Detection stage

1. Add 200 μL of 30% probe hybridization buffer on top of the tissue sample.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
2. Pre-hybridize for 10 min inside the 37 °C humidified chamber.
3. Prepare probe solution by adding 0.8 pmol of each probe mixture (e.g. 0.8 μL of 1 μM stock) to 200 μL of 30% probe hybridization buffer at 37 °C.
   NOTE: *For single-molecule RNA imaging, use higher probe concentration (e.g., 16 nM) to increase probe hybridization yield. If desired, this approach can also be used to increase signal for subcellular quantitative RNA imaging.*
4. Remove the pre-hybridization solution and drain excess buffer on slide by blotting edges on a Kimwipe.
5. Add 200 μL of the probe solution on top of the tissue sample.
   NOTE: *Amount of probe solution depends on the size of the tissue.*
6. Incubate overnight (12–16 h) in the 37 °C humidified chamber.
7. Remove excess probes by incubating slide in 30% probe wash buffer at 37 °C for:
   (a) 1 × 5 min
   (b) 2 × 15 min

   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *Wash solutions should be pre-heated to 37 °C before use.*
8. Immerse slide in 5× SSCT for 5 min at room temperature.

Amplification stage

1. Dry slide by blotting edges on a Kimwipe.
2. 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.
3. Separately prepare 12 pmol of hairpin H1 and 12 pmol of hairpin H2 by snap cooling 4 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: *HCR hairpins H1 and H2 are provided in hairpin storage buffer ready for snap cooling. H1 and H2 should be snap cooled in separate tubes.*
4. Prepare hairpin solution by adding all snap-cooled hairpins to 200 μL of amplification buffer at room temperature.
5. Remove the pre-amplification solution and drain excess buffer on slide by blotting edges on a Kimwipe.
6. Add 200 μL of the hairpin solution on top of the tissue sample.
   NOTE: *Amount of hairpin solution depends on the size of the tissue.*
7. Incubate overnight (12–16 h) in a dark humidified chamber at room temperature.
   NOTE: *For dHCR imaging, amplify for a shorter period of time to ensure single-molecule dots are diffraction-limited.*

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

Sample mounting for microscopy

1. Dry slide by blotting edges on a Kimwipe.
2. Add 30 μL of SlowFade Gold antifade mountant with DAPI on top of human tissue section.
3. Place a 22 mm × 40 mm No. 1 coverslip on top and seal using clear nail polish.
4. A Zeiss Axio Observer Z1 fluorescent inverted microscope equipped with an Plan-Apochromat 63×/1.4 Oil DIC M27 objective was used in our lab to acquire tissue section images. Samples were illuminated with X-Cite Series 120 (EXFO Photonics) and images were collected with an ImagEM-1K EM-CCD digital camera (Hamamatsu).

HCR regents storage conditions

Probes, amplifiers, probe hybridization buffer, and probe wash buffer should be stored at -20 °C. Amplification buffer should be stored at 4 °C. Keep these reagents on ice at all times during probe and amplifier preparation. Make sure all solutions are well mixed before use.

General buffer recipes for HCR RNA-FISH

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

Reagents and supplies

Ethanol, 200 proof (VWR Cat. # V1001G)
20 mg/mL Proteinase K (Life Technologies Cat. # AM2546)
10% Tween 20 Solution (Bio-Rad Cat. # 161-0781)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
22 mm × 30 mm No. 1 coverslip (VWR Cat. # 48393-026)
SlowFade Gold antifade mountant with DAPI (Life Technologies Cat. # S36938)

Citation Notes

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- **10-plex HCR spectral imaging**
  HCR spectral imaging of any combination of 10 RNA and/or protein targets with 1-step quantitative HCR signal amplification for all targets simultaneously (Schulte *et al.*, 2024a).
  Software: HCR Imaging Python module including Dot Detection 2.0 and Unmix 1.0 packages.

- **HCR protein:protein complex imaging**
  Multiplexed, quantitative, high-resolution imaging of protein:protein complexes with/without HCR IF and/or HCR RNA-FISH with simultaneous HCR signal amplification for all protein:protein, protein, and RNA targets (Schulte *et al.*, 2024b).

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

- **HCR IF**
  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)**
  Automatic background suppression for dramatically enhanced performance (signal-to-background and quantitative precision) and ease-of-use (no probe set optimization for new targets) (Choi *et al.*, 2018). v3.0 supports three quantitative modes:

  qHCR imaging: analog mRNA relative quantitation with subcellular resolution in an anatomical context;

  qHCR flow cytometry: analog mRNA relative quantitation for high-throughput single-cell analysis;

  dHCR imaging: digital mRNA absolute quantitation with single-molecule resolution in an anatomical context.

  Protocols for v3.0 in diverse organisms are adapted from the zoo paper.
  Software: Dot Analysis 1.0 package.

- **Quantitative HCR (qHCR) imaging with subcellular resolution**
  Analog mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos. The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (Trivedi *et al.*, 2018).
  Software: Read-out/Read-in 1.0 package.

- **Zoo paper**
  Protocols for multiplexed mRNA imaging in diverse sample types (Choi *et al.*, 2016):

bacteria in suspension	whole-mount zebrafish embryos and larvae
whole-mount fruit fly embryos	whole-mount chicken embryos
whole-mount worm larvae	whole-mount mouse embryos
whole-mount sea urchin embryos	FFPE human tissue sections

- **Digitial HCR (dHCR) imaging with single-molecule resolution**
  Digital mRNA absolute quantitation with single-molecule resolution in thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah *et al.*, 2016).

- **Multiplexed quantitative HCR (qHCR) northern blots**
  Simultaneous quantification of RNA target size and abundance with signal amplification for up to 5 target RNAs (Schwarzkopf & Pierce, 2016).

- **HCR RNA-FISH (v2.0)**
  2nd generation HCR RNA-FISH technology (v2.0) using DNA 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)**
  1st generation HCR RNA-FISH technology (v1.0) using RNA 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 mechanism**
  The hybridization chain reaction (HCR) mechanism enables isothermal enzyme-free molecular signal amplification (Dirks & Pierce, 2004).

We are happy to provide advice and technical support: support@moleculartechnologies.org.

– The Molecular Technologies Team

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