Molecular Technologies
GENERIC SAMPLES IN SOLUTION

HCR IF/RNA-FISH for samples in solution

This protocol has not been validated for all mammalian cell types and should only be used as a template.
Technical support: support@moleculartechnologies.org

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.

HCR IF with/without HCR RNA-FISH

Unlabeled primary antibody probes and initiator-labeled secondary antibody probes for protein targets, split-initiator DNA probes for RNA targets, and simultaneous HCR signal amplification for all targets.

Protein detection stage

1. Block samples with 250 μL of antibody buffer for 4 h at 4 °C .
2. Prepare working concentration of primary antibodies in antibody buffer. Prepare 250 μL per chamber.
   NOTE: *follow manufacturer's guidelines for primary antibody working concentration.*
3. Replace 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.*
4. Remove excess antibodies by washing 4 × 30 min with 500 μL of 1× PBST at room temperature with gentle agitation.
5. Prepare 1 μg/mL working concentration of initiator-labeled secondary antibodies in antibody buffer. Prepare 250 μL per chamber.
   NOTE: *Concentration may be optimized depending on protein target and primary antibody.*
6. Remove PBST and add secondary antibody solution to sample.
7. Incubate sample for 3 h at room temperature with gentle agitation.
8. Remove excess antibodies by washing 5 × 5 min with 500 μL of 1× PBST at room temperature with gentle agitation.
9. Wash 1 × 5 min with 500 μL of 5× SSCT at room temperature
10. Proceed to **RNA detection stage** for co-detection of protein and RNA. Otherwise, proceed to **Amplification stage**.

RNA detection stage

1. Post-fix sample with 500 μL of 4% formaldehyde.
   CAUTION: *use formaldehyde with extreme care as it is a hazardous material.*
2. Incubate for 10 min at room temperature.
3. Remove fixative and wash each chamber with 2 $\times$ 500 μL of PBST.
4. Wash sample with 500 μL of 2$\times$ SSCT.
5. Pre-hybridize samples in 250 μL of probe hybridization buffer for 30 min at 37 °C.
   CAUTION: *Probe hybridization buffer contains formamide, a hazardous material.*
   NOTE: *pre-heat probe hybridization buffer to 37 °C before use.*
6. Prepare a 16 nM probe solution by adding 4 pmol of each probe mixture (e.g. 4 μL of 1 μM stock) to 250 μL of probe hybridization buffer at 37 °C.
   NOTE: *This is the amount of probe set needed for each target on a single sample using 250 μL of incubation volume.*
7. Remove the pre-hybridization solution and add the probe solution.
8. Incubate samples overnight (>12 h) at 37 °C.
9. Remove excess probes by washing 4 $\times$ 5 min with 500 μL of probe wash buffer at 37 °C.
   CAUTION: *Probe wash buffer contains formamide, a hazardous material.*
   NOTE: *pre-heat probe wash buffer to 37 °C before use.*
10. Wash with 500 μL of 5$\times$ SSCT at room temperature for 5 min.
11. Proceed to **Amplification stage**.

Amplification stage

1. Pre-amplify samples in 250 μL of amplification buffer for 30 min at room temperature.
   NOTE: *Equilibrate amplification buffer to room temperature before use.*
2. Separately prepare 15 pmol of hairpin h1 and 15 pmol of hairpin h2 by snap cooling 5 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. This is the amount of hairpins needed for each target in a single sample using 300 μL of incubation volume.*
3. Prepare a 60 nM hairpin solution by adding all snap-cooled h1 hairpins and snap-cooled h2 hairpins to 250 μL of amplification buffer at room temperature per sample.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate the slide overnight (>12 h) protected from light at room temperature.
6. Remove excess hairpins by washing with 500 μL of 5$\times$ SSCT at room temperature:
   (a) 2 $\times$ 5 min
   (b) 2 $\times$ 30 min
   (c) 1 $\times$ 5 min
7. Samples can be stored at 4 °C protected from light before microscopy.

Sample mounting for microscopy

1. Remove final wash and add 150 μL of mounting medium (e.g., Fluoromount-G with DAPI).
2. Slides can be stored at 4 °C protected from light prior to imaging.

Buffers for HCR IF with/without HCR RNA-FISH

HCR probes (initiator-labeled antibody probes, split-initiator DNA probes), amplifiers, and buffers (antibody buffer, probe hybridization buffer, probe wash buffer, amplification buffer) are available from Molecular Technologies (www.moleculartechnologies.org). Probe hybridization buffer and probe wash buffer should be stored at -20 °C. Antibody buffer and amplification buffer should be stored at 4 °C. Make sure all solutions are well mixed before use.

1× PBST	For 40 mL of solution
1× phosphate-buffered saline (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

NOTE: *avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the samples.*

5× SSCT	For 40 mL of solution
5× saline 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

ibidi μ-slide ibitreat (ibidi Cat. # 80826)
Poly-D-lysine hydrobromide (Sigma-Aldrich Cat. # P7280)
Molecular biology grade H_2O (Corning Cat. # 46-000-CV)
DPBS, no calcium, no magnesium (Life Technologies Cat. # 14190144)
Image-iT Fixative Solution 4% (Thermo Fisher Scientific Cat. # FB002)
10× PBS (Ambion Cat. # AM9624)
10% Tween 20 (Teknova Cat. # T0710)
20× saline sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
DAPI Fluoromount-G (SouthernBiotech Cat. # 0100-20)

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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