In situ HCR 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.
   NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K.*
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 in situ HCR assay.
   NOTE: *Do not let tissues dry out.*

Multiplexed in situ HCR 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 0.4 pmol of each probe mixture (odd & even: 0.2 μL of 2 μM stock per probe mixture) to 100 μL of 30% probe hybridization buffer at 37 °C.
   NOTE: *For dHCR imaging, use higher concentration of probe to improve probe hybridization efficiency.*
4. Remove the pre-hybridization solution and drain excess buffer on slide by blotting edges on a Kimwipe.
5. Add 50–100 μL of the probe solution on top of the tissue sample.
   NOTE: *Amount of probe solution depends on the size of the tissue.*
6. Place a coverslip on the sample and incubate overnight (12–16 h) in a 37 °C humidified chamber.
7. Immerse slide in 30% probe wash buffer at 37 °C to float off coverslip.
   CAUTION: *Probe hybridization buffer contains formamide, a hazardous material.*
8. Remove excess probes by incubating slide at 37 °C in:
   (a) 75% of probe wash buffer / 25% 5 × SSCT for 15 min
   (b) 50% of probe wash buffer / 50% 5 × SSCT for 15 min
   (c) 25% of probe wash buffer / 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.*
9. 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 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: *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 snap-cooled H1 hairpins snap-cooled H2 hairpins to 100 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 50–100 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 $\times$ 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$\times$/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).

Buffer recipes for in situ HCR v3.0

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.

30% probe hybridization buffer	For 40 mL of solution
30% formamide	12 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
1× Denhardt's solution	800 μL of 50× Denhardt's solution
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill up to 40 mL with ultrapure H_2O

30% probe wash buffer	For 40 mL of solution
30% formamide	12 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
	Fill up to 40 mL with ultrapure H_2O

Amplification buffer	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
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill up to 40 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

50% dextran sulfate	For 40 mL of solution
50% dextran sulfate	20 g of dextran sulfate powder
	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)
Formamide (Deionized) (Ambion Cat. # AM9342)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
50× Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)
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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- **In situ HCR 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; qHCR flow cytometry – analog mRNA relative quantitation for high-throughput single-cell analysis; dHCR imaging – digital mRNA absolute quantitation. Protocols for v3.0 in diverse organisms are adapted from the zoo paper.

- **Quantitative HCR (qHCR) imaging**
  mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos; read-out/read-in analysis framework (Trivedi *et al.*, 2018).

- **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

- **Single-molecule HCR imaging**
  Single-molecule mRNA imaging 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).

- **In situ HCR v2.0**
  2nd generation in situ HCR 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).

- **Shielded covalent (SC) probes**
  Highly selective covalent capture of DNA and RNA targets, overcoming the longstanding tradeoff between selectivity and durable target capture (Vieregg *et al.*, 2013).

- **In situ HCR v1.0**
  1st generation in situ HCR 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).

- **Hybridization chain reaction (HCR) mechanism**
  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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