In situ HCR v2.0 protocol for samples in solution

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

Sample pre-treatment

Samples should be prepared in the same manner as for a traditional in situ hybridization, up to the probe hybridization step. This may include permeabilization and protease digestion. Then proceed with the protocol described below.

Multiplexed in situ HCR v2.0 protocol

Detection stage

1. Pre-hybridize samples in 500 μL of probe hybridization buffer for 30 min at 45 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
2. Prepare probe solution by adding 1 pmol of each probe (1 μL of 1 μM stock per probe) to 500 μL of probe hybridization buffer at 45 °C.
3. Remove the pre-hybridization solution and add the probe solution.
4. Incubate samples overnight (12–16 h) at 45 °C.
5. Remove excess probes by washing with 500 μL of probe wash buffer at 45 °C:
   (a) 2 × 5 min
   (b) 2 × 30 min

   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *Wash solutions should be pre-heated to 45 °C before use.*
6. Wash samples 3 × 5 min with 5× SSCT at room temperature.

Amplification stage

1. Pre-amplify samples in 500 μL of amplification buffer for 30 min at room temperature.
2. Separately prepare 30 pmol of hairpin H1 and 30 pmol of hairpin H2 by snap cooling 10 μL of 3 μM stock in hairpin storage buffer (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.
3. Prepare hairpin solution by adding all snap-cooled hairpins to 500 μL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate samples overnight (12–16 h) in the dark at room temperature.
6. Remove excess hairpins by washing with 500 μL of 5× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 30 min
   (c) 1 × 5 min

Sample mounting for microscopy

Samples can be mounted in SlowFade Gold antifade mountant inside an imaging chamber tailored for the sample of interest. For whole-mount embryos or larvae, a Zeiss 710 NLO inverted confocal microscope equipped with either an LD LCI Plan-Apochromat 25×/0.8 Imm Corr DIC objective or an LD C-Apochromat 40×/1.1 W Korr M27 objective was used in our lab to acquire images.

Buffer recipes for in situ HCR v2.0

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.

Probe hybridization buffer	For 40 mL of solution
50% formamide	20 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

Probe wash buffer	For 40 mL of solution
50% formamide	20 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

Formamide (Deionized) (Ambion Cat. # AM9342)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
50% Tween 20 (Life Technologies Cat. # 00-3005)
50× Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)
SlowFade Gold antifade mountant (Life Technologies Cat. # S36936)

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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