Molecular Technologies
WORM LARVAE

In situ HCR v2.0 protocol for whole-mount worm larvae (*Caenorhabditis elegans*)

This protocol has been optimized for larvae at stages L1–L4.
Technical support: support@moleculartechnologies.org

Sample preparation

1. Wash worm larvae off plate with 1 mL of M9 buffer and transfer to a 1.5 mL eppendorf tube.
2. Centrifuge at 200 × g for 2 min to bring larvae to the bottom of the tube and remove the M9 buffer.
3. Wash larvae 3 times with 1 mL of M9 buffer each. Centrifuge at 200 × g for 2 min between washes.
4. Centrifuge and remove ≈ 800 μL of M9 buffer.
5. Aliquot larvae sufficent for an in situ reaction into 1.5 mL eppendorf tubes.
6. Add 1 mL of 4% paraformaldehyde (PFA).
   CAUTION: *Use PFA with extreme care as it is a hazardous material.*
   NOTE: *Use fresh PFA to avoid increased autofluorescence.*
7. Immediately freeze sample at -80 °C overnight before use. Larvae could stay in -80 °C freezer for long-term storage.

Buffer recipes for sample preparation

M9 buffer

22 mM KH_2PO_4
42 mM Na_2HPO_4
20.5 mM NaCl
1 mM $MgSO_4$

For 1 L of solution
3 g of KH_2PO_4
6 g of Na_2HPO_4
5 g of NaCl
1 mL of 1 M $MgSO_4$
Fill up to 1 L with ultrapure H_2O
Sterilize by autoclaving
Store buffer at 4 °C before use

4% Paraformaldehyde (PFA)

4% PFA
1× PBS

For 40 mL of solution
10 mL of 16% PFA solution
4 mL of 10× PBS
Fill up to 40 mL with ultrapure H_2O

PBST

1× PBS
0.1% Tween 20

For 50 mL of solution
5 mL of 10× PBS
500 μL of 10% Tween 20
Fill up to 50 mL with ultrapure H_2O

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

Multiplexed in situ HCR v2.0 protocol

Detection stage

1. Fix larvae by thawing at room temperature for 45 min.
2. Wash larvae 2 times with 1 mL of PBST each. Bring larvae to the bottom of the tube with centrifugation at 200 × g for 2 min in between washes.
3. Treat larvae with 1 mL of proteinase K (100 μg/mL) for 10 min at 37 °C.
   NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K.*
4. Wash larvae 2 times with 1 mL of PBST each.
5. Incubate in 1 mL of 2 mg/mL of glycine solution for 15 min on ice.
6. Wash larvae 2 times with 1 mL of PBST each.
7. Incubate larvae in 1 mL of 50% PBST / 50% probe hybridization buffer for 5 min at room temperature.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
8. Centrifuge at 200 × g for 2 min to remove solution.
9. Pre-hybridize larvae in 300 μL of probe hybridization buffer at 45 °C for 1 h.
10. Prepare probe solution by adding 1 pmol of each probe (1 μL of 1 μM stock per probe) to 200 μL of probe hybridization buffer at 45 °C.
11. Add the probe solution to reach a final hybridization volume of 500 μL.
12. Incubate larvae overnight (12–16 h) at 45 °C.
13. Remove excess probes by washing with 1 mL 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.*
    NOTE: *Before each wash, bring larvae to the bottom of the tube with centrifugation at 500 × g for 2 min.*
14. Wash larvae 2 × 5 min with 1 mL of 5× SSCT.

Amplification stage

1. Pre-amplify larvae with 300 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 200 μL of amplification buffer at room temperature.
4. Add the hairpin solution to reach a final amplification volume of 500 μL.
5. Incubate larvae overnight (12–16 h) in the dark at room temperature.
6. Remove excess hairpins by washing with 1 mL of 5× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 30 min
   (c) 1 × 5 min

   NOTE: *Before each wash, bring larvae to the bottom of the tube with centrifugation at 500 × g for 2 min.*

Sample mounting for microscopy

1. Remove 5× SSCT and add ≈50 μL of SlowFade Gold antifade mountant to each tube.
2. Pipette 50 μL of larvae on a 25 mm × 75 mm glass slide.
3. Place a 22 mm × 60 mm No. 1 coverslip on top of the solution to close the chamber.
4. A Zeiss 710 NLO inverted confocal microscope equipped with an LD C-Apochromat 40×/1.1 W Korr M27 objective was used in our lab to acquire worm 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.

Proteinase K solution	For 1 mL of solution
100 μg/mL proteinase K	5 μL of 20 mg/mL proteinase K Fill up to 1 mL with PBST
Glycine solution	For 50 mL of solution
2 mg/mL glycine PBST	100 mg of glycine Fill up to 50 mL with PBST
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

Paraformaldehyde, 16% w/v aq. soln., methanol free (Alfa Aesar Cat. # 30525-89-4)
10× PBS (Life Technologies Cat.# AM9625)
Proteinase K, molecular biology grade (NEB Cat. # P8107S)
Glycine (Sigma Cat. # G7126)
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)
25 mm × 75 mm glass slide (VWR Cat. # 48300-025)
22 mm × 60 mm No. 1 coverslip (VWR Cat. # 48393-070)
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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