In situ HCR v2.0 protocol for whole-mount sea urchin embryos (*Strongylocentrotus purpuratus*)

This protocol has been optimized for embryos at 45 hpf.
Technical support: support@moleculartechnologies.org

Sample preparation

1. Incubate fertilized embryos with fresh filtered seawater in a 24-well plate at 16 °C until 45 hpf (hours post-feritilization).
2. Transfer embryos from the 24-well plate into a 50 mL falcon conical tube.
3. Centrifuge at 100 × g for 2 min to bring embryos to the bottom of the tube.
4. Remove seawater to reach 20 mL volume and add 2.4 mL of 4.45 M NaCl to deciliate embryos.
5. Incubate on ice until embryos settle to the bottom of the tube.
6. Remove seawater (with NaCl) and add 20 mL of filtered sea water.
7. Incubate on ice until embryos settle to the bottom of the tube.
8. Repeat steps 6 and 7.
9. Aspirate as much sea water as possible without removing embryos.
10. Fix embryos in 40 mL of 4% paraformaldehyde (PFA) overnight at 4 °C on a nutator.
    CAUTION: *Use PFA with extreme care as it is a hazardous material.*
    NOTE: *Use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.*
11. Remove fixative to reach a volume of ≈ 6 mL.
12. Transfer embryos to six 1.5 mL eppendorf tubes (1 mL each).
13. Centrifuge at 100 × g for 2 min.
14. Wash embryos five times with 1 mL of PBST each. Centrifuge at 100 × g for 2 min to bring embryos to the bottom of a tube in between washes.
15. Wash embryos three times with 1 mL of 70% ethanol each. Centrifuge at 100 × g for 2 min to bring embryos to the bottom of a tube in between ethanol washes.
16. Store embryos in 1 mL of 70 % ethanol at -20 °C before use.

Buffer recipes for sample preparation

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

PBST	For 50 mL of solution
1× PBS	5 mL of 10× PBS
0.1% Tween 20	500 μL of 10% Tween 20
	Fill up to 50 mL with ultrapure H_2O

NOTE: *16% PFA solution is filter sterilized using a 25 mm syringe filter with 0.2 μm membrane before use.*
NOTE: *Avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the embryos.*

Multiplexed in situ HCR v2.0 protocol

Detection stage

1. Transfer ≈200 embryos to each well of a 96 well plate.
   NOTE: *Adding and removing solution should be performed under a dissecting scope to avoid losing a large number of embryos.*
2. Rehydrate embryos 3 × 5 min with 250 μL of 5× SSCT.
3. Aspirate with care to reach ≈10 μL of volume.
4. Add 50 μL of probe hybridization buffer and pre-hybridize at 45 °C for 30 min.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
5. Prepare probe solution by adding 0.2 pmol of each probe (0.2 μL of 1 μM stock per probe) to 50 μL of probe hybridization buffer at 45 °C.
6. Add the probe solution to reach a final hybridization volume of 100 μL.
7. Gently stir the solution with a 10 μL pipette tip.
8. Cover plate with Bio-Rad Microseal 'A' film and incubate embryos overnight (12–16 h) at 45 °C.
9. Add 150 μL of probe wash buffer (pre-heated to 45 °C) to each well of embryos.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
10. Incubate at 45 °C for 5 min.
11. Remove excess probes by washing with ≈200 μL of probe wash buffer at 45 °C:
    (a) 2 × 5 min
    (b) 2 × 30 min

    NOTE: *Wash solutions should be pre-heated to 45 °C before use.*
    NOTE: *Fill wash solution to top of each well but do not overfill.*
    NOTE: *It is important to maintain plate temperature at 45 °C during probe washing steps. This can be achieved by placing the 96-well plate containing the embryos on a heat plate while removing solution from each well.*
12. Wash embryos 2 × 5 min with ≈200 μL of 5× SSCT.

Amplification stage

1. Separately prepare 6 pmol of hairpin H1 and 6 pmol of hairpin H2 by snap cooling 2 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.*
2. Prepare hairpin solution by adding all snap-cooled hairpins to 100 μL of amplification buffer at room temperature.
3. Aspirate as much 5× SSCT as possible without removing embryos.
4. Add the hairpin solution and incubate embryos overnight (12–16 h) in the dark at room temperature.
5. Add 150 μL of 5× SSCT and incubate for 5 min at room temperature.
6. Remove excess hairpins by washing with ≈200 μL of 5× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 30 min

Sample mounting for microscopy

1. Remove 5× SSCT and add ≈50 μL of SlowFade Gold antifade mountant to each well.
2. A chamber for mounting embryos was made by aligning two stacks of double-sided tape (3 pieces per stack) 1.5 cm apart on a 25 mm × 75 mm glass slide to prevent crushing the embryos.
3. Pipette 50 μL of embryos between the tape stacks on the slide.
4. Place a 22 mm × 30 mm No. 1 coverslip on top of the stacks to close the chamber.
5. 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 sea urchin images.

Buffer recipes for in situ HCR v2.0

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)
Sterile Acrodisc 25 mm syringe filters with 0.2 μm HT Tuffryn membrane (Pall Cat. # PN4192)
10$\times$ PBS (Life Technologies Cat.# AM9625)
Ethanol, 200 proof (VWR Cat. # V1001G)
Formamide (Deionized) (Ambion Cat. # AM9342)
20$\times$ sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
50% Tween 20 (Life Technologies Cat. # 00-3005)
50$\times$ Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)
Microseal 'A' Film (Bio-Rad Cat. # MSA5001)
25 mm $\times$ 75 mm glass slide (VWR Cat. # 48300-025)
22 mm $\times$ 30 mm No. 1 coverslip (VWR Cat. # 48393-026)
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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