Molecular Technologies
CHICKEN EMBRYOS

In situ HCR v2.0 protocol for whole-mount chicken embryos (*Gallus gallus domesticus*)

This protocol has been optimized for embryos at stage HH 11–12.
Technical support: support@moleculartechnologies.org

Sample preparation

1. Collect stage HH10–11 chick embryos on 3M paper circles and rinse in 1× DEPC-treated PBS.
2. Immediately transfer into a petri dish containing 4% paraformaldehyde (PFA).
   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.*
3. Transfer all embryos into a new petri dish with fresh 4% PFA after dissection.
   NOTE: *This is to avoid fixing embryos in PFA diluted by egg yolk.*
4. Fix the samples at room temperature for 1 h.
5. Dissect the embryos off the paper, preferably in PFA.
6. Remove the fixed vitelline membrane (opaque) and cut the squares around area pellucida, without leaving excess of extra-embryonic tissue.
7. Transfer embryos into a 2 mL eppendorf tube containing PBST.
8. Nutate for 5 mins with the tube positioned horizontally in a small ice bucket.
9. Dehydrate embryos into methanol (MeOH) with a series of graded 2 mL MeOH / PBST washes, each time nutating for 5 min on ice:
   (a) 25% MeOH / 75% PBST
   (b) 50% MeOH / 50% PBST
   (c) 75% MeOH / 25% PBST
   (d) 100% MeOH
   (e) 100% MeOH.
10. Store embryos at -20 °C overnight before use.
    NOTE: *Embryos can be stored for six months at -20 °C.*

Buffer recipes for sample preparation

4% Paraformaldehyde (PFA)

4% PFA
1× PBS

For 25 mL of solution
1 g of PFA powder
25 mL of 1× PBS
Heat solution at 50–60 °C to dissolve powder

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 embryos.*

Multiplexed in situ HCR v2.0 protocol

Detection stage

1. Transfer the required number of embryos for an experiment to a 2 mL eppendorf tube.
   NOTE: *Do not place more than 4 embryos in each 2 mL eppendorf tube.*
2. Rehydrate with a series of graded 2 mL MeOH/PBST washes for 5 min each on ice:
   (a) 75% MeOH / 25% PBST
   (b) 50% MeOH / 50% PBST
   (c) 25% MeOH / 75% PBST
   (d) 100% PBST
   (e) 100% PBST.
3. Treat embryos with 2 mL of 20 μg/mL proteinase K solution for 30 min at room temperature.
   NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage.*
4. Postfix with 2 mL of 4% PFA for 20 min at room temperature.
5. Wash embryos 2 $\times$ 5 min with 2 mL of PBST on ice.
6. Wash embryos with 2 mL of 50% PBST / 50% 5$\times$ SSCT for 5 min on ice.
7. Wash embryos with 2 mL of 5$\times$ SSCT for 5 min on ice.
8. For each sample, transfer 1-4 embryos to a 2 mL eppendorf tube.
9. Incubate embryos in 1 mL of probe hybridization buffer on ice for 5 min.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
10. Remove the buffer and pre-hybridize with 1 mL of probe hybridization buffer for 30 min at 45 °C.
11. Prepare probe solution by adding 2 pmol of each probe (2 μL of 1 μM stock per probe) to 1 mL of probe hybridization buffer at 45 °C.
12. Remove the pre-hybridization solution and add the probe solution.
13. Incubate embryos overnight (12–16 h) at 45 °C.
14. Remove excess probes by washing with 1 mL of probe wash buffer at 45 °C:
    (a) 2 $\times$ 5 min
    (b) 2 $\times$ 30 min

    CAUTION: *probe wash buffer contains formamide, a hazardous material.*
    NOTE: *Wash solutions should be pre-heated to 45 °C before use.*

Amplification stage

1. Pre-amplify embryos with 1 mL of amplification buffer for 5 min at room temperature.
2. Separately prepare 60 pmol of hairpin H1 and 60 pmol of hairpin H2 by snap cooling 20 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 1 mL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate the embryos 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

Sample mounting for microscopy

1. A chamber for mounting each embryo was made by aligning two stacks of double-sided tape (2 pieces per stack) 1 cm apart on a 25 mm × 75 mm glass slide.
2. Place an embryo between the tape stacks on the slide and remove as much solution as possible.
3. Align the embryo for dorsal imaging and carefully touch the slide with a kimwipe to further dry the area around the embryo.
4. Add two drops of SlowFade Gold antifade mountant on top of the embryo.
5. Place a 22 mm × 30 mm No. 1 coverslip on top of the stacks to close the chamber.
6. A Zeiss 710 NLO inverted confocal microscope equipped with an LD LCI Plan-Apochromat 25×/0.8 Imm Corr DIC objective was used in our lab to acquire chicken 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
20 μg/mL proteinase K	1 μL of 20 mg/mL proteinase K Fill up to 1 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 (PFA) (Sigma Cat. # P6148)
Methanol (Mallinckrodt Chemicals Cat. # 3016-16)
Proteinase K, molecular biology grade (NEB Cat. # P8107S)
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 × 30 mm No. 1 coverslip (VWR Cat. # 48393-026)
SlowFade Gold antifade mountant (Life Technologies Cat. # S36937)

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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