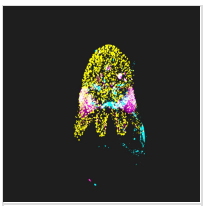

May 10, 2022

Hybridization Chain Reaction combined with Immunohistochemistry for Whole-Mount Embryos

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dx.doi.org/10.17504/protocols.io.bxz6pp9e

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Here, we are providing an optimized, step-by-step clearing protocol that retains the signal generated by HCR v3.0 in whole mount *Octopus vulgaris* embryos, even in combination with immunohistochemistry. Please cite (Elagoz et al., 2022) if you use this protocol.

For designing HCR v3.0 probe pairs, we have developed an automated tool called Easy_HCR which can be found at https://github.com/SeuntjensLab/Easy_HCR.

DOI

dx.doi.org/10.17504/protocols.io.bxz6pp9e

Ali M Elagoz, Ruth Styfhals, Sofia Maccuro, Luca Masin, Lieve Moons, Eve Seuntjens 2022. Hybridization Chain Reaction combined with Immunohistochemistry for Whole-Mount Embryos. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bxz6pp9e

HCR, hybridization chain reaction, wholemount, immunohistochemistry, HCR v3.0, cephalopod, octopus

protocol ,

Sep 07, 2021

May 10, 2022

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The bench, lids etc. were cleaned thoroughly with EtOH and RNase away. Work as RNase-free as possible during the duration of the experiment.

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.

Buffer recipes for sample preparation

4% paraformaldehyde (PFA)	*For 25 mL of solution*
4% PFA	1 g of PFA powder
1x PBS	25 mL of 1x PBS
	Heat solution at 50–60 °C to dissolve the powder

PBST	*For 50 mL of solution*
1x PBS	5 mL of 10x PBS
0.1% Tween 20	500 uL of 10% Tween 20
	Fill up to 50 mL with ultrapure H2O

Proteinase K solution	*For 2 mL of solution*
10 ug/mL proteinase K	1,08 uL of 20 mg/mL proteinase K
	Fill up to 2 mL with PBST

Probe hybridization buffer (aliquot and store at -20°C) => Prepare in 50 ml falcon

Final concentration	*For 40 ml*
30% formamide	12 ml formamide
5x SSC	10 ml of 20 x SSC
9 mM citric acid pH 6	360 ul 1 M citric acid pH 6
0,1% Tween 20	400 ul of 10% Tween 20
50 ug/ml heparin	200 ul of 10 mg/ml heparin
1 x Denhardts solution	800 ul of 50x Denhardt's solution
10% dextran solution	8 ml of 50% dextran sulfate
	Fill up to 40 ml with ultrapure H20

Probe wash buffer (100 ml per experiment/ freeze in 50 ml aliquots) Store at -20°C => Prepare in 0.5l bottle

Final concentration	*For 400 ml*
30% formamide	120 ml formamide

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5x SSC	100 ml of 20 x SSC
9 mM citric acid pH 6	3,6 ml of 1 M citric acid pH 6
0.1% Tween 20	4 ml of 10% Tween 20
50 ug/ml heparin	2 ml of 10 mg/ml heparin (4°C)
	Fill up to 400 ml with ultrapure H20

(170,4 mls)

Amplification buffer (4°C) - prepare in 50 ml falcon - make 10 ml aliquots

Final concentration	*For 40 ml*
5 x SSC	10 ml of 20 x SSC
0,1% Tween 20	400 ul of 10% Tween 20
10% Dextran sulfate	8 ml of 50% Dextran sulfate
	Fill up to 40 ml with ultrapure H20

5x SSCT (RT) - prepare 1 L

Final concentration	*For 1 L*
5x SSC	250 ml of 20 x SSC
0,1% Tween 20	10 ml of 10% Tween 20
	Fill up to 1L with ultrapure H20 (740 ml)

50% Dextran sulfate => best to dissolve overnight (add water gradually) - prepare in falcon

20 g of dextran sulfate powder
Fill up to 40 ml with ultrapure H20.
=> make aliquots of 8 ml and store at -20°C.

10 mg/ml Heparin (50 ml) => aliquot per 10 ml store at 4°C

500 mg heparin
Fill up to 50 ml with ultrapure H20.
Filter 0.2 uM and store at 4°C.
! store powder at room T, store solution at 4°C.

1M citric acid pH 6 (50 ml)

10.5 g in 30 ml
adjust pH to 6.0 with NaOH : make 20 ml of conc NaOH in Rnase-Dnase free water, let dissolve and add slowly.
Fill up to 50 ml

Hairpins

Ordered from MI:
B1 546 600 pmol
B2 647 600 pmol
B3 488 600 pmol
300 pmol (100 ul of 3 pmol/ul = 3 uM)
Aliquot per 15 ul and store at -20°C.

Probes

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Ordered DNA oPools from IDT (without 5' mod): 50 pmol
Dissolved in 100 ul ultrapure water (or Tris pH 7.5). Final concentration is 0.5 pmol/ul. Aliquot per 10 ul and store at -20°C. We use 0.3 pmol = 0.6 ul per slide.

Reagents and supplies
Heparin (Sigma cat H3393): powder stored at room T, aliquots are stored at 4°C.
20x Sodium Chloride Sodium Citrate (SSC: Invitrogen) 1 L, stored at room T.
10 % Tween 20 (Bio-Rad). Protect from light. Stored at Room T.
50x Denhardt's solution (Aliquot and store at -20°C)
Dextran sulfate (Sigma cat D8906): powder stored at 4°C, aliquots are stored at -20°C.

Formamide is toxic substance so make sure to take the appropriate measures.

Embryo Fixation and Preparation

1

DAY 0

Fix tissue overnight in 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS-DEPC) at 4°C.

2 **DAY 1**

Wash with PBS-DEPC.

3 Dechorionate the octopus embryos in PBST.

Dehydration

4 Dehydrate embryos into methanol (MeOH) with a series of graded MeOH/PBST washes for 10 min on ice:
(a) 25% MeOH / 75% PBST
(b) 50% MeOH / 50% PBST
(c) 75% MeOH / 25% PBST
(d) 100% MeOH
(e) 100% MeOH.

5

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Incubate embryos at -20 °C overnight (> 16 h) or until use.

NOTE: Embryos can be stored for six months at -20 °C.

Rehydration and Permeabilization

6 **DAY 2**

Transfer the required number of embryos for an experiment to a 0.5 ml Eppendorf tube. “Thaw” on ice and gradually move them to room temperature (approx. in half an hour).

7 Rehydrate with a series of graded MeOH/PBST washes for 10 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

8 Immerse embryos in 10 ug/mL proteinase K solution for 15 min at room temperature (1,08 µl of proteinase K in 2 ml of PBS-DEPC (Proteinase K rec PCR grade, art. 3115887001, Roche)).

NOTE: Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage.

9 Wash embryos 2 x 5 min with PBST.

10 Postfix with 4% PFA for 20 min at room temperature.

11 Wash embryos 3 x 5 min with PBST.

Hybridization

12 Pre-warm oven and hybridization buffer to 37°C.

13 Remove the buffer and pre-hybridize with 100 ul of probe hybridization buffer for 30 min at 37°C.

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14 Thaw probes on ice, spin down before using.

15 Prepare probe solution by adding 0.4 pmol of each probe mixture to 100 ul of probe hybridization buffer at 37°C.

16 

Remove the pre-hybridization solution and add 100 ul of the probe solution and incubate embryos overnight (12–16 h) at 37°C (no shaker is used during this step).

Wash Steps

17 **DAY 3**

Remove excess probes by washing embryos 4 x 15 min with 100 ul of probe wash buffer at 37°C

NOTE: Pre-warm the wash solution in the oven to 37°C before use.

18 Wash samples 2 x 5 min with 5 x SSCT at room temperature. *(Thaw hairpins on ice in the dark and move the amplification buffer to room temp.)*

Amplification

19 Remove the solution and Pre-amplify embryos with 100 ul of amplification buffer for at least 30 min at room temperature.

NOTE: Equilibrate amplification buffer to room temperature before use.

20 Separately prepare 6 pmol of H1 and H2 in separate PCR tubes. Specifically, pipet 2 µl of each hairpin [3 µM stock (3 pmol for H1 and 3 pmol for H2) in hairpin storage buffer] in a separate PCR tube.

21 In a PCR thermocycler: heat hairpins at 95 °C for 90s. Immediately put hairpins on ice for 5 minutes, and then leave hairpins at room temperature for 30 minutes IN THE DARK.

22 Prepare the hairpin solution by adding all snap-cooled hairpins to 100 ul of amplification buffer at room T. (Add equal amounts of amplification buffer to both hairpin 1 and 2 and then add hairpin 1 to hairpin 2)

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23

Remove the pre-amplification solution and add the hairpin solution. Incubate the embryos/larvae overnight (12–16 h) in the dark at room temperature (no shaker is used during this step).

Wash Steps and Imaging

24 **DAY 4**

Remove excess hairpins by washing with 100 uL of 5x SSCT at room temperature in the dark:
(a) 2 x 5 min
(b) 2 x 30 min
(c) 1 x 5 min
(d) 1 x 2hrs with DAPI (1:2000)
(e) 1 x 5 mins

25 Samples can be transferred to the Fructose-Glycerol clearing solution described in Dekkers et al., 2019 for at least 2 days.

Fructose-Glycerol clearing solution was prepared by dissolving 29,72 grams of fructose in 33 ml of glycerol and 7 ml of distilled water on a magnetic stirrer. The solution can be stored at 4 °C for a month.

Immunohistochemistry (IHC)

26 Incubate embryos with the primary antibody (1:1000 rabbit anti-phosphohistone H3 (Ser10) (Millipore 06-570) for the following 2 days after the HCR protocol.

IMPORTANT NOTE: When HCR is combined with IHC, the incubation in DAPI is skipped and the embryos are directly processed for IHC after the last excess hairpin removal wash.

IMPORTANT NOTE: The whole protocol of IHC is carried out at 4°C.

27 **DAY 6**

Wash embryos with 5xSSCT three times for 2 hours.

28 Add the secondary antibody donkey anti-rabbit Alexa 488 (Life Technologies) at a final concentration of 1:300 diluted in antibody diluent (Roche) and incubate O/N.

29 **DAY 7**

Wash with 5xSSCT twice for 2 hours.

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30 Incubate embryos in 1:2000 DAPI in 5xSSCT for 2 hours followed by 5xSSCT wash for 5 minutes.

31 Incubate embryos in Fructose-Glycerol clearing as described above before imaging.

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