HCR v3.0 protocol for whole-mount sea urchin embryos (*Strongylocentrotus purpuratus*)

This protocol has not been optimized for all stages and should only be used as a template.
Technical support: support@molecularinstruments.com
Safety data sheets (SDS): www.molecularinstruments.com/safety
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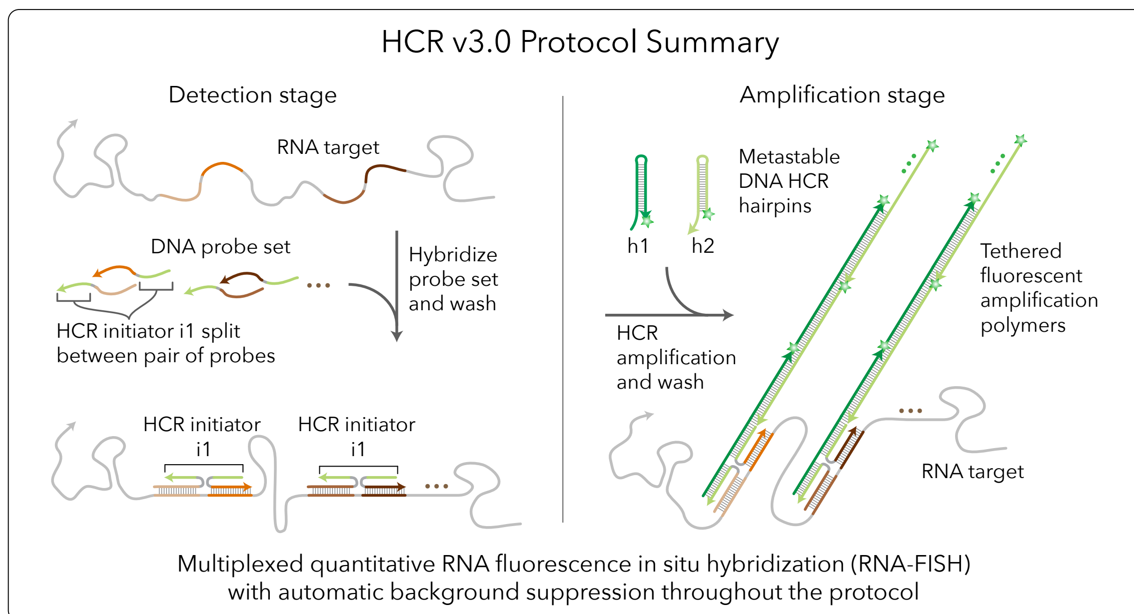
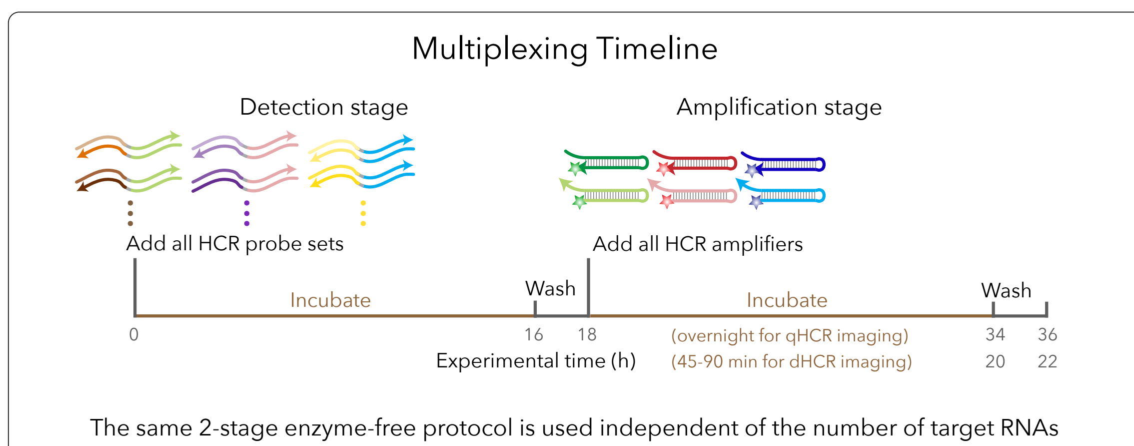
Storage conditions

Store HCR probe sets, HCR amplifiers, HCR probe hybridization buffer, and HCR probe wash buffer at -20 °C. Store HCR amplification buffer at 4 °C. On the bench top, keep stock solutions on ice. Make sure all solutions are well mixed before use.





Preparation of fixed whole-mount sea urchin embryos

1. Incubate fertilized embryos with fresh filtered seawater in a 24-well plate at 16 °C until 45 hpf.
2. Transfer embryos from the 24-well plate into a 50 mL 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 tubes (1 mL each).
13. Centrifuge at 100 × g for 2 min.
14. Wash embryos 5 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 3 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 HCR v3.0 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 for 30 min at 37 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*

5. Prepare probe solution by adding 0.4 pmol of each probe mixture (e.g. 0.4 μL of 1 μM stock) to 50 μL of probe hybridization buffer at 37 °C.
   NOTE: *For dHCR imaging, use higher concentration of probe to improve probe hybridization efficiency.*

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 37 °C.

9. Add 150 μL of probe wash buffer to each well of embryos.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *pre-heat probe wash buffer to 37 °C before use.*

10. Incubate at 37 °C for 5 min.

11. Remove excess probes by washing with ≈200 μL of probe wash buffer at 37 °C:

    (a) 2 × 5 min
    (b) 2 × 30 min

    NOTE: *Fill probe wash buffer to top of each well but do not overfill.*
    NOTE: *It is important to maintain plate temperature at 37 °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 (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. Equilibrate amplification buffer to room temperature.

3. Prepare hairpin mixture by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 100 μL of amplification buffer at room temperature.

4. Aspirate as much 5× SSCT as possible without removing embryos.

5. Add the hairpin mixture and incubate embryos overnight (12–16 h) in the dark at room temperature.
   NOTE: *For dHCR imaging, amplify for a shorter period of time to ensure single-molecule dots are diffraction-limited.*

6. Add 150 μL of 5× SSCT and incubate for 5 min at room temperature.

7. Remove excess hairpins by washing with ≈200 μL of 5× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 30 min

8. Samples can be stored at 4 °C protected from light before microscopy.

Citation Notes

For citation, please select from the list below as appropriate for your application:

- **HCR v3.0**
  Automatic background suppression for dramatically enhanced performance (signal-to-background, qHCR precision, dHCR fidelity) and ease-of-use (no probe set optimization for new targets and organisms) (Choi *et al.*, 2018). Quantitative analysis modes:

  qHCR imaging: analog mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples.

  qHCR flow cytometry: analog mRNA relative quantitation for high-throughput expression profiling of mammalian and bacterial cells.

  dHCR imaging: digital mRNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples.

  Protocols for v3.0 in diverse organisms are adapted from the Zoo paper.

- **qHCR imaging**
  mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos; read-out/read-in analysis framwork (Trivedi *et al.*, 2018).

- **Zoo paper**
  Protocols for multiplexed mRNA imaging in diverse sample types (Choi *et al.*, 2016):

  bacteria in suspension
  generic sample in suspension
  whole-mount chicken embryos
  whole-mount mouse embryos
  whole-mount worm larvae
  FFPE human tissue sections
  generic sample on slide
  whole-mount fruit fly embryos
  whole-mount sea urchin embryos
  whole-mount zebrafish embryos and larvae

- **dHCR imaging**
  Single-molecule mRNA imaging in thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah *et al.*, 2016).

- **qHCR northern blot**
  Simultaneous quantification of RNA target size and abundance for up to 5 target RNAs (Schwarzkopf & Pierce, 2016).

- **HCR v2.0**
  2nd generation in situ HCR technology (v2.0) using DNA HCR probes and DNA HCR amplifiers: 10× increase in signal, 10× reduction in cost, dramatic increase in reagent durability (Choi *et al.*, 2014).

- **HCR v1.0**
  1st generation in situ HCR technology (v1.0) using RNA HCR 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).

- **HCR mechanism**
  Isothermal enzyme-free molecular signal amplification (Dirks & Pierce, 2004).

References

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