

## 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.

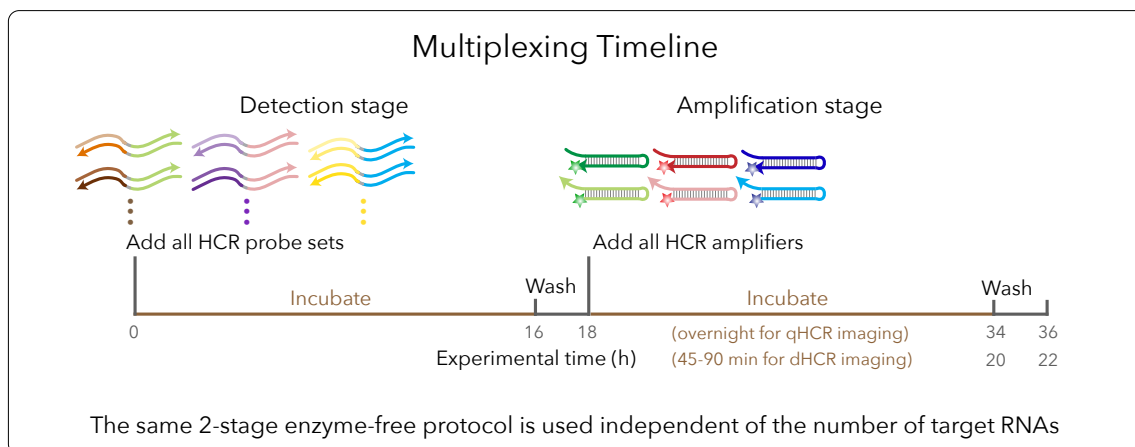
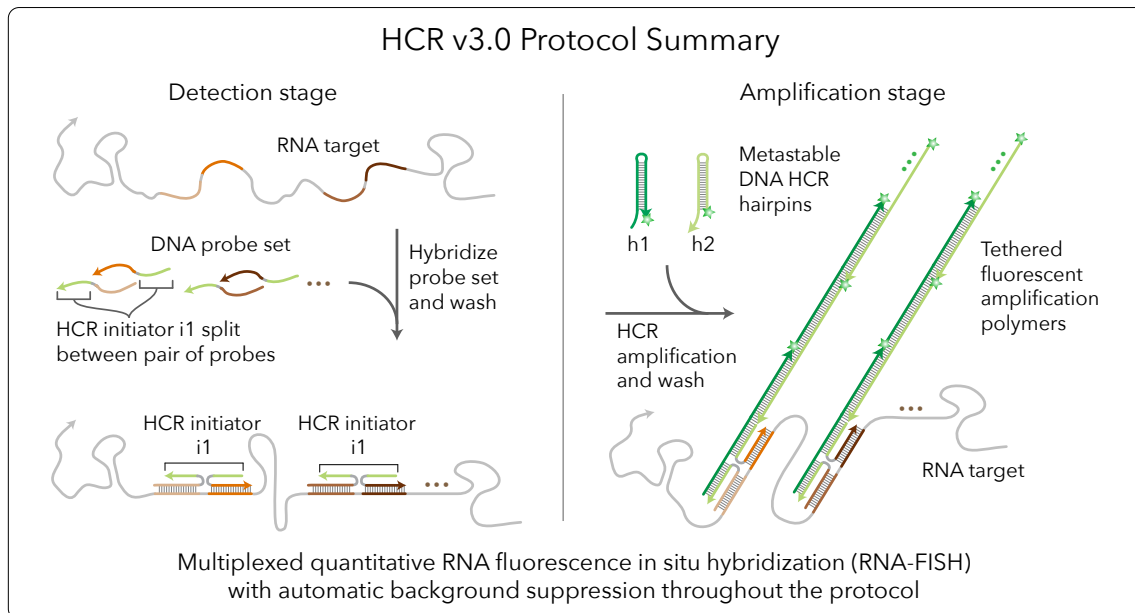
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### Storage conditions

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  $100 \times g$  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  $\approx 6$  mL.
12. Transfer embryos to six 1.5 mL tubes (1 mL each).
13. Centrifuge at  $100 \times g$  for 2 min.
14. Wash embryos 5 times with 1 mL of PBST each. Centrifuge at  $100 \times g$  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  $100 \times g$  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)

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<sub>2</sub>O

### 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<sub>2</sub>O

*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  $\approx 200$  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  $3 \times 5$  min with  $250 \mu\text{L}$  of  $5\times$  SSCT.
3. Aspirate with care to reach  $\approx 10 \mu\text{L}$  of volume.
4. Add  $50 \mu\text{L}$  of probe hybridization buffer and pre-hybridize for 30 min at  $37^\circ\text{C}$ .  
*CAUTION: probe hybridization buffer contains formamide, a hazardous material.*
5. Prepare probe solution by adding  $0.4 \text{ pmol}$  of each probe set (e.g.  $0.4 \mu\text{L}$  of  $1 \mu\text{M}$  stock) to  $50 \mu\text{L}$  of probe hybridization buffer at  $37^\circ\text{C}$ .  
*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  $100 \mu\text{L}$ .
7. Gently stir the solution with a  $10 \mu\text{L}$  pipette tip.
8. Cover plate with Bio-Rad Microseal 'A' film and incubate embryos overnight (12–16 h) at  $37^\circ\text{C}$ .
9. Add  $150 \mu\text{L}$  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  $37^\circ\text{C}$  before use.*
10. Incubate at  $37^\circ\text{C}$  for 5 min.
11. Remove excess probes by washing with  $\approx 200 \mu\text{L}$  of probe wash buffer at  $37^\circ\text{C}$ :
  - (a)  $2 \times 5$  min
  - (b)  $2 \times 30$  min

*NOTE: Fill probe wash buffer to top of each well but do not overflow.*

*NOTE: It is important to maintain plate temperature at  $37^\circ\text{C}$  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  $2 \times 5$  min with  $\approx 200 \mu\text{L}$  of  $5\times$  SSCT.

## Amplification stage

1. Separately prepare 6 pmol of hairpin h1 and 6 pmol of hairpin h2 by snap cooling 2  $\mu\text{L}$  of 3  $\mu\text{M}$  stock (heat at 95  $^{\circ}\text{C}$  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 solution by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 100  $\mu\text{L}$  of amplification buffer at room temperature.
4. Aspirate as much 5 $\times$  SSCT as possible without removing embryos.
5. Add the hairpin solution 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  $\mu\text{L}$  of 5 $\times$  SSCT and incubate for 5 min at room temperature.
7. Remove excess hairpins by washing with  $\approx 200$   $\mu\text{L}$  of 5 $\times$  SSCT at room temperature:
  - (a) 2  $\times$  5 min
  - (b) 2  $\times$  30 min
8. Samples can be stored at 4  $^{\circ}\text{C}$  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 framework (Trivedi *et al.*, 2018).
- **Zoo paper**  
Protocols for multiplexed mRNA imaging in diverse sample types (Choi *et al.*, 2016):
 

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