***HCR In situ* hybridization protocol, Kit Version 3**

Adapted From: Choi, H.M.T., M. Schwarzkopf, M.E. Fornace, A. Acharya, G. Artavanis, J. Stegmaier, A. Cunha, and N.A. Pierce. 2018. Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. BioRxiv, doi: https://doi.org/10.1101/285213. Available at: https://www.biorxiv.org/content/early/2018/03/19/285213

Fixation and Dehydration of Specimens

1. Dechorinate embryos with fine tweezers or a pair of needles.
2. Transfer embryos to an eppendorf tube and fix them with 1 mL of 4 %paraformaldehyde and keep them at 4 °C for at least 24 hours.
3. Wash embryos 3 times for 5 minutes with 1mL of 1X PBS **(DEPC treated/RNase free)** to stop fixation. Fixed embryos can be stored at 4°C at this stage.
4. Dehydrate embryos in a series of methanol (MeOH) washes with 1 mL each (100% MeOH 4 times for 10 min and 100% MeOH once for 50 min
5. Store embryos at -20 °C.

Rehydrate specimens with a series of 1 mL MeOH/PBST washes

1. Set-up 6 washes
    a. 3:1 MeOH: 1X PBST 5 min
    b. 1:1 MeOH: 1X PBST 5 min
    c. 1:3 MeOH: 1X PBST 5 min
    d. 100% 1X PBST 5 min three times

Proteinase K

1. Treat embryos with 5 *µL* of PK in 100 *µL o*f 1X PBST for 10 min at room temperature.
2. Stop PK by replacing the solution with 250 *µL* of 4% PFA in 100 *µL* of PBST for 20 min at room temperature.
3. Wash embryos twice in 250 *µL* of 1X PBST for 5 min at room temperature.

(While the washes are going heat up the water bath to 37 °C and warm up 250 µL of probe hybridization buffer)

Probe Hybridization

1. Pre-hybridize the embryos in 250 µL of 30% of probe hybridization buffer for 3 hours at 37°C.
2. Prepare probe solution by adding 2 pmol of each probe solution (odd and even: 0.5 µL of 2 µM stock per probe mixture to 250 µL of 30% probe hybridization buffer at 37°C).
3. After 3 hours the embryos are finished pre-hybridizing.
4. Remove the pre-hybridization solution and add the probe solution.

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5. Incubate embryos in probe solution for at least (**12-16) hours at 37°C in the dark**.
6. Remove the excess probes by washing with 250 µL of the following solutions at 37°C **(Pre-heat wash solutions to 37 °C before use)**
    a. Wash embryos 4 times for 15 minutes with 250 µL of 30% probe wash buffer at 37 °C
7. Wash embryos 3 times for 5 minutes with 5X SSCT at room temperature.

Amplification (When working with hairpin stocks remember to change tips between stock tubes! Do not contaminate the amplifiers! Keep embryos and amplifiers in the dark.)

1. Pre-amplify the embryos in 250 µL of amplification buffer for **30 min** at room temperature.
2. Prepare 30 pmol of each fluorescently labeled hairpin by snap cooling 5 µL of 3 µM stock in hairpin storage buffer (heat at 95°C for 90 seconds and cool to room temperature in a dark for 30 min).
3. Prepare hairpin solution by adding all snap-cooled hairpins to 250 µL of amplification buffer at room temperature.
4. Replace pre-amplification buffer with the hairpin and amplification buffer solution.
5. Incubate embryos in the amplifiers for at least (**12-16) hours at room temperature in the dark**.
6. Remove excess probes by washing at room temperature with 250 µL of the following solutions:
    a. Wash twice in 5X SSCT for 5 min.
    b. Wash twice in 5X SSCT for 30 min.
    c. Wash one time in 5X SSCT for 5 min.

Mounting Embryos

1. Mount embryos in depression slides (or use slides with a glass ring, nylon washer, or well made out of scotch tape).
2. Apply a drop of Aquamount or similar mounting solution.
3. Using a transfer pipet, pipet an embryo into the mounting solution.
4. Orient the embryo in the mounting solution using a nylon loop (e.g. fishing line) or similar.
5. Attach a glass coverslip.

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Reagents

-4% Paraformaldehyde (store at 4°C)
-Methanol
-Proteinase K
-1X PBS
-1X PBST
-5X SSCT
-5X SSC
-Probe Hybridization Buffer (Molecular Instruments)
-Probe Wash Buffer (Molecular Instruments)
-Amplification Buffer (Molecular Instruments)
-DNA Probes (Molecular Instruments)
-Amplifiers (Molecular Instruments)
-Aquamount (Fisher - Catnum: 14-390-5)

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