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

**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 of 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.
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)