HCR™ Pro RNA-ISH User Guide

This User Guide enables HCR™ Pro RNA-ISH, which integrates enzymatic signal amplification for extreme-sensitivity RNA imaging with chromogenic or fluorescent staining (RNA-CISH/RNA-FISH). Technical support: support@molecularinstruments.com

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The HCR™ Pro RNA-ISH kit is used with an HRP chromogen for RNA-CISH (e.g., Matisse® Green or Matisse® Brown) or an HRP fluorogenic substrate for RNA-FISH (e.g., any of a variety of recommended third-party fluorescent tyramides).

Safety Data Sheets (SDS)

- www.molecularinstruments.com/safety

Patents

- www.molecularinstruments.com/patents

HCR™ Pro RNA-ISH Storage Conditions and Shelf Life

Upon receiving your HCR™ Pro RNA-ISH kit, please check storage conditions for each reagent. HCR™ reagents should be thawed and mixed before use. We recommend aliquoting the HCR™ HiFi Probes to minimize freeze-thaw cycles and maximize shelf life. On the bench top, keep stock solutions on ice. Make sure all solutions are well mixed before use.

HCR™ Reagent	Storage Temperature (°C)	Storage Condition	Shelf Life (yr)	Comments
HCR™ HiFi Probe	−20	—	2	
HCR™ HiFi Probe Hybridization Buffer	−20	—	1	
HCR™ HiFi Probe Wash Buffer	4	—	1	Provided at 4×; dilute to 1× with UltraPure H_2O before use
Pretreat	4	Shield from light	1	
HCR™ Pro Detect A	4	Shield from light	0.5	
HCR™ Pro Detect B	4	Shield from light	0.5	
HCR™ Pro Detect C	4	Shield from light	0.5	
HCR™ Pro Detect D	4	Shield from light	0.5	
HCR™ Pro Detect E	4	Shield from light	0.5	
HCR™ Pro Detect F HRP	4	Shield from light	0.5	
Post-Process	4	Shield from light	1	Use only if HRP-based IHC/IF follows
HCR™ Pro Amplifier Wash Buffer	4	—	1	Provided at 10×; dilute to 1× with UltraPure H_2O before use
Matisse® Brown	4	Shield from light	1	HRP chromogen
Matisse® Green	4	Shield from light	1	HRP chromogen
Matisse® Brown Buffer	4	Shield from light	1	
Matisse® Green Buffer	4	Shield from light	1	

User-Supplied Materials

Reagent†	Supplier	Comments
UltraPure H_2O	Any	Type I water
ProLong™ Gold Antifade Mountant with DAPI	ThermoFisher	Use for HCR™ Pro RNA-FISH. This is our recommended antifade mountant but any antifade mountant, with or without DAPI, is acceptable (e.g., Fluoromount-G).
Hematoxylin	Any	Use for HCR™ Pro RNA-CISH in tissue sections
Bluing Reagent	Any	Use for HCR™ Pro RNA-CISH in tissue sections
Xylene or xylene alternative	Any	Use for HCR™ Pro RNA-CISH in tissue sections
200-Proof Ethanol (EtOH)	Any	Use for HCR™ Pro RNA-CISH in tissue sections
Cytoseal	Any	Use to mount tissue sections for HCR™ Pro RNA-CISH
Fluorescent tyramide	See recommended	Use to perform fluorescent staining for HCR™ Pro RNA-FISH

†All user-supplied reagents should be DNase- and RNase-free.

HCR™ Pro RNA-ISH Workflow

Below is a general overview of the steps involved and their purposes in the HCR™ Pro RNA-CISH/FISH assay.

Sample Preparation

1. **Fixation:** Preserve morphology and cellular components of a sample (e.g., DNA, RNA, proteins). Fixation is sensitive to several factors including type of fixative, concentration, and duration, and must be optimized for each sample type.

2. **Permeabilization:** Ensure the sample is sufficiently permeable for HCR™ reagents to diffuse into and out of the sample.

Pretreatment

3. **Pretreatment:** Minimize non-specific amplification by blocking off-target binding sites and suppressing endogenous background.

Probe Hybridization

4. **Pre-Hybridization:** Pre-condition the sample in HCR™ HiFi Probe Hybridization Buffer to block non-specific binding sites and minimize potential background signal. The buffer used in this step is identical to the probe hybridization step, but it does not contain any HCR™ HiFi Probe. Minimizing background is critical for maximizing signal-to-background.

5. **Probe Hybridization:** Introduce HCR™ HiFi Probes complementary to the RNA targets of interest to localize initiation sites at cognate targets.

6. **Probe Washing:** Remove any unbound or non-specifically bound probes through a series of washing steps to ensure they do not generate amplified background in the next stage.

Amplification

7. **Amplification:** Introduce HCR™ Pro Detect reagents that diffuse to the cognate target where the initiation sites trigger growth of tethered amplification polymers.

8. **Amplifier Washing:** Remove any unbound HCR™ Pro Detect reagents from the sample through a series of washing steps to minimize background.

9. **Catalytic Reporter Deposition:** Introduce an HRP (horseradish peroxidase) chromogen to perform RNA-CISH (e.g., Matisse® Green or Matisse® Brown) or an HRP fluorogenic substrate to perform RNA-FISH (e.g., any of a variety of recommended third-party fluorescent tyramides), leading to enzyme-mediated catalytic reporter deposition at the site of the target.

Counterstain and Imaging

10. **Counterstain (optional):** Apply a nuclear counterstain (e.g., hematoxylin for RNA-CISH or DAPI for RNA-FISH) to provide morphological context and facilitate image analysis.

11. **Imaging:** Use a mounting solution compatible with chromogenic or fluorescent staining to preserve the integrity of the HCR™ Pro RNA-CISH/FISH signal. Mounting media also serves to secure the sample during imaging to prevent it from drying and helps match the refractive index of an objective lens. Choose an imaging modality suited to your detection method: brightfield microscopy for chromogenic staining with RNA-CISH and fluorescence microscopy (e.g., epifluorescence, confocal, or light sheet) for fluorescent staining with RNA-FISH.

HCR™ Pro RNA-ISH Protocol: Sample on a Slide

This protocol is suitable for use with FFPE tissue sections, fresh/frozen tissue sections, FFPE cell pellets, adherent cells, etc on a slide. This protocol has not been validated for all sample types and should only be used as a template. While a reaction volume of 100 µL is used as the default throughout this protocol, please apply sufficient volume of each reagent to fully cover the sample to ensure uniform treatment and prevent tissue drying.

Sample Preparation

Samples should be prepared in the same manner as for traditional in situ hybridization up to the probe hybridization step. Then proceed with the protocol described below.

Pretreatment

1. Pre-warm Pretreat to room temperature.
2. Pre-warm HCR™ HiFi Probe Wash Buffer to room temperature.
3. Add 100 µL of Pretreat on top of sample and incubate at room temperature for 10 min inside a humidified chamber.
4. Immerse 3 × 5 min in HCR™ HiFi Probe Wash Buffer at room temperature. Dry slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.

Probe Hybridization

1. Pre-heat a humidified chamber and HCR™ HiFi Probe Hybridization Buffer to 37 °C.
   CAUTION: *HCR™ HiFi Probe Hybridization Buffer contains formamide, a hazardous material.*
2. Pre-hybridize by adding 200 µL of HCR™ HiFi Probe Hybridization Buffer on top of sample and incubating at 37 °C for 10 min inside the humidified chamber.
3. Prepare probe solution by adding 2 µL of each HCR™ HiFi Probe to 100 µL of HCR™ HiFi Probe Hybridization Buffer at 37 °C.
4. Remove the pre-hybridization solution and drain excess buffer on slide by blotting edges on a Kimwipe.
5. Hybridize by adding 50–100 µL of the probe solution on top of sample, placing a coverslip on sample, and incubating at 37 °C for >3 h in the humidified chamber.
   NOTE: *Use enough probe solution to fully cover sample.*
   NOTE: *Increase incubation time for thicker samples (e.g., >12 h for thick brain slices).*
6. Pre-heat HCR™ HiFi Probe Wash Buffer to 37 °C.
   NOTE: *HCR™ HiFi Probe Wash Buffer provided at 4×; dilute to 1× with UltraPure H_2O before use.*
7. Immerse slide in HCR™ HiFi Probe Wash Buffer at 37 °C to float off coverslip.
8. Remove excess probe by immersing 4 × 15 min in HCR™ HiFi Probe Wash Buffer at 37 °C.

Amplification

HCR™ Pro Detect A

1. Pre-warm HCR™ Pro Detect A to room temperature.
   NOTE: *Use enough HCR™ Pro Detect A to fully cover sample.*
2. Drain slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.
3. Add 100 µL of HCR™ Pro Detect A on top of sample.
4. Incubate at room temperature for 30 min in a humidified chamber.
5. Remove the HCR™ Pro Detect A reagent and drain excess solution on slide by blotting edges on a Kimwipe.

HCR™ Pro Detect B

1. Add 100 µL of HCR™ Pro Detect B on top of sample.
   NOTE: *Use enough HCR™ Pro Detect B to fully cover sample.*
2. Place a coverslip on the sample and incubate at room temperature for >3 h in the humidified chamber.
   NOTE: *Increase incubation time for thicker samples (e.g., >12 h for thick brain slices).*
3. Pre-warm HCR™ Pro Amplifier Wash Buffer to room temperature.
   NOTE: *HCR™ Pro Amplifier Wash Buffer provided at 10×; dilute to 1× with UltraPure H_2O before use.*
4. Immerse slide in HCR™ Pro Amplifier Wash Buffer at room temperature to float off coverslip.
5. Immerse 4 × 15 min in HCR™ Pro Amplifier Wash Buffer at room temperature. Dry slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.

HCR™ Pro Detect C

1. Add 100 µL of HCR™ Pro Detect C on top of sample.
   NOTE: *Use enough HCR™ Pro Detect C to fully cover sample.*
2. Incubate at room temperature for 15 min in a humidified chamber.
3. Remove the HCR™ Pro Detect C reagent and drain excess solution on slide by blotting edges on a Kimwipe.

HCR™ Pro Detect D

1. Add 100 µL of HCR™ Pro Detect D on top of sample.
   NOTE: *Use enough HCR™ Pro Detect D to fully cover sample.*
2. Incubate at room temperature for 30 min in a humidified chamber.
3. Immerse 3 × 5 min in HCR™ Pro Amplifier Wash Buffer at room temperature. Dry slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.
   NOTE: *HCR™ Pro Amplifier Wash Buffer provided at 10×; dilute to 1× with UltraPure H_2O before use.*

HCR™ Pro Detect E

1. Add 100 µL of HCR™ Pro Detect E on top of sample.
   NOTE: *Use enough HCR™ Pro Detect E to fully cover sample.*

2. Incubate at room temperature for 10 min in a humidified chamber.
   NOTE: *Assay sensitivity can be tuned by adjusting the incubation time with HCR™ Pro Detect E. The recommended range is 5–30 min.*
   NOTE: *This step is time sensitive. Begin washing immediately once incubation is complete.*

3. Immerse 3 × 5 min in HCR™ Pro Amplifier Wash Buffer at room temperature. Dry slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.
   NOTE: *HCR™ Pro Amplifier Wash Buffer provided at 10×; dilute to 1× with UltraPure H_2O before use.*

HCR™ Pro Detect F HRP

1. Add 100 µL of HCR™ Pro Detect F on top of sample.
   NOTE: *Use enough HCR™ Pro Detect F to fully cover sample.*

2. Incubate at room temperature for 15 min in a humidified chamber.

3. Immerse 3 × 5 min in HCR™ Pro Amplifier Wash Buffer at room temperature. Dry slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.
   NOTE: *HCR™ Pro Amplifier Wash Buffer provided at 10×; dilute to 1× with UltraPure H_2O before use.*

4. Proceed to either:

   - Chromogenic Reporter Deposition to perform chromogenic staining for RNA-CISH.
   - Fluorogenic Reporter Deposition to perform fluorescent staining for RNA-FISH.

Chromogenic Reporter Deposition

1. Pre-warm Matisse® chromogen (Brown or Green) and Matisse® Buffer (Brown or Green) to room temperature.
   NOTE: *Matisse® Green is sensitive to TBST/PBST. Use Matisse® Brown if continuing with a traditional IHC assay.*

2. Prepare Matisse® solution by adding 5 µL of Matisse® chromogen (Brown or Green) to 95 µL of Matisse® Buffer (Brown or Green).
   NOTE: *Solution must be used within 30 min of preparation.*

3. Add 100 µL of Matisse® solution on top of sample.

4. Incubate at room temperature for 15 min in a humidified chamber.

5. Immerse 3 × 5 min in UltraPure H_2O at room temperature. Dry slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.
   NOTE: *If continuing with a traditional IHC assay, add 100 µL of Post-Process solution on top of sample and incubate at 37 °C for 30 min in a humidified chamber. Following incubation, remove the Post-Process reagent and drain excess solution on slide by blotting edges on a Kimwipe. You may stop the HCR™ Pro RNA-ISH assay at this point and proceed directly to your traditional IHC workflow.*

6. (Optional Counterstain): Add 100 µL of hematoxylin on top of sample and incubate at room temperature for 30 seconds. Remove hematoxylin and drain excess solution on slide by blotting edges on a Kimwipe. Rinse slides with UltraPure H_2O. Dry slide by blotting edges on a Kimwipe.
   NOTE: *For a more intense counterstain, increase the incubation time as needed.*

7. (Optional Counterstain): Add 100 µL of bluing reagent on top of sample and incubate at room temperature for 5 min in a humidified chamber. Remove bluing reagent and drain excess solution on slide by blotting edges on a Kimwipe. Rinse slides with UltraPure H_2O. Dry slide by blotting edges on a Kimwipe.

8. Immerse 2 × 3 min in 95% ethanol at room temperature.

9. Immerse 3 min in 100% ethanol at room temperature.

10. Immerse 5 min in a xylene (or xylene alternative) solution at room temperature.

11. Mount one slide at a time with Cytoseal (or any other xylene-based mounting medium).

12. Air dry for 5 min before storing sample at 4 °C prior to imaging (brightfield microscopy).

Fluorescent Reporter Deposition

1. Prepare fluorescent tyramide solution following the manufacturer's instructions.
   NOTE: *See list of recommended third-party fluorescent tyramides.*

2. Add 100 µL of fluorescent tyramide solution.

3. Incubate according to the manufacturer's instructions in a humidified chamber.

4. Immerse 3 × 5 min in HCR™ Pro Amplifier Wash Buffer at room temperature. Dry slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.
   NOTE: *HCR™ Pro Amplifier Wash Buffer provided at 10×; dilute to 1× with UltraPure H_2O before use.*
   NOTE: *If continuing with a traditional IF assay, add 100 µL of Post-Process solution to sample and incubate at 37 °C for 30 min in a humidified chamber. Following incubation, remove the Post-Process reagent and drain excess solution on slide by blotting edges on a Kimwipe. You may stop the HCR™ Pro RNA-ISH assay at this point and proceed directly to your traditional IF workflow.*

5. Add antifade mounting reagent on top of sample and place coverslip on sample.
   NOTE: *Use antifade mounting reagent with a counterstain (e.g., DAPI) if preferred.*

6. Store sample at 4 °C and shield from light prior to imaging (fluorescence microscopy).

HCR™ Pro RNA-ISH Protocol: Sample in Solution

This protocol is suitable for use with whole-mount embryos, larvae, thick brain slices, etc in a tube or for adherent mammalian cells on a chambered slide. This protocol has not been validated for all sample types and should only be used as a template.

Sample Preparation

Samples should be prepared in the same manner as for traditional in situ hybridization up to the probe hybridization step. Then proceed with the protocol described below.

Pretreatment

1. Pre-warm Pretreat to room temperature.
2. Pre-warm HCR™ HiFi Probe Wash Buffer to room temperature.
3. Add 500 µL of Pretreat to sample and incubate at room temperature for 10 min.
4. Remove the Pretreat solution and wash 4 × 5 min with 500 µL of HCR™ HiFi Probe Wash Buffer at room temperature.

Probe Hybridization

1. Pre-heat HCR™ HiFi Probe Hybridization Buffer to 37 °C.
   CAUTION: *HCR™ HiFi Probe Hybridization Buffer contains formamide, a hazardous material.*
2. Remove the HCR™ HiFi Probe Wash Buffer and pre-hybridize samples in 500 µL of HCR™ HiFi Probe Hybridization Buffer for 30 min at 37 °C.
   NOTE: *Use enough HCR™ HiFi Probe Hybridization Buffer to immerse sample.*
3. Prepare probe solution by adding 10 µL of each HCR™ HiFi Probe to 500 µL of HCR™ HiFi Probe Hybridization Buffer at 37 °C.
4. Remove the pre-hybridization solution and add the probe solution.
5. Incubate sample for >3 h at 37 °C.
   NOTE: *Use enough probe solution to immerse sample.*
   NOTE: *Increase incubation time for thicker samples (e.g., >12 h for whole-mount vertebrate embryos).*
6. Pre-heat HCR™ HiFi Probe Wash Buffer to 37 °C.
   NOTE: *HCR™ HiFi Probe Wash Buffer provided at 4×; dilute to 1× with UltraPure H_2O before use.*
7. Remove excess probe by washing 4 × 15 min with 500 µL of HCR™ HiFi Probe Wash Buffer at 37 °C.

Amplification

HCR™ Pro Detect A

1. Pre-warm HCR™ Pro Detect A to room temperature.
2. Remove HCR™ HiFi Probe Wash Buffer from samples and add 500 µL of HCR™ Pro Detect A.
   NOTE: *Use enough HCR™ Pro Detect A to immerse sample.*

3. Incubate sample for 30 min at room temperature.

HCR™ Pro Detect B

1. Remove the HCR™ Pro Detect A solution and add 500 µL of HCR™ Pro Detect B.
   NOTE: *Use enough HCR™ Pro Detect B to immerse sample.*
2. Incubate at room temperature for >3 h.
   NOTE: *Increase incubation time for thicker samples (e.g., >12 h for thick brain slices).*
3. Pre-warm HCR™ Pro Amplifier Wash Buffer to room temperature.
   NOTE: *HCR™ Pro Amplifier Wash Buffer provided at 10×; dilute to 1× with UltraPure H_2O before use.*
4. Remove the HCR™ Pro Detect B reagent and wash 4 × 15 min with 500 µL of HCR™ Pro Amplifier Wash Buffer at room temperature.

HCR™ Pro Detect C

1. Remove the HCR™ Pro Amplifier Wash Buffer and add 500 µL of HCR™ Pro Detect C to sample.
   NOTE: *Use enough HCR™ Pro Detect C to immerse sample.*
2. Incubate for 1 h at room temperature.

HCR™ Pro Detect D

1. Remove the HCR™ Pro Detect C reagent and add 500 µL of HCR™ Pro Detect D to sample.
   NOTE: *Use enough HCR™ Pro Detect D to immerse sample.*
2. Incubate at room temperature for 2 h with gentle agitation.
3. Remove the HCR™ Pro Detect D reagent and wash 3 × 5 min with 500 µL of HCR™ Pro Amplifier Wash Buffer at room temperature.
   NOTE: *HCR™ Pro Amplifier Wash Buffer provided at 10×; dilute to 1× with UltraPure H_2O before use.*

HCR™ Pro Detect E

1. Remove the HCR™ Pro Amplifier Wash Buffer and add 500 µL of HCR™ Pro Detect E.
   NOTE: *Use enough HCR™ Pro Detect E to immerse sample.*
2. Incubate at room temperature for 20 min.
   NOTE: *Assay sensitivity can be tuned by adjusting the incubation time with HCR™ Pro Detect E. The recommended range is 5–30 min.*
   NOTE: *This step is time sensitive. Begin washing immediately once incubation is complete.*
3. Remove the HCR™ Pro Detect E reagent and wash 3 × 5 min with 500 µL of HCR™ Pro Amplifier Wash Buffer at room temperature.
   NOTE: *HCR™ Pro Amplifier Wash Buffer provided at 10×; dilute to 1× with UltraPure H_2O before use.*

HCR™ Pro Detect F HRP

1. Remove the HCR™ Pro Amplifier Wash Buffer and add 500 µL of HCR™ Detect F to sample.
   NOTE: *Use enough HCR™ Pro Detect F to immerse sample.*
2. Incubate for 30 min at room temperature .
3. Remove the HCR™ Pro Detect F solution and wash 3 × 5 min with 500 µL of HCR™ Pro Amplifier Wash Buffer at room temperature.
   NOTE: *HCR™ Pro Amplifier Wash Buffer provided at 10×; dilute to 1× with UltraPure H_2O before use.*
4. Proceed to either:
   - Chromogenic Reporter Deposition to perform chromogenic staining for RNA-CISH.
   - Fluorogenic Reporter Deposition to perform fluorescent staining for RNA-FISH.

Chromogenic Reporter Deposition

1. Pre-warm Matisse® chromogen (Brown or Green) and Matisse® Buffer (Brown or Green) to room temperature.
   NOTE: *Matisse® Green is sensitive to TBST/PBST. Use Matisse® Brown if continuing with a traditional IHC assay.*
2. Prepare Matisse® solution by adding 25 µL of Matisse® chromogen (Brown or Green) to 475 µL of Matisse® Buffer (Brown or Green).
   NOTE: *Solution must be used within 30 min of preparation.*
3. Add 500 µL of Matisse® solution to sample.
4. Incubate for 15 min at room temperature.
5. Remove Matisse® solution and wash 3 × 5 min with 500 µL of UltraPure H_2O at room temperature.
   NOTE: *If continuing with a traditional IHC assay, remove UltraPure H_2O and add 500 µL of Post-Process solution to sample. Incubate at 37 °C for 30 min. Remove the Post-Process solution following the incubation. You may stop the HCR™ Pro RNA-ISH assay at this point and proceed directly to your traditional IHC workflow.*
6. Transfer samples to glycerol through a graded series of UltraPure H_2O/glycerol washes:
   (a) 75% UltraPure H_2O / 25% glycerol
   (b) 50% UltraPure H_2O / 50% glycerol
   (c) 25% UltraPure H_2O / 75% glycerol
7. Store samples in 100% glycerol at 4 °C prior to imaging.

Fluorescent Reporter Deposition

1. Prepare fluorescent tyramide solution following the manufacturer's instructions.
   NOTE: *See list of recommended third-party fluorescent tyramides.*
2. Add 500 µL of fluorescent tyramide solution.

3. Incubate according to the manufacturer's instructions.

4. Remove the fluorescent tyramide solution and wash 3 × 5 min with HCR™ Pro Amplifier Wash Buffer at room temperature.
   NOTE: *HCR™ Pro Amplifier Wash Buffer provided at 10×; dilute to 1× with UltraPure H_2O before use.*
   NOTE: *If continuing with a traditional IF assay, remove HCR™ Pro Amplifier Wash Buffer and add 500 µL of Post-Process solution to sample. Incubate at 37 °C for 30 min. Remove the Post-Process solution following the incubation. You may stop the HCR™ Pro RNA-ISH assay at this point and proceed directly to your traditional IF workflow.*

5. Store sample at 4 °C and shield from light prior to imaging.

6. Use antifade mounting reagent to mount sample for imaging.
   NOTE: *Use antifade mounting reagent with a counterstain (e.g., DAPI) if preferred.*

Sample Preparation Protocols

This section provides MI-validated sample preparation protocols for:

- FFPE Tissue Sections on a Slide
- Fresh/Fixed Frozen Tissue Sections on a Slide
- Whole-Mount Zebrafish Embryos and Larvae in Solution
- Whole-Mount Mouse Embryos in Solution
- Whole-Mount Chicken Embryos in Solution
- Mammalian Cells on a Chambered Slide

Please note that these sample preparation protocols have not been validated for all sample variations and should only be used as a template.

For more information regarding your specific sample type, please refer to HCR™ RNA-FISH User Examples by Organism and Sample Type, which contains a table of selected user publications that used HCR™ RNA-FISH in diverse organisms and sample types, often including detailed sample preparation protocols. If you do not find your sample type listed, please reach out to our support team for guidance.

If you have previously used another RNA-FISH method on your sample, you can start with the same sample preparation protocol and then perform HCR™ Pro RNA-ISH using a protocol provided above.

Sample Preparation: FFPE Tissue Sections on a Slide

User-Supplied Materials

Reagent†	Supplier	Comments
Pro-Par Clearant	Fisher Scientific	Dewaxing solution
200-Proof Ethanol (EtOH)	Any	—
Target-Retrieval Solution	Any	1× citrate buffer (pH 6.0) or 1× Tris-EDTA buffer (pH 9.0)
UltraPure H_2O	Any	Type I water
10× PBS	Any	Avoid using PBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.
10% Tween-20	Any	—
1× PBST	—	See Common Recipes

†All user-supplied reagents should be DNase and RNase-free.

Sample Preparation Protocol

1. Bake slides in a dry oven for 1 h at 60 °C to improve sample adhesion to the slide.
2. In a fume hood, deparaffinize FFPE tissue by immersing slide in dewaxing solution (e.g., xylene, Pro-Par Clearant etc.) for 3 × 5 min. Move slides up and down occasionally.
   CAUTION: *Dewaxing solution may contain hazardous material, use with care.*
   NOTE: *Each 50 mL tube can fit two outward-facing slides. A volume of 30 mL is sufficient to immerse sections in the tube. If desired, a larger number of slides can be processed together using a Coplin jar.*
3. Incubate slides in 100% ethanol (EtOH) for 2 × 3 min at room temperature. Move slides up and down occasionally.
4. Rehydrate with a series of graded EtOH washes at room temperature.
   (a) 95% EtOH for 3 min
   (b) 70% EtOH for 3 min
   (c) 50% EtOH for 3 min
   (d) UltraPure H_2O for 3 min
5. Bring 500 mL of 1× Tris-EDTA buffer (pH 9.0) in a beaker to boil in a microwave.
   NOTE: *1× citrate buffer (pH 6.0) can be used in place of Tris-EDTA buffer (pH 9.0).*
6. Maintain Tris-EDTA buffer temperature at 95 °C on a hot plate.
7. Immerse slides for 15 min.
8. Remove beaker from hot plate and add 100 mL of UltraPure H_2O every 5 min to allow temperature to decrease to 45 °C in 20 min.
9. Immerse slides in 400 mL of UltraPure H_2O in a separate container for 10 min at room temperature.
10. Immerse slides in 1× PBST for 2 × 2 min at room temperature.
11. Dry slide using a Kimwipe. Avoid touching the tissue.

12. Draw a barrier around the tissue using a hydrophobic pen.
13. Wash slides by immersing in 1× PBST.
14. Repeat with fresh 1× PBST.
15. Proceed to HCR™ Pro RNA-ISH assay.

Sample Preparation: Fresh/Fixed Frozen Tissue Sections on a Slide

User-Supplied Materials

Reagent†	Supplier	Comments
Paraformaldehye (PFA)	Millipore Sigma	—
4% Paraformaldehyde (PFA) Solution	—	See Common Recipes
200-Proof Ethanol (EtOH)	Any	—
UltraPure H_2O	Any	Type I water
10× PBS	Any	Avoid using PBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.
1× PBS	—	See Common Recipes

†All user-supplied reagents should be DNase and RNase-free.

Sample Preparation Protocol

1. Remove frozen sections on slides from -80 °C.
2. Fix tissues by immersing slides in ice-cold 4% paraformaldehye (PFA) for 15 min at 4 °C.
   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.*
   NOTE: *Each 50 mL tube can fit two outward-facing slides. A volume of 30 mL is sufficient to immerse sections in the tube. If desired, a larger number of slides can be processed together using a Coplin jar.*
3. Immerse slides in 50% EtOH for 5 min at room temperature.
4. Immerse slides in 70% EtOH for 5 min at room temperature.
5. Immerse slides in 100% EtOH for 5 min at room temperature.
6. Immerse slides in another fresh 100% EtOH for 5 min at room temperature.
7. Immerse slides in 1× PBS.
8. Dry slide using a Kimwipe. Avoid touching the tissue.
9. Draw a barrier around the tissue using a hydrophobic pen.
10. Wash slides by immersing in 1× PBS.
11. Repeat with fresh 1× PBS.
12. Proceed to HCR™ Pro RNA-ISH assay.

Sample Preparation: Whole-Mount Zebrafish Embryos and Larvae in Solution

User-Supplied Materials

Reagent†	Supplier	Comments
Paraformaldehye (PFA)	Millipore Sigma	—
4% Paraformaldehyde (PFA) Solution	—	See Common Recipes
Egg H_2O	—	See ZFIN General Methods for Zebrafish Care
1-Phenyl 2-thiourea (PTU)	Millipore Sigma	Optional treatment to inhibit pigment development
6% PTU Solution	—	See Common Recipes
0.003% PTU Solution	—	See Common Recipes
100% Methanol (MeOH)	Any	—
UltraPure H_2O	Any	Type I water
10× PBS	Any	Avoid using PBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.
10% Tween-20	Any	—
1× PBST	—	See Common Recipes
20 mg/mL Proteinase K	ThermoFisher Scientific	—
30 µg/mL Proteinase K Solution	—	See Common Recipes

†All user-supplied reagents should be DNase and RNase-free.

Sample Preparation Protocol

This protocol has not been optimized for all stages and should only be used as a template.

1. Collect zebrafish embryos and incubate at 28 °C in a petri dish with egg H_2O.
2. Exchange egg H_2O with 0.003% PTU solution when embryos reach 12 hpf (hours post-fertilization). Replace with fresh 0.003% PTU solution every day until the larvae reach 5 dpf (days post-fertilization).
   NOTE: *Skip this step for embryos that will be imaged younger than 30 hpf as PTU treatment is not necessary.*
   NOTE: *PTU inhibits melanogenesis but can be toxic at high concentrations. PTU treatment must start before the initial pigmentation occurs as PTU does not remove pigment that has already formed. PTU treatment is not necessary for nacre embryos.*
3. Transfer ∼40 embryos/larvae to a 2 mL eppendorf tube and remove excess egg H_2O.
   NOTE: *Dechorionate embryos that will be imaged younger than 72 hpf before fixation.*
4. Fix embryos/larvae in 2 mL of 4% paraformaldehyde (PFA) for 24 h at 4 °C.
   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.*
5. Wash embryos/larvae 3 × 5 min with 1 mL of 1× PBS to stop the fixation.
6. Dehydrate and permeabilize with a series of methanol (MeOH) washes (1 mL each):
   (a) 100% MeOH for 4 × 10 min
   (b) 100% MeOH for 1 × 50 min.
7. Store embryos/larvae at -20 °C before use.
   NOTE: *Embryos/larvae can be stored for six months at -20 °C.*

8. Transfer the required number of embryos/larvae for an experiment to a 2 mL eppendorf tube.
9. Rehydrate with a series of graded 1 mL MeOH/PBST washes for 5 min each at room temperature:
   (a) 75% MeOH / 25% PBST
   (b) 50% MeOH / 50% PBST
   (c) 25% MeOH / 75% PBST
   (d) 5 × 100% PBST.
10. Treat larvae (5 dpf) with 1 mL of proteinase K (30 µg/mL) for 45 min at room temperature.
    NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage. Skip proteinase K treatment and postfixation (steps 11–14) for embryos 30 hpf and younger.*
11. Wash embryos/larvae two times with PBST (1 mL each) without incubation.
12. Postfix with 1 mL of 4% PFA for 20 min at room temperature.
13. Wash embryos/larvae 5 × 5 min with 1 mL of PBST.
14. Proceed to HCR™ Pro RNA-ISH assay.

Sample Preparation: Whole-Mount Mouse Embryos in Solution

User-Supplied Materials

Reagent†	Supplier	Comments
Paraformaldehye (PFA)	Millipore Sigma	—
4% Paraformaldehyde (PFA) Solution	—	See Common Recipes
100% Methanol (MeOH)	Any	—
UltraPure H_2O	Any	Type I water
10× PBS	Any	Avoid using PBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.
10% Tween-20	Any	—
1× PBST	—	See Common Recipes
20 mg/mL Proteinase K	ThermoFisher Scientific	—
10 µg/mL Proteinase K Solution	—	See Common Recipes

†All user-supplied reagents should be DNase and RNase-free.

Sample Preparation Protocol

This protocol has been optimized for embryos at stage E9.5 and should only be used as a template for other stages.

1. Wipe all dissection equipment with RNaseZap.
2. Kill a pregnant female mouse using an IACUC-approved protocol.
3. Immediately remove the uterus and submerge it in 4% paraformaldehyde (PFA) in a fresh RNase-free petri dish.
   NOTE: *Use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.*
4. Dissect the mouse embryos from the uterus while it is submerged in 4% PFA.
   CAUTION: *Use PFA with extreme care as it is a hazardous material.*
   NOTE: *Each female mouse produces 6–9 embryos. We recommend using ≈2 mL of solution per group of 10 embryos.*
5. Transfer the embryos to a clean vial containing fresh 4% PFA and fix them overnight or longer at 4 °C.
   NOTE: *Make sure all embryos are submerged in PFA during fixation.*
6. Wash 2 × 5 min with PBST on ice.
7. 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.

8. Maintain embryos at -20 °C overnight (> 16 h) or until use.
   NOTE: *Embryos can be stored for six months at -20 °C .*

9. Transfer the required number of embryos for an experiment to a 2 mL tube.
   NOTE: *Make sure embryos are submerged during washes.*

10. 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

11. Wash embryos with PBST for 10 min at room temperature.

12. Immerse embryos in 10 µg/mL proteinase K solution for 15 min at room temperature.
    NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage.*

13. Wash embryos 2 × 5 min with PBST.

14. Postfix with 4% PFA for 20 min at room temperature.
    CAUTION: *Use PFA with extreme care as it is a hazardous material.*

15. Wash embryos 3 × 5 min with PBST.

16. Proceed to HCR™ Pro RNA-ISH assay.

Sample Preparation: Whole-Mount Chicken Embryos in Solution

User-Supplied Materials

Reagent†	Supplier	Comments
Ringer's Solution	—	See Common Recipes
Paraformaldehye (PFA)	Millipore Sigma	—
4% Paraformaldehyde (PFA) Solution	—	See Common Recipes
100% Methanol (MeOH)	Any	—
UltraPure H_2O Water	Any	Type I water
10× PBS	Any	Avoid using PBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.
10% Tween-20	Any	—
1× PBST	—	See Common Recipes
20 mg/mL Proteinase K	ThermoFisher Scientific	—
10 µg/mL Proteinase K Solution	—	See Common Recipes

†All user-supplied reagents should be DNase and RNase-free.

Sample Preparation Protocol

This protocol has been optimized for embryos at stage HH 8–11 and should only be used as a template for other stages.

1. Collect chick embryos on 3M paper circles and place in a petri dish containing Ringer's solution.
2. Transfer embryos into a new petri dish with fresh Ringer's solution to rinse away egg yolk before fixation.
3. Transfer into a petri dish containing 4% paraformaldehyde (PFA).
   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.*
4. Fix the samples at room temperature for 1 h.
5. Transfer embryos into a petri dish containing PBST.
6. Dissect the embryos off the filter paper. Remove the fixed vitelline membrane (opaque) and cut the squares around area pellucida, without leaving excess of extra-embryonic tissue.
7. Transfer embryos into a 2 mL tube containing PBST.
8. Nutate for 5 min with the tube positioned horizontally in a small ice bucket.
9. Wash embryos two additional times with 2 mL of PBST, each time nutating for 5 min on ice.
10. Dehydrate embryos with 2 × 5 min washes of 2 mL methanol (MeOH) on ice.
11. Store embryos at -20 °C before use.
    NOTE: *Embryos can be stored for six months at -20 °C.*
12. Transfer the required number of embryos for an experiment to a 2 mL tube.
    NOTE: *Do not place more than 4 embryos in each 2 mL tube.*

13. Rehydrate with a series of graded 2 mL MeOH/PBST washes for 5 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.
14. Treat embryos with 2 mL of 10 µg/mL proteinase K solution for 2 min (stage HH 8) or 2.5 min (stage HH 10–11) at room temperature.
    NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage.*
15. Postfix with 2 mL of 4% PFA for 20 min at room temperature.
    CAUTION: *Use PFA with extreme care as it is a hazardous material.*
16. Wash embryos 2 × 5 min with 2 mL of PBST on ice.
17. Wash embryos with 2 mL of 50% PBST / 50% 5× SSCT for 5 min on ice.
18. Wash embryos with 2 mL of 5× SSCT for 5 min on ice.
19. Proceed to HCR™ Pro RNA-ISH assay.

Sample Preparation: Mammalian Cells on a Chambered Slide

User-Supplied Materials

Reagent†	Supplier	Comments
DPBS	Gibco	Avoid using DPBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.
UltraPure H_2O	Any	Type I water
Poly-D-Lysine Hydrobromide	Millipore Sigma	—
0.01% (w/v) Poly-D-Lysine	—	See Common Recipes
16% Formaldehyde (FA), Methanol-free	Polysciences	—
4% Formaldehyde (FA) Solution	—	See Common Recipes
200-Proof Ethanol (EtOH)	Any	—
20× SSC	Any	—
2× SSC	—	See Common Recipes

†All user-supplied reagents should be DNase and RNase-free.

Sample Preparation Protocol

This protocol has not been validated for all mammalian cell types and should only be used as a template.

1. Coat bottom of each chamber by applying 300 µL of 0.01% (w/v) poly-D-lysine solution.
   NOTE: *A volume of 300 µL is sufficient per chamber on an 8-chamber slide.*
2. Incubate for at least 30 min at room temperature.
3. Aspirate the coating solution and wash each chamber twice with UltraPure H_2O.
4. Plate desired number of cells in each chamber.
5. Grow cells to desired confluency for 24–48 h.
6. Aspirate growth media and wash each chamber with 300 µL of DPBS.
7. Add 300 µL of 4% formaldehyde to each chamber.
   CAUTION: *Use formaldehyde with extreme care as it is a hazardous material.*
8. Incubate for 10 min at room temperature.
9. Aspirate fixative and wash each chamber 2 × 300 µL of DPBS.
10. Aspirate DPBS and add 300 µL of ice-cold 70% ethanol.
11. Permeabilize cells overnight at -20 °C.
    NOTE: *Cells can be stored at -20 °C or 4 °C until use.*
12. Prior to in situ hybridization, aspirate EtOH and wash samples 2 × 300 µL of 2× SSC.
13. Proceed to HCR™ Pro RNA-ISH assay.

Common Recipes

NOTE: *avoid using PBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.*

4% Paraformaldehyde (PFA)
4% PFA
1× PBS

For 25 mL of solution
1 g of PFA powder
25 mL of 1× PBS
Heat solution at 50–60 °C to dissolve powder

4% Formaldehyde (FA)
4% formaldehyde
1× PBS

For 10 mL of solution
2.5 mL of 16% formaldehyde
1 mL of 10× PBS
Fill up to 10 mL with UltraPure H_2O

1× PBS
1× PBS

For 50 mL of solution
5 mL of 10× PBS
Fill up to 50 mL with UltraPure H_2O

1× 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_2O

2× SSC
2× sodium chloride sodium citrate (SSC)

For 40 mL of solution
4 mL of 20× SSC
Fill up to 40 mL with UltraPure H_2O

6% PTU Solution
6% PTU

For 100 mL of solution
6 g of 1-phenyl 2-thiourea (PTU) powder
Fill up to 100 mL with egg H_2O
Heat solution at 50–60 °C overnight to dissolve powder

0.003% PTU Solution
0.003% PTU

For 50 mL of solution
25 µL of 6% PTU
Fill up to 50 mL with egg H_2O

Ringer's Solution
123 mM NaCl
1.53 mM $CaCl_2$
4.96 mM KCl_2
0.81 mM Na_2HPO_4
0.15 mM KH_2PO_4

For 2 L of solution
14.4 g of NaCl
340 mg of $CaCl_2$
740 mg of KCl
230 mg of Na_2HPO_4
40 mg of KH_2PO_4
Bring volume up to 1.5 L with UltraPure H_2O
Adjust pH to 7.4 and fill up to 2 L with UltraPure H_2O
Filter sterilize with 0.22 µm bottle top filter

0.01% (w/v) Poly-D-Lysine
0.01% (w/v) Poly-D-Lysine

For 50 mL of solution
5 mg of Poly-D-Lysine Hydrobromide
Fill up to 50 mL with UltraPure H_2O

30 µg/mL Proteinase K Solution
30 µg/mL proteinase K

For 1 mL of solution
1.5 µL of 20 mg/mL proteinase K
Fill up to 1 mL with PBST

10 µg/mL Proteinase K Solution
10 µg/mL proteinase K

For 1 mL of solution
0.5 µL of 20 mg/mL proteinase K
Fill up to 1 mL with PBST

Recommended Third-Party Fluorescent Tyramides

Fluorescent Tyramides	Incubation Time (min)	Recommended Starting Concentration	Supplier	Catalog Number
CF488A	8–24	5 µM	Biotium	92171
CF550R	8–24	5 µM	Biotium	96077
CF555	8–24	5 µM	Biotium	96021
CF583R	8–24	5 µM	Biotium	96085
CF594	8–24	5 µM	Biotium	92174
CF640R	8–24	5 µM	Biotium	92175
CF754	8–24	5 µM	Biotium	96090
Alexa Fluor 488 - Tyramide	8–24	1×	ThermoFisher	B40953
Alexa Fluor 546 - Tyramide	8–24	1×	ThermoFisher	B40954
Alexa Fluor 647 - Tyramide	8–24	1×	ThermoFisher	B40958
Alexa Fluor 750 - Tyramide	8–24	1×	ThermoFisher	B56131

Frequently Asked Questions (FAQ)

I've never used HCR™ Pro RNA-ISH – what's the best way to get started?

- We offer new users a complimentary HCR™ Pro RNA-ISH Starter Kit. Please register for an account and fill out this questionnaire.

What comes in an HCR™ Pro RNA-ISH kit?

- HCR™ HiFi Probe backed by the HCR™ HiFi Probe Promise
- HCR™ HiFi Probe Hybridization Buffer
- HCR™ HiFi Probe Wash Buffer
- HCR™ Pro Amplifier kit
  - Pretreat
  - HCR™ Pro Detect A
  - HCR™ Pro Detect B
  - HCR™ Pro Detect C
  - HCR™ Pro Detect D
  - HCR™ Pro Detect E
  - HCR™ Pro Detect F HRP
  - Post-Process
- HCR™ Pro Amplifier Wash Buffer
- Matisse® chromogen kit
  - Matisse® Green or Matisse® Brown
  - Matisse® Green Buffer or Matisse® Brown Buffer

The HCR™ Pro RNA-ISH kit is used with an HRP chromogen for RNA-CISH (e.g., Matisse® Green or Matisse® Brown) or an HRP fluorogenic substrate for RNA-FISH (e.g., any of a variety of recommended third-party fluorescent tyramides).

Can I order a subset of these components?

- Yes. You can order any subset of the following:
  - HCR™ HiFi Probe
  - HCR™ HiFi Probe Hybridization Buffer
  - HCR™ HiFi Probe Wash Buffer
  - HCR™ Pro Amplifier kit
  - HCR™ Pro Amplifier Wash Buffer
  - Matisse® chromogen kit

Note that the components of the HCR™ Pro Amplifier kit are used at the same rate so they cannot be ordered individually.

Can I use HCR™ HiFi Probes with either HCR™ Gold or HCR™ Pro?
HCR™ HiFi Probes come in systems X1-X10. HCR™ Gold kits use X1-X10 probes (supporting 10-plex experiments). HCR™ Pro kits use X2 probes (supporting 1-plex experiments). So any X2 HCR™ HiFi Probe can be used for either HCR™ Gold or HCR™ Pro. Note that the forthcoming HCR™ Ultra will support multiplex experiments using a subset of X1-X10 probes (details TBA).

Can I use HCR™ HiFi Probe Hybridization Buffer and HCR™ HiFi Probe Wash Buffer with either HCR™ Gold or HCR™ Pro?
Yes, HCR™ HiFi Probe Hybridization Buffer and HCR™ HiFi Probe Wash Buffer are each the same between HCR™ Gold and HCR™ Pro kits. However, note that HCR™ Gold Amplifier Wash Buffer and HCR™ Pro Amplifier Wash Buffer are different, and must be used for HCR™ Gold and HCR™ Pro, respectively.

Do you recommend specific HRP substrates for use with HCR™ Pro?
Yes.

- **RNA-CISH:** For chromogenic staining, HCR™ Pro RNA-ISH protocols have been optimized for Matisse® Green and Matisse® Brown; other third-party HRP chromogens can be used as desired (follow the manufacturer's instructions).
- **RNA-FISH:** For fluorescent staining, HCR™ Pro RNA-ISH protocols have been validated for a variety of recommended third-party fluorescent tyramides.

Can I combine HCR™ Pro RNA-ISH with traditional IHC/IF for simultaneous RNA and protein imaging?
Yes, HCR™ Pro RNA-ISH is compatible with traditional immunohistochemistry (IHC) and immunofluorescence (IF). We recommend performing the HCR™ Pro RNA-ISH assay first followed by a Post-Process incubation before proceeding to traditional IHC/IF.

Is the HCR™ platform compatible with clearing techniques?
Yes, the HCR™ platform has been shown to be compatible with numerous clearing methods including PACT (Markman *et al.*, 2023), CLARITY (Shah *et al.*, 2016), Ce3D+ (Anderson *et al.*, 2020; Boylan *et al.*, 2020; Lex *et al.*, 2022), TDE (Kim *et al.*, 2023), and SDS (Inagaki *et al.*, 2022; Knoedler *et al.*, 2022; Kozareva *et al.*, 2021). Please contact the MI Team for additional information.

HCR™ Technology Citation Notes

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

- **HCR™ RNA-ISH**
  HCR™ RNA in situ hybridization (RNA-ISH) offers unmatched performance, robustness, and versatility imaging RNA targets in diverse organisms and sample types (Choi et al., 2010, Choi et al., 2014, Choi et al., 2018):
  - **HCR™ RNA-FISH**
    HCR™ RNA-FISH enables 10-plex, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) with automatic background suppression throughout the protocol for dramatically enhanced performance (signal-to-background, quantitative precision, single-molecule fidelity) and ease-of-use (no probe set optimization for new targets and organisms).
  - **Enzymatic HCR™ RNA-CISH/RNA-FISH**
    Enzymatic HCR™ RNA-ISH integrates enzymatic signal amplification to enable extreme-sensitivity RNA imaging using either chromogenic or fluorescent staining (RNA-CISH/RNA-FISH). In tissue sections, entirely protease-free workflows preserve sample morphology and maintain protein target integrity, enabling seamless compatibility with existing immunohistochemistry (IHC)/immunofluorescence (IF) assays. HCR™ RNA-CISH offers the convenience of bright-field microscopy and the option of archival staining.
- **10-Plex HCR™ Spectral Imaging**
  HCR™ RNA-FISH/IF enables quantitative high-resolution imaging of 10 RNA and/or protein targets with 1-step HCR™ signal amplification for all targets simultaneously. The method is suitable even for whole-mounts and delicate samples as it requires no repeated staining, imaging, registration, or stripping (Schulte et al., 2024).
- **HCR™ RNA-FISH/IF**
  HCR™ RNA-FISH/IF enables a unified approach to multiplex, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) and protein immunofluorescence (IF), with quantitative 1-step enzyme-free signal amplification performed for all RNA and protein targets simultaneously (Schwarzkopf et al., 2021).
- **HCR™ IF**
  HCR™ IF enables multiplex, quantitative, high-resolution protein immunofluorescence (IF) in highly autofluorescent samples (e.g., FFPE brain tissue sections) (Schwarzkopf et al., 2021).
- **Subcellular Quantitative RNA and Protein Imaging**
  HCR™ RNA-FISH enables analog relative quantitation of RNA and/or protein targets with subcellular resolution in the anatomical context of thick autofluorescent samples (e.g., whole-mount vertebrate embryos) (Trivedi et al., 2018, Choi et al., 2018, Schwarzkopf et al., 2021).
- **Single-Molecule Quantitative RNA Imaging**
  HCR™ RNA-FISH enables digital RNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah et al., 2016, Choi et al., 2018).

- **Read-Out/Read-In Analysis Framework**
  The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (Trivedi et al., 2018).

- **Protocols in Diverse Sample Types**
  Protocols for HCR™ RNA-FISH and/or IF in diverse sample types are adapted from the zoo paper (Choi et al., 2016):
  - bacteria in suspension
  - FFPE human tissue sections
  - generic sample in solution
  - generic sample on a slide
  - mammalian cells on a slide
  - mammalian cells in suspension
  - whole-mount chicken embryos
  - whole-mount fruit fly embryos
  - whole-mount mouse embryos
  - whole-mount nematode larvae
  - whole-mount sea urchin embryos
  - whole-mount zebrafish embryos and larvae

- **HCR™ RNA Flow Cytometry**
  HCR™ RNA Flow Cytometry enables analog RNA relative quantitation for high-throughput expression profiling of mammalian cells and bacteria without the need to engineer reporter lines (Choi et al., 2018).

- **HCR™ Northern Blots**
  HCR™ Northern Blots enable simultaneous quantification of RNA target size and abundance with automatic background suppression throughout the protocol (Schwarzkopf & Pierce, 2016).

- **HCR™ Amplifiers**
  HCR™ Amplifiers enable multiplex, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings (Dirks & Pierce, 2004).

HCR™ RNA-FISH User Examples by Organism and Sample Type

Organism	Sample types*
African clawed frog	whole-mount embryos (Lee *et al.*, 2023; Leigh *et al.*, 2020; Sun *et al.*, 2022a; Zhao *et al.*, 2022), whole-mount limb and tail (Aztekin *et al.*, 2021)
Amphipod crustacean	whole-mount embryos (Bruce & Patel, 2020, 2022; Bruce *et al.*, 2021)
Ant	whole-mount larvae (Qiu *et al.*, 2022), whole-mount ovaries (Qiu *et al.*, 2022), whole-mount brain (Fetter-Pruneda *et al.*, 2021; Li *et al.*, 2022a; Nagel *et al.*, 2020)
Arabidopsis	whole-mount root (Nobori *et al.*, 2023)
Axolotl	thin tissue sections (Freitas *et al.*, 2019), thin spinal cord tissue section (Duerr *et al.*, 2022), thin lung tissue sections (Jensen *et al.*, 2021), limb buds (Schloissnig *et al.*, 2021; Trofka *et al.*, 2021), thin limb tissue sections (Zhong *et al.*, 2023), thick brain tissue sections (Woych *et al.*, 2022), thin brain tissue sections (Woych *et al.*, 2022)
Bacteria	bacteria on termite gut protozoa (Rosenthal *et al.*, 2013), bacteria in environmental samples (Jia *et al.*, 2021; Yamaguchi *et al.*, 2015), cultured bacteria (Choi *et al.*, 2016; Lassinantti *et al.*, 2021; Needham *et al.*, 2022; Rammohan *et al.*, 2021, 2022), cultured bacterial flow cytometry (Choi *et al.*, 2018; Grieb *et al.*, 2020; Rammohan *et al.*, 2021, 2022), aggregates (Jorth *et al.*, 2019), bacterial symbionts within whole-mount juvenile squid light organ (Bennett *et al.*, 2020), bacteria on cleared plant roots (Dar *et al.*, 2020), bacteria in cleared whole-mount mouse intestines (Gallego-Hernandez *et al.*, 2020), symbionts of the giant tube worms (Hinzke *et al.*, 2021), bacteria in fungal tissue (Dahlstrom & Newman, 2022; Morales *et al.*, 2022; Robinson *et al.*, 2021), bacteria symbionts within sea anemone (Goffredi *et al.*, 2021), bacteria in backskins of mice with clearing (Lay *et al.*, 2018), agar block biofilm (Livingston *et al.*, 2022)
Basal chordate	whole-mount (Kourakis *et al.*, 2019)
Beetle	whole-mount embryos (Bruce & Patel, 2020, 2022; Bruce *et al.*, 2021; Kobayashi *et al.*, 2022; Tidswell *et al.*, 2021), whole-mount heads (Crabtree *et al.*, 2020), whole-mount genitalia (Crabtree *et al.*, 2020), whole-mount ovaries (Luo *et al.*, 2020; Tidswell *et al.*, 2021), thin thoracic tissue sections (Hu *et al.*, 2019), dorsal abdominal segments (Brückner *et al.*, 2021)
Blood fluke	whole-mount (Diaz Soria *et al.*, 2020; Rawlinson *et al.*, 2021)
Brine shrimp	naupili and adults (Bruce & Patel, 2020; Bruce *et al.*, 2021)
Butterfly	whole-mount embryos and imaginal discs (Bruce *et al.*, 2021)
Cavefish	whole-mount embryo (Kozol *et al.*, 2023; O'Gorman *et al.*, 2021)
Chicken	whole-mount embryos (Asmar *et al.*, 2023; Choi *et al.*, 2016, 2018; Galton *et al.*, 2022; Gandhi *et al.*, 2020, 2021; Hutchins *et al.*, 2021; Kim *et al.*, 2022; Ling & Sauka-Spengler, 2019; McLennan *et al.*, 2015; Monroy *et al.*, 2022; Nandagopal *et al.*, 2018; Piacentino & Bronner, 2018; Piacentino *et al.*, 2021; Williams *et al.*, 2019, 2022), thin whole-embryo tissue sections (Askary *et al.*, 2020; Kim *et al.*, 2022), thick cochlea tissue sections (Benkafadar *et al.*, 2021; Janesick *et al.*, 2021), FFPE embryo tissue sections (Rees & Gillis, 2022), utricle tissue sections (Scheibinger *et al.*, 2022)
Daddy long legs	whole-mount embryos (Gainett *et al.*, 2021)
Deep-sea anemone	thin tissue sections (Goffredi *et al.*, 2021)

Organism	Sample types*
Fruit fly	whole-mount embryos (Beaven & Denholm, 2022; Bruce *et al.*, 2021; Choi *et al.*, 2016; Clark *et al.*, 2022b; Domsch *et al.*, 2021; Duk *et al.*, 2021; Graham *et al.*, 2021; Karunaraj *et al.*, 2022; Sankaranarayanan *et al.*, 2021; Sharrock *et al.*, 2022; Surkova *et al.*, 2019; Velten *et al.*, 2022; Zechini *et al.*, 2022), whole-mount pupae (Rose *et al.*, 2022), whole-mount imaginal discs (Bruce *et al.*, 2021; Worley *et al.*, 2022),whole-mount larvae (Ali *et al.*, 2019), whole-mount brains (Lacin *et al.*, 2019; Michki *et al.*, 2021; Sgammeglia *et al.*, 2023; Tang *et al.*, 2022), whole-mount nervous system (Duckhorn *et al.*, 2022a,b), whole-mount ventral nerve cord (Shao *et al.*, 2019), whole-mount ovaries (Dunipace *et al.*, 2022; Lin *et al.*, 2023; Slaidina *et al.*, 2020, 2021; Soriano *et al.*, 2023; Tatapudy *et al.*, 2021; Tu *et al.*, 2021; Zhao *et al.*, 2019), whole-mount testes (Chen *et al.*, 2021b,c), cells (Liu *et al.*, 2023), antenna tissue sections (Task *et al.*, 2022), whole-mount eye-optic lobe (Ali *et al.*, 2019), whole-mount heart (Zechini *et al.*, 2022), whole-mount eye imaginal disc (Kozlov *et al.*, 2022), whole-mount salivary gland (Li *et al.*, 2022b)
Green foxtail	half-mount root (Guillotin *et al.*, 2023)
Hemichordata	whole-mount larva (López *et al.*, 2023)
Honey bee	whole-mount ovary (Cullen *et al.*, 2023)
Human	FFPE thin breast tissue sections (Choi *et al.*, 2016), FFPE thin brain tissue sections (Glineburg *et al.*, 2021), FFPE thin tumor tissue sections (Tanaka *et al.*, 2020), FFPE clear-cell renal-cell carcinoma tissue sections (Kufukihara *et al.*, 2022), cultured cells on a slide (Choi *et al.*, 2018; Fang *et al.*, 2023; Gerbin *et al.*, 2021; Glineburg *et al.*, 2021; Grancharova *et al.*, 2021; Hildebrandt *et al.*, 2023; Kelley *et al.*, 2022; Nandagopal *et al.*, 2019; Pond *et al.*, 2022; Rafiee *et al.*, 2020; Rinaldi *et al.*, 2022; Shilo *et al.*, 2022; Sil *et al.*, 2023; Wen *et al.*, 2021; Zhu *et al.*, 2023), cultured cell flow cytometry (Choi *et al.*, 2018; Gasperini *et al.*, 2019; Reilly *et al.*, 2021), thin brain tissue sections (Fernandez-Cerado *et al.*, 2021; Kamath *et al.*, 2022; Kamermans *et al.*, 2019; Mayerl *et al.*, 2022), thin intestine tissue sections (May-Zhang *et al.*, 2021, 2022), thin kidney tissue sections (Marshall *et al.*, 2022), expanded cultured cells on a slide (Alon *et al.*, 2021), expanded clear-cell renal-cell carcinoma tissue sections (Kufukihara *et al.*, 2022), thin tumor tissue sections and microarrays (Tanaka *et al.*, 2020), organoids (Albanese *et al.*, 2020; Sanaki-Matsumiya *et al.*, 2022; Tanaka *et al.*, 2020; Yamanaka *et al.*, 2022), thin auditory nerve sections (Chen *et al.*, 2022)
Hydra	whole-mount (Vogg *et al.*, 2022)
Killifish	thin coronal sections (van Houcke *et al.*, 2021)
Lancelet	whole-mount embryos (Andrews *et al.*, 2020; Herrera-Úbeda *et al.*, 2019; Zawisza-Álvarez *et al.*, 2020), whole-mount larva (Zawisza-Álvarez *et al.*, 2020), FFPE tissue sections (Sackville *et al.*, 2022), thick tissue sections (Andrews *et al.*, 2020)
Little skate	thin FFPE tissue sections (Criswell & Gillis, 2020; Hirschberger & Gillis, 2022; Marconi *et al.*, 2020; Rees *et al.*, 2023)
Lizard	whole-mount embryo (Sanger *et al.*, 2021)
Maize	half-mount root (Guillotin *et al.*, 2023)
Marmoset	thick brain tissue sections (Krienen *et al.*, 2020), FFPE thin brain tissue sections (Lin *et al.*, 2022)
Minipig	FFPE muscle tissue (Nikovics *et al.*, 2020; Sicherre *et al.*, 2021)
Mosquito	whole-mount embryos (Bui *et al.*, 2023), whole-mount antenna and maxillary palp (Herre *et al.*, 2022; Task *et al.*, 2022), whole-mount ovary (Venkataraman *et al.*, 2023)
Moth	whole-mount pupal wings (Bruce *et al.*, 2021)

Organism	Sample types*
Mouse	whole-mount embryos (Anderson *et al.*, 2020; Boylan *et al.*, 2020; Choi *et al.*, 2016; Huss *et al.*, 2015; Kim *et al.*, 2019; Kumar *et al.*, 2023; Lex *et al.*, 2022; Lohoff *et al.*, 2022; Ramachandran *et al.*, 2022; Trofka *et al.*, 2021; Tyser *et al.*, 2021; Zhu *et al.*, 2022), cleared thick brain tissue sections (Condylis *et al.*, 2022; Greenbaum *et al.*, 2017; Inagaki *et al.*, 2022; Knoedler *et al.*, 2022; Kozareva *et al.*, 2021; Kramer *et al.*, 2018; Mich *et al.*, 2021; Shah *et al.*, 2016; Sylwestrak *et al.*, 2016; Takatoh *et al.*, 2022; Wang *et al.*, 2021), thin brain tissue sections (Askary *et al.*, 2020; Ben-Simon *et al.*, 2022; Carriere *et al.*, 2020; Cleary *et al.*, 2021; Frank *et al.*, 2023; Hu *et al.*, 2022; Ibarra *et al.*, 2021; Kamath *et al.*, 2022; Lui *et al.*, 2021; Mayerl *et al.*, 2021; Osorno *et al.*, 2022; Ren *et al.*, 2019; Shi *et al.*, 2023; Sun *et al.*, 2022b; Young & Song, 2020; Zhang *et al.*, 2022), thin nose tissue sections (Baxter *et al.*, 2020), cultured cells on a slide (Alon *et al.*, 2021; Brookes *et al.*, 2022; Denes *et al.*, 2021; Glineburg *et al.*, 2021; Jain *et al.*, 2022; Nandagopal *et al.*, 2019; Raina *et al.*, 2021; Rodriguez *et al.*, 2020; Shah *et al.*, 2016), expanded thick brain tissue sections (Alon *et al.*, 2021; Arshadi *et al.*, 2021), thick brain tissue sections (Chen *et al.*, 2021d; Hsueh *et al.*, 2023; Kim *et al.*, 2023; Krienen *et al.*, 2020; Michael *et al.*, 2020; Mohan *et al.*, 2023; Norman *et al.*, 2021; Tabata *et al.*, 2022), whole-mount retina (Anderson *et al.*, 2019, 2022), thin spinal cord tissue sections (Li *et al.*, 2020), thin intestine tissue sections (Duque-Correa *et al.*, 2022; May-Zhang *et al.*, 2021), thin retina tissue sections (Zhuang *et al.*, 2020), thin whole-embryo tissue sections (Deal *et al.*, 2021; Liu *et al.*, 2020), thin trigeminal ganglia tissue sections (Emrick *et al.*, 2020; von Buchholtz *et al.*, 2020, 2021), gastruloids (van den Brink *et al.*, 2020; Vianello *et al.*, 2021), expanded thin brain tissue sections (Alon *et al.*, 2021), thin FFPE liver tissue sections (Wells *et al.*, 2021), whole-mount mouse cochlea (Diaz & Heller, 2021), thin cochlear tissue sections (Chen *et al.*, 2022; Ebeid *et al.*, 2022, 2023), thin testes tissue sections (Chen *et al.*, 2021a; Green *et al.*, 2018; Luca *et al.*, 2023), thin kidney tissue sections (Clark *et al.*, 2022a; Marshall *et al.*, 2022), thin digit tissue sections (Johnson *et al.*, 2022), thin tibia tissue sections (Rubin *et al.*, 2021), thin tendon sections (Vinestock *et al.*, 2022), thin optic nerve tissue sections (Wang *et al.*, 2020), thin FFPE digit sections (Castilla-Ibeas *et al.*, 2023), thin FFPE cervical ganglia tissue sections (Ge *et al.*, 2022), thin lymph node tissue section (Koning *et al.*, 2021), thin muscle tissue sections (Pinheiro *et al.*, 2021), whole mount embryo tail (Sanchez & Miyazawa, 2019), whole-mount ganglia (von Buchholtz *et al.*, 2021), thin molar and incisor tissue sections (Nottmeier *et al.*, 2021), whole-mount forelimbs (Markman *et al.*, 2023), whole-mount ear (Jia *et al.*, 2023)
Multi-kingdom	whole-mount juvenile squid light organ and bacterial symbionts (Nikolakakis *et al.*, 2015), consortia of archaea and bacteria (Metcalfe *et al.*, 2021)
Nematode	whole-mount larvae (Belew *et al.*, 2021; Choi *et al.*, 2016; Wong *et al.*, 2018), whole-mount oocyte (Trimmer *et al.*, 2023)
Quail	whole-mount embryo (Huss *et al.*, 2019; Monroy *et al.*, 2022)
Rat	thin rat brain tissue sections (Sui *et al.*, 2016), thin rat femur sections (Nikovics *et al.*, 2022), thick rat brain tissue sections (Chen *et al.*, 2021d)
Rhesus macaque	FFPE thin brain tissue sections (Redmayne & Chavez, 2019)
Sea lamprey	whole-mount embryos (Bedois *et al.*, 2024; Hockman *et al.*, 2019)
Sea urchin	whole-mount embryos (Choi *et al.*, 2016; Ka *et al.*, 2021; Rodríguez-Sastre *et al.*, 2022; Thomas *et al.*, 2023)
Snake	whole-mount embryo (Tzika *et al.*, 2023)
Sorghum	half-mount root (Guillotin *et al.*, 2023)
Sponge	whole-mount sponge (Colgren & Nichols, 2022)
Tarantula	whole-mount embryos (Bruce *et al.*, 2021)
Virus	SARS-CoV-2 in human cells (Kula-Pacurar *et al.*, 2020; Milewska *et al.*, 2020), SARS-Cov2 lateral flow assay (Schulte *et al.*, 2023)
Wasp	whole-mount embryo (Taylor & Dearden, 2022), whole-mount ovaries (Inwood *et al.*, 2023)
Water flea	whole-mount embryos (Bruce & Patel, 2022; Bruce *et al.*, 2021)

Organism	Sample types*
Xenopus	whole-mount embryos (Leigh *et al.*, 2020; Naert *et al.*, 2021; Yoon *et al.*, 2021; Zhao *et al.*, 2022), whole-mount tadpole tails and limbs (Aztekin *et al.*, 2021; Trofka *et al.*, 2021)
Zebrafish	whole-mount embryos (Attardi *et al.*, 2018; Barker *et al.*, 2022; Britto *et al.*, 2022; Bruce *et al.*, 2021; Caviglia *et al.*, 2022; Cayuso *et al.*, 2019; Choi *et al.*, 2010, 2014; Edwards *et al.*, 2023; Fulton *et al.*, 2020; Gallagher *et al.*, 2017; Hageter *et al.*, 2021; Hason *et al.*, 2022; Howard *et al.*, 2021; Ibarra-García-Padilla *et al.*, 2021; Kappel *et al.*, 2022; Kinney *et al.*, 2020; Lencer *et al.*, 2021; Maili *et al.*, 2023; McLaren & Steventon, 2021; Meinecke *et al.*, 2018; Nandamuri *et al.*, 2022; Peloggia *et al.*, 2021; Pond *et al.*, 2021; Rocha *et al.*, 2021; Shah *et al.*, 2016; Sharma *et al.*, 2019; Tao *et al.*, 2021; Thomson *et al.*, 2021; Ton *et al.*, 2018; Trivedi *et al.*, 2018; Truong *et al.*, 2023; Tsai *et al.*, 2020; Wang *et al.*, 2019; Zhao *et al.*, 2022), whole-mount larvae (Basnakova *et al.*, 2021; Bhandiwad *et al.*, 2022; Callahan *et al.*, 2019; Choi *et al.*, 2016; Corradi *et al.*, 2022; Denans *et al.*, 2022; Farrell *et al.*, 2021; Howard *et al.*, 2021, 2022; Ibarra-García-Padilla *et al.*, 2021; Jimenez *et al.*, 2021; Kappel *et al.*, 2022; Lovett-Barron *et al.*, 2017; Martin *et al.*, 2022; Ogawa *et al.*, 2021; Parab *et al.*, 2023; Ruiz *et al.*, 2022; Shainer *et al.*, 2023; Shi *et al.*, 2023; Weinberger *et al.*, 2020; Whitesell *et al.*, 2019; Wurster *et al.*, 2021), embryo tissue sections (Tsai *et al.*, 2020), thin brain tissue sections (O'Brown *et al.*, 2019), FFPE thin heart tissue sections (Simões *et al.*, 2020), whole-mount embryo tails (Thomson *et al.*, 2021), whole-mount heart (Cao *et al.*, 2022; Xia *et al.*, 2022), thin heart tissue sections (Xia *et al.*, 2022), whole-mount ovary (Liu *et al.*, 2022), FFPE ovary tissue sections (Liu *et al.*, 2022)

*Tissue sections are classified as "thick" for thickness $\geq$ 50 µm and "thin" otherwise.

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