HCR™ Gold RNA-FISH/IF User Guide

This User Guide enables HCR™ Gold RNA-FISH/IF for multiplex, quantitative, high-resolution RNA and protein imaging in diverse organisms and sample types.

Contents

HCR™ Gold RNA-FISH/IF Kit Information

Ordering for Multiplex Experiment

- Order one HCR™ Gold RNA-FISH kit per target RNA
- Order one HCR™ Gold IF kit per target protein

Example 4-Plex Experiment (2 RNAs, 2 proteins):

- HCR™ Gold RNA-FISH kit for target RNA1:
  - HCR™ HiFi Probe: target RNA1 for use with amplifier X3
  - HCR™ HiFi Probe Hybridization Buffer (for use with all RNA-FISH kits)
  - HCR™ HiFi Probe Wash Buffer (for use with all RNA-FISH kits)
  - HCR™ Gold Amplifier: X3 with label 647
  - HCR™ Gold Amplifier Buffer (for use with all RNA-FISH and IF kits)
  - HCR™ Gold Amplifier Wash Buffer (for use with all RNA-FISH and IF kits)
- HCR™ Gold RNA-FISH kit for target RNA2:
  - HCR™ HiFi Probe: target RNA2 for use with amplifier X4
  - HCR™ Gold Amplifier: X4 with label 750
- HCR™ Gold IF kit for target Protein1:
  - 1° antibody: Rabbit Anti-Protein1 (your own 1° antibody)
  - HCR™ 2° Antibody Probe: Donkey Anti-Rabbit for use with amplifier X1
  - HCR™ Antibody Buffer (for use with all IF kits)
  - HCR™ Gold Amplifier: X1 with label 488
- HCR™ Gold IF kit for target Protein2:
  - 1° antibody: Mouse Anti-Protein2 (your own 1° antibody)
  - HCR™ 2° Antibody Probe: Donkey Anti-Mouse for use with amplifier X2
  - HCR™ Gold Amplifier: X2 with label 546

HCR™ Gold RNA-FISH and IF Storage Conditions and Shelf Life

Upon receiving your HCR™ Gold RNA-FISH and IF kits, please check storage conditions for each reagent. HCR™ reagents should be thawed and mixed before use. We recommend aliquoting the HCR™ HiFi Probes, HCR™ 2° Antibody Probes, and HCR™ Gold Amplifiers 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
HCR™ 2° Antibody Probe	−20	—	2	
HCR™ Antibody Buffer	4	—	1	
HCR™ Gold Amplifier	−20	Shielded from light	2	Comes in two separate tubes (h1 and h2)
HCR™ Gold Amplifier Buffer	4	—	1	
HCR™ Gold Amplifier Wash Buffer	4	—	1	Provided at 4×; dilute to 1× with UltraPure H_2O before use

User-Supplied Materials

Reagent†	Supplier	Comments
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
1° antibody	Any	1° antibody probes targeting proteins of interest. Each target must be detected using a 1° antibody raised in a different host species to enable subsequent detection by an anti-host HCR™ 2° Antibody Probe.
16% Formaldehyde (FA), Methanol-free	Polysciences	—
4% Formaldehyde (FA) Solution	—	See Common Recipes
ProLong™ Gold Antifade Mountant with DAPI	ThermoFisher	This is our recommended antifade mountant but any antifade mountant, with or without DAPI, is acceptable (e.g., Fluoromount-G).

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

HCR™ Gold RNA-FISH/IF Workflow

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

Antibody Binding

3. **Blocking:** Pre-condition the sample in HCR™ Antibody Buffer to block non-specific binding sites and minimize potential background signal. The buffer used in this step is identical to the antibody binding step, but it does not contain any 1° antibodies or HCR™ 2° Antibody Probes. Minimizing background is critical for maximizing signal-to-background.

4. **1° Antibody Binding:** Introduce 1° antibodies targeting the proteins of interest. Each target must be detected using a 1° antibody raised in a different host species to enable subsequent detection by an anti-host HCR™ 2° Antibody Probe.

5. **1° Antibody Washing:** Remove any unbound or non-specifically bound 1° antibodies through a series of washing steps.

6. **HCR™ 2° Antibody Probe Binding:** Introduce HCR™ 2° Antibody Probes targeting the 1° antibody host species to localize initiation sites at cognate targets.

7. **HCR™ 2° Antibody Probe Washing:** Remove any unbound or non-specifically bound probes through a series of washing steps to ensure they do not generate amplified background during amplification.

8. **Fixation:** Stabilize antibodies bound to target proteins before probe hybridization.

Probe Hybridization

9. **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.

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

11. **Probe Washing:** Remove any unbound or non-specifically bound probes through a series of washing steps to ensure they do not generate amplified background during amplification.

Amplification

12. **Pre-Amplification:** Equilibrate in HCR™ Gold Amplifier Buffer to facilitate diffusion of HCR™ Gold Amplifiers into the sample.

13. **Amplification:** Each HCR™ Gold Amplifier comprises two hairpins (h1 and h2) that are snap-cooled separately before use to ensure proper folding. Introduce HCR™ Gold Amplifiers that diffuse to the cognate target where the initiation sites trigger growth of tethered fluorescent amplification polymers.

14. **Amplifier Washing:** Remove any unbound HCR™ Gold Amplifiers from the sample through a series of washing steps to minimize background.

Counterstain and Imaging

15. **Counterstain (optional):** Apply a counterstain such as DAPI or Hoechst to visualize nuclei in the sample. Counterstaining provides morphological context to facilitate image analysis.

16. **Imaging:** Use an appropriate mounting solution to safeguard the integrity of the HCR™ Gold RNA-FISH/IF 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. Image using a fluorescent microscope (e.g., epifluorescence, confocal, light sheet) suitable for your sample type.

HCR™ Gold RNA-FISH/IF 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.

Sample Preparation

Samples should be prepared in the same manner as for traditional in situ hybridization and immunohistochemistry, up to either the 1° antibody binding step or the probe hybridization step, whichever occurs first. Then proceed with the protocol described below.

Antibody Binding

1. Pre-warm HCR™ Antibody Buffer to room temperature.
2. Block sample by applying 200 µL of HCR™ Antibody Buffer on top of sample. Incubate at room temperature for 1 h inside a humidified chamber.
3. Prepare working concentration of 1° antibodies in HCR™ Antibody Buffer. Prepare 50–100 µL per slide.
   NOTE: *Follow manufacturer's guidelines for 1° antibody working concentration.*
4. Drain slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.
5. Add 50–100 µL of the 1° antibody solution to each slide and incubate overnight (>12 h) at 4 °C in a humidified chamber.
   NOTE: *Use enough 1° antibody solution to fully cover sample.*
   NOTE: *Incubation may be optimized (e.g., 1–2 h at room temperature) depending on sample type and thickness.*
6. Remove excess antibodies by immersing slide in 1× PBST at room temperature for 3 × 5 min.
7. Prepare 1 µg/mL working concentration of HCR™ 2° Antibody Probe in HCR™ Antibody Buffer. Prepare 50–100 µL per slide.
   NOTE: *Concentration may be optimized depending on protein target and 1° antibody.*
8. Drain slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.
9. Add 50–100 µL of the HCR™ 2° Antibody Probe solution to each slide and incubate for 1 h at room temperature in a humidified chamber.
10. Remove excess antibodies by immersing slide in 1× PBST at room temperature for 3 × 5 min.
11. Drain slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.
12. Post-fix sample by adding 200 µL of 4% formaldehyde on the tissue.
    CAUTION: *Use formaldehyde with extreme care as it is a hazardous material.*
13. Incubate slides for 10 min at room temperature.
14. Immerse slides for 2 × 5 min in 1× PBST.

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. Drain slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.
3. 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.
4. Prepare probe solution by adding 2 µL of each HCR™ HiFi Probe to 100 µL of HCR™ HiFi Probe Hybridization Buffer at 37 °C.
5. Remove the pre-hybridization solution and drain excess buffer on slide by blotting edges on a Kimwipe.
6. 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).*
7. 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.*
8. Immerse slide in HCR™ HiFi Probe Wash Buffer at 37 °C to float off coverslip.
9. Remove excess probe by immersing 4 × 15 min in HCR™ HiFi Probe Wash Buffer at 37 °C. Dry slide by blotting edges on a Kimwipe.

Amplification

1. Pre-warm HCR™ Gold Amplifier Buffer to room temperature.
2. Pre-amplify by adding 200 µL of HCR™ Gold Amplifier Buffer on top of sample and incubating at room temperature for 30 min in a humidified chamber.
3. Separately prepare 2 µL of hairpin h1 and 2 µL of hairpin h2. Snap cool each hairpin separately by heating at 95 °C for 90 sec and cooling to room temperature in a dark drawer for 30 min.
4. Prepare amplifier solution by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 100 µL of HCR™ Gold Amplifier Buffer at room temperature.
5. Remove the pre-amplification solution and drain excess buffer on slide by blotting edges on a Kimwipe.
6. Add 50–100 µL of the amplifier solution on top of the sample.
   NOTE: *Use enough amplifier solution to fully cover sample.*
7. Place a coverslip on the sample and incubate at room temperature for >3 h in the humidified chamber in the dark.
   NOTE: *Increase incubation time for thicker samples (e.g., >12 h for thick brain slices).*

8. Pre-warm HCR™ Gold Amplifier Wash Buffer to room temperature.
   NOTE: *HCR™ Gold Amplifier Wash Buffer provided at 4×; dilute to 1× with UltraPure H_2O before use.*
9. Immerse slide in HCR™ Gold Amplifier Wash Buffer at room temperature to float off coverslip.
10. Remove excess amplifier by immersing 4 × 15 min in HCR™ Gold Amplifier Wash Buffer at room temperature in the dark. Dry slide by blotting edges on a Kimwipe.
11. 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.*
12. Store sample at 4 °C and shield from light prior to imaging.

HCR™ Gold RNA-FISH/IF 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 and immunofluorescence up to either the 1° antibody binding step or the probe hybridization step, whichever occurs first. Then proceed with the protocol described below.

Antibody Binding

1. Pre-warm HCR™ Antibody Buffer to room temperature.
2. Block samples with 500 µL of HCR™ Antibody Buffer for 4 h at 4 °C.
3. Prepare working concentration of 1° antibodies in HCR™ Antibody Buffer. Prepare 500 µL per sample.
   NOTE: *Follow manufacturer's guidelines for 1° antibody working concentration.*
4. Remove HCR™ Antibody Buffer and add 1° antibody solution to sample.
5. Incubate samples overnight at 4 °C with gentle rotation.
   NOTE: *Use enough 1° antibody solution to immerse sample.*
   NOTE: *Incubation may be optimized (e.g., 3 h at room temperature) depending on sample type and thickness.*
6. Remove excess antibodies by washing 4 × 30 min with 500 µL of 1× PBST at room temperature.
7. Prepare 1 µg/mL working concentration of HCR™ 2° Antibody Probe in HCR™ Antibody Buffer. Prepare 500 µL per sample.
   NOTE: *Concentration may be optimized depending on protein target and 1° antibody.*
8. Remove PBST and add HCR™ 2° Antibody Probe solution to samples.
9. Incubate samples for 3 h at room temperature with gentle rotation.
10. Remove excess antibodies by washing 5 × 5 min with 500 µL of 1× PBST at room temperature.
11. Remove 1× PBST and post-fix sample with 500 µL of 4% formaldehyde.
    CAUTION: *Use formaldehyde with extreme care as it is a hazardous material.*
12. Incubate for 10 min at room temperature.
13. Remove fixative and wash sample twice with 500 µL of 1× PBST.

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 1× PBST from samples.
3. 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.*
4. Prepare probe solution by adding 10 µL of each HCR™ HiFi Probe to 500 µL of HCR™ HiFi Probe Hybridization Buffer at 37 °C.
5. Remove the pre-hybridization solution and add the probe solution.
6. 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).*
7. 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.*
8. Remove excess probe by washing 4 × 15 min with 500 µL of HCR™ HiFi Probe Wash Buffer at 37 °C.

Amplification

1. Pre-warm HCR™ Gold Amplifier Buffer to room temperature.
2. Remove HCR™ HiFi Probe Wash Buffer from samples.
3. Pre-amplify samples in 500 µL of HCR™ Gold Amplifier Buffer for 30 min at room temperature.
   NOTE: *Use enough HCR™ Gold Amplifier Buffer to immerse sample.*
4. Separately prepare 10 µL of hairpin h1 and 10 µL of hairpin h2. Snap cool each hairpin separately by heating at 95 °C for 90 sec and cooling to room temperature in a dark drawer for 30 min.
5. Prepare amplifier solution by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 500 µL of HCR™ Gold Amplification Buffer at room temperature.
6. Remove the pre-amplification solution and add the amplifier solution.
7. Incubate for >3 h at room temperature in the dark.
   NOTE: *Use enough amplifier solution to immerse sample.*
   NOTE: *Increase incubation time for thicker samples (e.g., >12 h for whole-mount vertebrate embryos).*
8. Pre-warm HCR™ Gold Amplifier Wash Buffer to room temperature.
   NOTE: *HCR™ Gold Amplifier Wash Buffer provided at 4×; dilute to 1× with UltraPure H_2O before use.*
9. Remove excess amplifier by washing 4 × 15 min with 500 µL of HCR™ Gold Amplifier Wash Buffer at room temperature.
10. Store sample at 4 °C and shield from light prior to imaging.
11. 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
- 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. 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/IF method on your sample, you can start with the same preparation protocol and then perform HCR™ Gold RNA-FISH/IF using one of the protocols provided in previous sections.

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). Optimal antigen retrieval method may differ depending on the antigen/antibody used.*
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™ Gold RNA-FISH/IF 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™ Gold RNA-FISH/IF assay.

Common Recipes

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

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

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

Frequently Asked Questions (FAQ)

What comes in an HCR™ Gold RNA-FISH kit?

- HCR™ HiFi Probe
- HCR™ HiFi Probe Hybridization Buffer
- HCR™ HiFi Probe Wash Buffer
- HCR™ Gold Amplifier
- HCR™ Gold Amplifier Buffer
- HCR™ Gold Amplifier Wash Buffer

What comes in an HCR™ Gold IF kit?

- HCR™ 2° Antibody Probe
- HCR™ Antibody Buffer
- HCR™ Gold Amplifier
- HCR™ Gold Amplifier Buffer
- HCR™ Gold Amplifier Wash Buffer

Can I order a subset of these components?

- Yes. You can order kits containing any subset of the above components.

What HCR™ Gold Amplifiers are available for multiplexing?

- For multiplex experiments, choose a different HCR™ Gold Amplifier (X1, X2, . . . , X10) and label (405, 425, 488, 514, 546, 594, 633, 647, 700, 750, 800) for each target that will be imaged in the same sample (for example, amplifier X3-647 for target 1, amplifier X2-546 for target 2, . . .).
- We recommend labels (488, 546, 647, 750) for robust 4-plex bandpass imaging.
- We recommend labels (405, 425, 488, 514, 546, 594, 633, 700, 750, 800) for robust 10-plex spectral imaging.
- For flexibility, amplifiers X1, X2, and X3 are available with multiple label options (488, 546, 647), while for expanded multiplexing, amplifiers X4–X10 are each available with one label option.
- We recommend using a longer-wavelength label (e.g., 647 or 750) for targets that are more difficult to detect (e.g., low-expression target and/or short target sequence), as autofluorescence tends to be higher in shorter-wavelength channels (e.g., 405 or 488).

What do I order for a multiplex HCR™ Gold RNA-FISH/IF experiment?

- Order one HCR™ Gold RNA-FISH kit for each target RNA
- Order one HCR™ Gold IF kit for each target protein

- Example 4-Plex Experiment (2 RNAs, 2 proteins):
  - HCR™ Gold RNA-FISH kit for target RNA1:
    - HCR™ HiFi Probe: target RNA1 for use with amplifier X3
    - HCR™ HiFi Probe Hybridization Buffer (for use with all RNA-FISH kits)
    - HCR™ HiFi Probe Wash Buffer (for use with all RNA-FISH kits)
    - HCR™ Gold Amplifier: X3 with label 647
    - HCR™ Gold Amplifier Buffer (for use with all RNA-FISH and IF kits)
    - HCR™ Gold Amplifier Wash Buffer (for use with all RNA-FISH and IF kits)
  - HCR™ Gold RNA-FISH kit for target RNA2:
    - HCR™ HiFi Probe: target RNA2 for use with amplifier X4
    - HCR™ Gold Amplifier: X4 with label 750
  - HCR™ Gold IF kit for target Protein1:
    - 1° antibody: Rabbit Anti-Protein1 (your own 1° antibody)
    - HCR™ 2° Antibody Probe: Donkey Anti-Rabbit for use with amplifier X1
    - HCR™ Antibody Buffer (for use with all IF kits)
    - HCR™ Gold Amplifier: X1 with label 488
  - HCR™ Gold IF kit for target Protein2:
    - 1° antibody: Mouse Anti-Protein2 (your own 1° antibody)
    - HCR™ 2° Antibody Probe: Donkey Anti-Mouse for use with amplifier X2
    - HCR™ Gold Amplifier: X2 with label 546

Are HCR™ Gold Amplifiers interchangeable between HCR™ Gold RNA-FISH kits and HCR™ Gold IF kits?

- Yes! The HCR™ Gold Amplifiers (488, 546, 647) are interchangeable between HCR™ Gold RNA-FISH and HCR™ Gold IF kits. Likewise, HCR™ Gold Amplifier Buffer and HCR™ Gold Amplifier Wash Buffer are interchangeable between HCR™ Gold RNA-FISH and HCR™ Gold IF kits.

HCR™ Technology Citation Notes

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

- **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).
- **HCR™ RNA-FISH**
  - Third-generation HCR™ RNA-FISH (v3.0) enables multiplex, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) with automatic background suppression throughout the protocol for dramatically enhanced performance (signal-to-background, subcellular quantitative RNA imaging precision, single-molecule quantitative RNA imaging fidelity) and ease-of-use (no probe set optimization for new targets and organisms) (Choi et al., 2018).
  - Second-generation HCR™ RNA-FISH (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).
  - First-generation HCR™ RNA-FISH (v1.0) using RNA HCR™ Probes and RNA HCR™ Amplifiers: multiplex mRNA imaging in whole-mount vertebrate embryos with simultaneous signal amplification for up to 5 target mRNAs (Choi et al., 2010).
- **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).