HCR™ Gold IF/2°IF User Guide

This User Guide enables HCR™ Gold IF/2°IF for multiplex, quantitative, high-resolution protein imaging in diverse organisms and sample types. HCR™ Gold IF uses the HCR™ HiFi Encoder to robustly encode best-in-class quantitative signal amplification to your trusted 1° antibodies for same-species/isotype multiplexing in diverse organisms and sample types. Technical support: support@molecularinstruments.com

Contents

HCR™ Gold IF/2°IF Kit Information **3**
- HCR™ HiFi Encoder Highlights . . . 3
- Ordering for Multiplex Experiment . . . 4
- Example 4-Plex Experiment (2 encoded proteins, 2 unencoded proteins) . . . 4
- Safety Data Sheets (SDS) . . . 4
- Patents . . . 4
- HCR™ Gold IF/2°IF Storage Conditions and Shelf Life . . . 5
- User-Supplied Materials . . . 6

HCR™ Gold IF/2°IF Workflow **7**
- Sample Preparation . . . 7
- Encoding . . . 7
- 1° Antibody Binding . . . 7
- 2° Antibody Binding . . . 7
- Amplification . . . 7
- Counterstain and Imaging . . . 8

HCR™ Gold IF/2°IF Protocol: Sample on a Slide **9**
- Sample Preparation . . . 9
- Encoding and Preparation . . . 9
- 1° Antibody Binding . . . 10
- 2° Antibody Binding . . . 10
- Amplification . . . 11

HCR™ Gold IF/2°IF Protocol: Sample in Solution **12**
- Sample Preparation . . . 12
- Encoding and Preparation . . . 12
- 1° Antibody Binding . . . 13
- 2° Antibody Binding . . . 13
- Amplification . . . 13

Sample Preparation Protocols **15**
- FFPE Tissue Sections on a Slide . . . 16
- Fixed Frozen Tissue Sections on a Slide . . . 18
- Fresh Frozen Tissue Sections on a Slide . . . 20
- Whole-Mount Zebrafish Embryos and Larvae in Solution . . . 21

Whole-Mount Mouse Embryos in Solution 23
Whole-Mount Chicken Embryos in Solution 25
Mammalian Cells on a Chambered Slide 27

Example Encoding Protocol Calculations **28**
Sample in Solution for a 1-Plex Experiment 28
Sample on a Slide for a 3-Plex Experiment 28

Common Recipes **29**

Frequently Asked Questions (FAQ) **30**

HCR™ Technology Citation Notes **33**

HCR™ Gold IF/2°IF Kit Information

HCR™ HiFi Encoder Highlights

The HCR™ HiFi Encoder enables you to encode your favorite 1° antibodies for use with HCR™ Gold Amplifiers. Encoders are available for use with four host species/isotypes of primary antibodies (mouse IgG1, mouse IgG2a, rabbit IgG, and human IgG). Rabbit IgG and mouse IgG1 Encoders are available across X1-X10 HCR™ Gold Amplifiers, while mouse IgG2a and human IgG Encoders are available with a single X system: X2 for mouse IgG2a and X4 for human IgG. Coming soon, additional Encoders will be released to support more primary antibody host species/isotypes across more HCR™ Gold Amplifier systems.

Key Properties:

- **Robust Amplification Encoding:** Robustly encode your favorite primary antibodies for use with HCR™ Gold Amplifiers without affecting target-binding affinity.
- **Same-Species/Isotype Multiplexing:** Perform multiplex IF using multiple 1° antibodies from the same host species/isotype with no cross-reactivity. For example, perform N-plex HCR™ Gold IF using rabbit IgG primary antibodies for all N target proteins at the same time.
- **Best-In-Class Amplification:** Exploit 1-step quantitative HCR™ Gold signal amplification for protein imaging, leveraging the dynamic nanotechnology that has redefined the state-of-the-art for RNA imaging.
- **Compatibility with HCR™ Gold RNA-FISH:** Perform unified multiplex, quantitative, high-resolution RNA and protein imaging by combining HCR™ Gold RNA-FISH with HCR™ Gold IF.

Ordering for Multiplex Experiment

- Order one HCR™ Gold IF kit per encoded target protein (*same* host species/isotype for each target protein if desired)
- Order one HCR™ Gold 2°IF kit per unencoded target protein (each unencoded 1° antibody raised in a different host organism)

Example 4-Plex Experiment (2 encoded proteins, 2 unencoded proteins)

- HCR™ Gold IF kit for encoded target Protein1:
  - 1° antibody: Rabbit IgG Anti-Protein1 (your own 1° antibody)
  - HCR™ HiFi Encoder: Rabbit IgG for amplifier X3
  - HCR™ HiFi Antibody Buffer (for use with all kits)
  - HCR™ HiFi Antibody Wash Buffer (for use with all kits)
  - HCR™ Gold Amplifier: X3 with label 647
  - HCR™ Gold Amplifier Buffer (for use with all kits)
  - HCR™ Gold Amplifier Wash Buffer (for use with all kits)
- HCR™ Gold IF kit for encoded target Protein2:
  - 1° antibody: Rabbit IgG Anti-Protein2 (your own 1° antibody)
  - HCR™ HiFi Encoder: Rabbit IgG for amplifier X2
  - HCR™ Gold Amplifier: X2 with label 546
- HCR™ Gold 2°IF kit for unencoded target Protein3:
  - 1° antibody: Mouse Anti-Protein3 (your own 1° antibody raised in a different host organism from encoded Protein1 and Protein2)
  - HCR™ HiFi 2° Antibody Probe: Donkey Anti-Mouse for use with amplifier X1
  - HCR™ Gold Amplifier: X1 with label 488
- HCR™ Gold 2°IF kit for unencoded target Protein4:
  - 1° antibody: Rat Anti-Protein4 (your own 1° antibody raised in a different host organism from encoded Protein1 and Protein2 and unencoded Protein3)
  - HCR™ HiFi 2° Antibody Probe: Donkey Anti-Rat for use with amplifier X4
  - HCR™ Gold Amplifier: X4 with label 750

Safety Data Sheets (SDS)

- www.molecularinstruments.com/safety

Patents

- www.molecularinstruments.com/patents

HCR™ Gold IF/2°IF Storage Conditions and Shelf Life

Upon receiving your HCR™ Gold IF/2°IF kit, please check storage conditions for each reagent. HCR™ reagents should be thawed and mixed before use. We recommend aliquoting HCR™ HiFi Encoders, HCR™ HiFi 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	Comments
HCR™ HiFi Encoder A	−20	—	6 mo	Reactivity is specific to both the 1° antibody species/isotype (e.g., mouse IgG1 or rabbit IgG) and HCR™ Gold Amplifier (X1–X10)
HCR™ HiFi Encoder B	−20	—	6 mo	Reactivity is specific to both the 1° antibody species/isotype (e.g., mouse IgG1 or rabbit IgG) and HCR™ Gold Amplifier (X1–X10)
HCR™ HiFi 2° Antibody Probe	−20	—	2 yr	
HCR™ HiFi Antibody Buffer	4	—	1 yr	
HCR™ HiFi Antibody Wash Buffer	4	—	1 yr	
HCR™ Gold Amplifier†‡	−20	Shielded from light	2 yr	Comes in two separate tubes (h1 and h2)
HCR™ Gold Amplifier Buffer	4	—	1 yr	
HCR™ Gold Amplifier Wash Buffer	4	—	1 yr	Provided at 4×; dilute to 1× with UltraPure H_2O before use

†Use a matching HCR™ Gold Amplifier and HCR™ HiFi Encoder (X1–X10) for a given target.

‡Use a matching HCR™ Gold Amplifier and HCR™ HiFi 2° Antibody Probe (X1–X10) for a given target.

User-Supplied Materials

Reagent[†]	Supplier	Comments
Antibody blocking buffer	Any	Use standard antibody blocking buffer (to protect RNA targets, add RNase inhibitor if not RNase-free) or optionally substitute with HCR™ HiFi Antibody Buffer
1° antibody for use with the HCR™ HiFi Encoder	Any	One 1° antibody (must match the same species/isotype as your selected Encoder) per target protein
1° antibody for use with the HCR™ HiFi 2° Antibody Probe	Any	Each target must be detected using a 1° antibody raised in a different host species to enable subsequent detection by an anti-host HCR™ HiFi 2° Antibody Probe
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
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 IF/2°IF Workflow

Below is a general overview of the steps involved and their purposes in the HCR™ Gold IF/2°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.

Encoding

3. **Encoding:** Encode unmodified 1° antibodies to provide them with the ability to trigger HCR™ Gold amplification.

1° Antibody Binding

4. **Blocking:** Pre-condition the sample in standard antibody blocking buffer (or optionally use HCR™ HiFi Antibody Buffer) to block non-specific binding sites and minimize potential background signal. Minimizing background is critical for maximizing signal-to-background.
5. **1° Antibody Binding:** Introduce 1° antibodies targeting the proteins of interest. Each unencoded target must be detected using a 1° antibody raised in a different host species to enable subsequent detection by an anti-host HCR™ HiFi 2° Antibody Probe.
6. **1° Antibody Washing:** Remove any unbound or non-specifically bound 1° antibodies through a series of washing steps.

2° Antibody Binding

7. **HCR™ HiFi 2° Antibody Probe Binding:** Introduce HCR™ HiFi 2° Antibody Probes targeting the 1° antibody host species to localize initiation sites at cognate targets.
8. **HCR™ HiFi 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 in the next stage.

Amplification

9. **Pre-Amplification:** Equilibrate in HCR™ Gold Amplifier Buffer to facilitate diffusion of HCR™ Gold Amplifiers into the sample.
10. **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.
11. **Amplifier Washing:** Remove any unbound HCR™ Gold Amplifiers from the sample through a series of washing steps to minimize background.

Counterstain and Imaging

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

13. **Imaging:** Use an appropriate mounting solution to safeguard the integrity of the HCR™ Gold 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 IF/2°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 immunohistochemistry, up to the HCR™ HiFi Antibody Probe binding step. Then proceed with the protocol described below.

Encoding and Preparation

Before starting, calculate the total volume of HCR™ HiFi Antibody Probe solution required. As a general guideline, we recommend preparing 100 µL per sample on a slide. However, this volume may be adjusted to best suit your specific samples. Example encoding calculations based on this protocol can be found on page 28.

***Note**: We recommend using your primary antibody at a working concentration of ~1 µg/mL.*

1. Pre-warm HCR™ HiFi Antibody Buffer to room temperature.
2. Based on your normal dilution ratio, aliquot the required volume of stock 1° antibody into a clean tube without diluting.
3. Add 1 µL of HCR™ HiFi Encoder A for every 100 µL of desired final HCR™ HiFi Antibody Probe solution. Mix thoroughly by pipetting the full solution up and down 30 times. Incubate at room temperature for at least 5 min.
   NOTE: *If the total volume is less than 10 µL, add HCR™ HiFi Antibody Buffer to bring the final volume to 10 µL.*
4. Add 1 µL of HCR™ HiFi Encoder B for every 100 µL of desired final HCR™ HiFi Antibody Probe solution. Mix thoroughly by pipetting the full solution up and down 30 times. Incubate at room temperature for at least 5 min.
5. Dilute the encoded 1° antibody with HCR™ HiFi Antibody Buffer to reach the desired final volume of HCR™ HiFi Antibody Probe solution.
   NOTE: *For multiplexing, repeat Steps 1-3 for each 1° antibody. In Step 4, **add each encoded antibody directly** to the HCR™ HiFi Antibody Buffer.*
   NOTE: *We recommend encoding shortly before your experiment, ideally within 48 hours.*
6. Prepare working concentration of unencoded 1° antibodies.
   NOTE: *Follow manufacturer's guidelines for 1° antibody working concentration.*
7. Add the unencoded 1° antibodies directly to the HCR™ HiFi Antibody Probe solution to prepare the final 1° antibody cocktail. Prepare 100 µL per slide.
   NOTE: *When multiplexing HCR™ HiFi Encoded primaries with 2°IF secondaries, choose encoded primaries that are not recognized by the HiFi 2° Antibody Probe. For example, avoid pairing a mouse IgG1 encoded primary with a mouse primary detected by an anti-mouse HiFi 2° Antibody, as the anti-mouse secondary may also bind the encoded primary and cause cross-reactivity.*

1° Antibody Binding

Please ensure all reagents fully cover the sample(s) during each step.

1. Block sample by applying 200 µL of your standard antibody blocking buffer on top of sample. Incubate at room temperature for 1 h in a humidified chamber.
   NOTE: *Alternatively, use HCR™ HiFi Antibody Buffer for blocking.*
2. Drain slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.
3. Add 100 µL of 1° antibody cocktail solution to each slide. Place a coverslip on sample and incubate at room temperature for >3 h in a humidified chamber.
   NOTE: *If you have previous experience using these 1° antibodies, use your standard incubation conditions. Otherwise, follow supplier's recommended protocol.*
   NOTE: *Incubation may be optimized (e.g., >12 h at 4 °C) depending on sample type and thickness.*
4. Immerse slide in 1× PBST at room temperature to float off coverslip.
5. Remove excess antibodies by immersing slide in 1× PBST at room temperature for 3 × 5 min.
6. Drain slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.

2° Antibody Binding

Please ensure all reagents fully cover the sample(s) during each step.

1. Prepare HCR™ HiFi 2° Antibody Probe solution at a working concentration of 1 µg/mL (i.e., 0.2 µL of HCR™ HiFi 2° Antibody Probe per 100 µL of HCR™ HiFi Antibody Buffer). Prepare 100 µL per slide.
   NOTE: *Concentration may be optimized depending on protein target and 1° antibody.*
2. Add 100 µL of the HCR™ HiFi 2° Antibody Probe solution to each slide. Place a coverslip on sample and incubate at 37 °C for 1 h in a humidified chamber.
3. Immerse slide in 1× PBST at room temperature to float off coverslip.
4. Remove excess antibodies by immersing slide in 1× PBST at room temperature for 3 × 15 min.
5. Drain slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.
6. Pre-warm HCR™ HiFi Antibody Wash Buffer to room temperature.
7. Add 200 µL of HCR™ HiFi Antibody Wash Buffer on top of sample and incubate at room temperature for 30 min. Drain slide by blotting edges on a Kimwipe. Repeat this step once.
   NOTE: *Do not substitute other wash buffers or in-house formulations in place of HCR™ HiFi Antibody Wash Buffer. Alternative wash buffers are not compatible with the HCR™ Gold IF/2°IF workflow and will compromise assay performance.*
8. Immerse slide in 1× PBST at room temperature for 3 × 5 min.
9. Drain slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.

Amplification

Please ensure all reagents fully cover the sample(s) during each step.

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 100 µL of the HCR™ Gold Amplifier solution on top of the 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 IF/2°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 immunohistochemistry, up to the HCR™ HiFi Antibody Probe binding step. Then proceed with the protocol described below.

Encoding and Preparation

Before starting, calculate the total volume of HCR™ HiFi Antibody Probe solution required. As a general guideline, we recommend preparing 200 µL per sample in solution. However, this volume may be adjusted to best suit your specific samples. Example encoding calculations based on this protocol can be found on page 28.

***Note**: We recommend using your primary antibody at a working concentration of ~1 µg/mL.*

1. Pre-warm HCR™ HiFi Antibody Buffer to room temperature.
2. Based on your normal dilution ratio, aliquot the required volume of stock 1° antibody into a clean tube without diluting.
3. Add 1 µL of HCR™ HiFi Encoder A for every 100 µL of desired final HCR™ HiFi Antibody Probe solution. Mix thoroughly by pipetting the full solution up and down 30 times. Incubate at room temperature for at least 5 min.
   NOTE: *If the total volume is less than 10 µL, add HCR™ HiFi Antibody Buffer to bring the final volume to 10 µL.*
4. Add 1 µL of HCR™ HiFi Encoder B for every 100 µL of desired final HCR™ HiFi Antibody Probe solution. Mix thoroughly by pipetting the full solution up and down 30 times. Incubate at room temperature for at least 5 min.
5. Dilute the encoded 1° antibody with HCR™ HiFi Antibody Buffer to reach the desired final volume of HCR™ HiFi Antibody Probe solution.
   NOTE: *For multiplexing, repeat Steps 1-3 for each 1° antibody. In Step 4, **add each encoded antibody directly** to the HCR™ HiFi Antibody Buffer.*
   NOTE: *We recommend encoding shortly before your experiment, ideally within 48 hours.*
6. Prepare working concentration of unencoded 1° antibodies.
   NOTE: *Follow manufacturer's guidelines for 1° antibody working concentration.*
7. Add the unencoded 1° antibodies directly to the HCR™ HiFi Antibody Probe solution to prepare the final 1° antibody cocktail. Prepare 200 µL per sample.
   NOTE: *When multiplexing HCR™ HiFi Encoded primaries with 2°IF secondaries, choose encoded primaries that are not recognized by the HiFi 2° Antibody Probe. For example, avoid pairing a mouse IgG1 encoded primary with a mouse primary detected by an anti-mouse HiFi 2° Antibody, as the anti-mouse secondary may also bind the encoded primary and cause cross-reactivity.*

1° Antibody Binding

Please ensure all reagents fully immerse the sample(s) during each step.

1. Block samples with 200 µL of your standard antibody blocking buffer for 2 h at 4 °C.
   NOTE: *Alternatively, use HCR™ HiFi Antibody Buffer for blocking.*
2. Remove antibody blocking buffer and add 200 µL of 1° antibody cocktail solution to sample. Incubate samples for >3 h at room temperature.
   NOTE: *If you have previous experience using these 1° antibodies, use your standard incubation conditions. Otherwise, follow the supplier's recommended protocol.*
   NOTE: *Incubation may be optimized (e.g., >12 h at 4 °C with gentle rotation) depending on sample type and thickness.*
3. Remove 1° antibody cocktail solution.
4. Remove excess antibodies by washing 4 × 15 min with 500 µL of 1× PBST at room temperature.

2° Antibody Binding

Please ensure all reagents fully immerse the sample(s) during each step.

1. Prepare HCR™ HiFi 2° Antibody Probe solution at a working concentration of 1 µg/mL (i.e., 0.2 µL of HCR™ HiFi 2° Antibody Probe per 100 µL of HCR™ HiFi Antibody Buffer). Prepare 200 µL per sample.
   NOTE: *Concentration may be optimized depending on protein target and 1° antibody.*
2. Remove 1× PBST and add HCR™ HiFi 2° Antibody Probe solution to samples.
3. Incubate samples for 3 h at room temperature with gentle rotation.
4. Remove the HCR™ HiFi 2° Antibody Probe solution.
5. Remove excess antibodies by washing 4 × 15 min with 500 µL of 1× PBST at room temperature.
6. Pre-warm HCR™ HiFi Antibody Wash Buffer to room temperature.
7. Remove 1× PBST and wash sample 2 × 30 min with 500 µL of HCR™ HiFi Antibody Wash Buffer at room temperature.
   NOTE: *Do not substitute other wash buffers or in-house formulations in place of HCR™ HiFi Antibody Wash Buffer. Alternative wash buffers are not compatible with the HCR™ Gold IF/2°IF workflow and will compromise assay performance.*
8. Remove HCR™ HiFi Antibody Wash Buffer and wash sample 3 × 5 min with 500 µL of 1 × PBST at room temperature.

Amplification

Please ensure all reagents fully immerse the sample(s) during each step.

1. Pre-warm HCR™ Gold Amplifier Buffer to room temperature.
2. Remove 1× PBST and pre-amplify samples in 200 µL of HCR™ Gold Amplifier Buffer for 30 min at room temperature.

3. Separately prepare 4 µL of hairpin h1 and 4 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 200 µL of HCR™ Gold Amplification Buffer at room temperature.
5. Remove the pre-amplification solution and add the HCR™ Gold Amplifier solution.
6. Incubate for >3 h at room temperature in the dark.
   NOTE: *Increase incubation time for thicker samples (e.g., >12 h for whole-mount vertebrate embryos).*
7. 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.*
8. Remove excess amplifier by washing 4 × 15 min with 500 µL of HCR™ Gold Amplifier Wash Buffer at room temperature.
9. Store sample at 4 °C and shield from light prior to imaging.
10. 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
- Fixed Frozen Tissue Sections on a Slide
- Fresh 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. If you do not find your sample type listed, please reach out to our support team for guidance. If you have previously used another IF method on your sample, you can start with the same preparation protocol and then perform HCR™ Gold IF/2°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 IF/2°IF assay.

Sample Preparation: 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	—
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. 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. 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).*
9. Maintain Tris-EDTA buffer temperature at 95 °C on a hot plate.
10. Immerse slides for 5 min.
11. 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.
12. Immerse slides in 400 mL of UltraPure H_2O in a separate container for 10 min at room temperature.
13. Immerse slides in 1× PBST for 2 × 2 min at room temperature.
14. Dry slide using a Kimwipe. Avoid touching the tissue.

15. Draw a barrier around the tissue using a hydrophobic pen.
16. Wash slides by immersing in 1× PBST.
17. Repeat with fresh 1× PBST.
18. Proceed to HCR™ Gold IF/2°IF assay.

Sample Preparation: Fresh 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™ Gold IF/2°IF 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 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	—
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™ Gold IF/2°IF 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™ Gold IF/2°IF 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™ Gold IF/2°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 IF/2°IF assay.

Example Encoding Protocol Calculations

Sample in Solution for a 1-Plex Experiment

As previously mentioned, our general guideline is to prepare 200 µL of HCR™ HiFi Antibody Probe solution per sample in solution. In this example, we will stain two tubes of whole-mount embryos using an unmodified rabbit IgG primary antibody (optimal working dilution: 1:200) with the X1 HCR™ Gold Amplifier. Since each tube requires 200 µL, the total volume of HCR™ HiFi Antibody Probe solution needed is 400 µL.

1. Aliquot 2 µL of stock rabbit IgG primary antibody into a clean tube.
2. Add 4 µL of HCR™ HiFi Encoder A (rabbit IgG-X1). Because the total volume is 6 µL, add 4 µL of HCR™ HiFi Antibody Buffer to bring the volume to 10 µL. Mix thoroughly by pipetting the full solution up and down 30 times. Incubate at room temperature for $>$ 5 min.
3. Add 4 µL of HCR™ HiFi Encoder B (rabbit IgG-X1). Mix thoroughly by pipetting the full solution up and down 30 times. Incubate at room temperature for $>$ 5 min.
4. Add the encoded primary antibody to 386 µL of HCR™ HiFi Antibody Buffer to reach a final volume of 400 µL HCR™ HiFi Antibody Probe solution.

Sample on a Slide for a 3-Plex Experiment

As previously mentioned, our general guideline is to prepare 100 µL of HCR™ HiFi Antibody Probe solution per sample on a slide. In this example, we will stain 10 slides so the total volume of HCR™ HiFi Antibody Probe solution needed is 1 mL. This multiplex experiment uses three encoded primary antibodies with the following optimal working dilutions: 1:100 for Antibody 1 (rabbit IgG with amplifier X1), 1:500 for Antibody 2 (mouse IgG1 with amplifier X2), and 1:1000 for Antibody 3 (mouse IgG1 with amplifier X3).

1. Aliquot 10 µL of Antibody 1, 2 µL of Antibody 2, and 1 µL of Antibody 3 into three separate clean tubes.
2. Add 10 µL of the appropriate HCR™ HiFi Encoder A (rabbit IgG-X1, mouse IgG1-X2, mouse IgG1-X3) to each tube. Mix thoroughly by pipetting the full solution up and down 30 times. Incubate at room temperature for $>$ 5 min.
3. Add 10 µL of the appropriate HCR™ HiFi Encoder B (rabbit IgG-X1, mouse IgG1-X2, mouse IgG1-X3) to each tube. Mix thoroughly by pipetting the full solution up and down 30 times. Incubate at room temperature for $>$ 5 min.
4. Add all 3 encoded primaries to 927 µL of HCR™ HiFi Antibody Buffer to reach a final volume of 1 mL HCR™ HiFi Antibody Probe solution.

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

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

Frequently Asked Questions (FAQ)

Can I multiplex with same-species/isotype primaries?

- Yes! With the HCR™ HiFi Encoder, same-species/isotype primary antibodies can be multiplexed in parallel without cross-reactivity. Each encoded antibody is uniquely paired with its corresponding HCR™ Gold Amplifier system (X1–X10), allowing multiple same-species primaries to be used simultaneously in the same sample.

Are HCR™ Gold Amplifiers interchangeable between HCR™ Gold IF kits and HCR™ Gold 2°IF kits?

- Yes! The HCR™ Gold Amplifiers (X1–X10) are interchangeable between HCR™ Gold IF and HCR™ Gold 2° kits. Likewise, HCR™ Gold Amplifier Buffer and HCR™ Gold Amplifier Wash Buffer are interchangeable between HCR™ Gold IF and HCR™ Gold 2°IF kits.

Do carriers or amine-containing buffers interfere with HCR™ HiFi Encoding?

- Encoding is compatible with common carriers and buffers, including BSA, glycerol, Tris buffer, and preservatives such as sodium azide.

Can encoded antibodies be stored after labeling?

- Encoded antibodies are best used soon after preparation. We recommend encoding shortly before your experiment, ideally within 48 hours. However, because the stability of different primary antibodies can vary, you may wish to assess storage conditions empirically for your antibodies.

What comes in an HCR™ Gold IF kit?

- HCR™ HiFi Encoder
- HCR™ HiFi Antibody Buffer
- HCR™ HiFi Antibody Wash Buffer
- HCR™ Gold Amplifier
- HCR™ Gold Amplifier Buffer
- HCR™ Gold Amplifier Wash Buffer

What comes in an HCR™ Gold 2°IF kit?

- HCR™ HiFi 2° Antibody Probe
- HCR™ HiFi Antibody Buffer
- HCR™ HiFi Antibody Wash 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–X10 are available with multiple label options (488, 546, 594, and 647). For expanded multiplexing, select amplifiers also offer an additional unique 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 IF/2°IF experiment?

- Use your own trusted 1° antibodies (*same* host species/isotype for each target protein, if desired; e.g., multiplex experiment using Rabbit IgG 1° antibodies for all encoded target proteins)
- Use your own trusted 1° antibodies for each unencoded target protein (each unencoded 1° antibody raised in a different host organism)
- Order one HCR™ Gold IF kit for each encoded target protein
- Order one HCR™ Gold 2°IF kit for each unencoded target protein
- Example 4-plex experiment (2 encoded proteins, 2 unencoded proteins):
    - HCR™ Gold IF kit for encoded target Protein1:
        - 1° antibody: Rabbit IgG Anti-Protein1 (your own 1° antibody)
        - HCR™ HiFi Encoder: Rabbit IgG for amplifier X3
        - HCR™ HiFi Antibody Buffer (for use with all kits)
        - HCR™ HiFi Antibody Wash Buffer (for use with all kits)
        - HCR™ Gold Amplifier: X3 with label 647
        - HCR™ Gold Amplifier Buffer (for use with all kits)
        - HCR™ Gold Amplifier Wash Buffer (for use with all kits)

- HCR™ Gold IF kit for encoded target Protein2:
  - 1° antibody: Rabbit IgG Anti-Protein2 (your own 1° antibody)
  - HCR™ HiFi Encoder: Rabbit IgG for amplifier X2
  - HCR™ Gold Amplifier: X2 with label 546
- HCR™ Gold 2°IF kit for unencoded target Protein3:
  - 1° antibody: Mouse Anti-Protein3 (your own 1° antibody raised in a different host organism from encoded Protein1 and Protein2)
  - HCR™ HiFi 2° Antibody Probe: Donkey Anti-Mouse for use with amplifier X1
  - HCR™ Gold Amplifier: X1 with label 488
- HCR™ Gold 2°IF kit for unencoded target Protein4:
  - 1° antibody: Rat Anti-Protein4 (your own 1° antibody raised in a different host organism from encoded Protein1 and Protein2 and unencoded Protein3)
  - HCR™ HiFi 2° Antibody Probe: Donkey Anti-Rat for use with amplifier X4
  - HCR™ Gold Amplifier: X4 with label 750

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).