HCR™ Gold IF User Guide

This User Guide enables HCR™ Gold IF for multiplex, quantitative, high-resolution protein imaging in diverse organisms and sample types. Technical support: support@molecularinstruments.com

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HCR™ Gold IF Kit Information

Ordering for Multiplex Experiment

- Order one HCR™ Gold IF kit per target protein

Example 2-Plex Experiment

- 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 X3
  - HCR™ Antibody 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 target Protein2
  - 1° antibody: Mouse Anti-Protein2 (your own 1° antibody)
  - HCR™ 2° Antibody Probe: Donkey Anti-Mouse for use with amplifier X1
  - HCR™ Gold Amplifier: X1 with label 488

Safety Data Sheets (SDS)

- www.molecularinstruments.com/safety

Patents

- www.molecularinstruments.com/patents

HCR™ Gold IF Storage Conditions and Shelf Life

Upon receiving your HCR™ Gold IF kit, please check storage conditions for each reagent. HCR™ reagents should be thawed and mixed before use. We recommend aliquoting the 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™ 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.
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 Workflow

Below is a general overview of the steps involved and their purposes in the HCR™ Gold 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 in the next stage.

Amplification

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

Counterstain and Imaging

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

12. **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 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 1° antibody binding step. 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.

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 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 1° antibody binding step. 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 (>12 h) 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.

Amplification

1. Pre-warm HCR™ Gold Amplifier Buffer to room temperature.
2. 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.*
3. 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.
4. 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.
5. Remove the pre-amplification solution and add the amplifier solution.

6. 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).*

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
- Whole-Mount Zebrafish Embryos and Larvae 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 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 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 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 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

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)

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 IF experiment?

- Use your own 1° antibody of choice for each target protein (each 1° antibody raised in a different host organism)
- Order one HCR™ Gold IF kit for each target protein:
  - HCR™ 2° Antibody Probe (for use with a different amplifier for each target)
  - HCR™ Antibody Buffer
  - HCR™ Gold Amplifier (for example, amplifier X1 with label 647 for target 1, amplifier X2 with label 546 for target 2, ...)
  - HCR™ Gold Amplifier Buffer
  - HCR™ Gold Amplifier Wash Buffer
  - Note: buffers only needed in one kit

- Example 3-plex experiment:
  - 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 X3
    - HCR™ Antibody 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 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 IF kit for target Protein3:
    - 1° antibody: Rat Anti-Protein3 (your own 1° antibody)
    - HCR™ 2° Antibody Probe: Donkey Anti-Rat for use with amplifier X1
    - HCR™ Gold Amplifier: X1 with label 488

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).