HCR™ Gold RNA Flow Cytometry User Guide

This User Guide enables HCR™ Gold RNA Flow Cytometry for multiplex, quantitative, high-throughput RNA expression profiling (or cell sorting) for mammalian cells and bacteria without the need to engineer reporter lines.

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HCR™ Gold RNA-FISH Storage Conditions and Shelf Life

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

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

HCR™ Gold RNA Flow Cytometry Workflow

Below is a general overview of the steps involved and their purposes in the HCR™ Gold RNA Flow Cytometry 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.

Probe Hybridization

3. **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.

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

5. **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

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

7. **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.

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

Flow Cytometry

9. **Flow Cytometry:** Prepare sample by resuspending cells in the desired buffer and volume. Filter sample to remove cellular debris before flow cytometry analysis (or cell sorting).

HCR™ Gold RNA Flow Cytometry Protocol

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 RNA flow cytometry up to the probe hybridization step. Then proceed with the protocol described below.

Probe Hybridization

1. Pre-heat the HCR™ HiFi Probe Hybridization Buffer to 37 °C.
   CAUTION: *HCR™ HiFi Probe Hybridization Buffer contains formamide, a hazardous material.*
2. Centrifuge cell sample for 5 minutes and remove supernatant.
   NOTE: *Centrifugation should be as gentle as possible to pellet cells. All centrifugation steps are done at 180 × g for mammalian cells and 4000 × g for bacteria.*
3. Wash cells with 500 µL of 1× PBST. Centrifuge for 5 min and remove supernatant. Repeat this step.
4. Resuspend the pellet with 400 µL of HCR™ HiFi Probe Hybridization Buffer and pre-hybridize for 30 min at 37 °C.
5. Prepare probe solution by adding 10 µL of each HCR™ HiFi Probe to 100 µL of HCR™ HiFi Probe Hybridization Buffer at 37 °C.
6. Add the probe solution directly to the sample to reach a final volume of 500 µL.
7. Incubate sample for >3 h at 37 °C.
   NOTE: *Incubation times can be adjusted for optimal results.*
8. 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.*
9. Add 1 mL of HCR™ HiFi Probe Wash Buffer to the sample to dilute the probes.
10. Centrifuge for 5 min and remove the wash solution.
11. Resuspend the cell pellet with 500 µL of HCR™ HiFi Probe Wash Buffer at 37 °C.
12. Incubate for 10 min at 37 °C and remove the wash solution by centrifugation for 5 min.
13. Repeat steps 11 and 12 two additional times.

Amplification

1. Pre-warm HCR™ Gold Amplifier Buffer to room temperature.
2. Resuspend the cell pellet with 400 µL of HCR™ Gold Amplifier Buffer and pre-amplify for 30 min at room temperature.
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 100 µL of HCR™ Gold Amplifier Buffer at room temperature.
5. Add the amplifier solution directly to the sample to reach a final volume of 500 µL.
6. Incubate for >3 h at room temperature in the dark.
   NOTE: *Incubation times can be adjusted for optimal results.*
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. Add 1 mL of HCR™ Gold Amplifier Wash Buffer to the sample to dilute the amplifiers.
9. Centrifuge for 5 min and remove the wash solution.
10. Resuspend the cell pellet with 500 µL of HCR™ Gold Amplifier Wash Buffer.
11. Without incubation, remove the wash solution by centrifugation for 5 min.
12. Repeat steps 10 and 11 five additional times.
13. Resuspend the cell pellet in the desired buffer and volume.
14. Store sample at 4 °C and shield from light prior to flow cytometry.
15. Filter cells before flow cytometry.
    NOTE: *Samples may also be mounted and imaged via microscopy.*

Sample Preparation Protocols

This section provides MI-validated sample preparation protocols for:

- Mammalian Cells in Suspension
- Bacteria in Suspension

Please note that these sample preparation protocols have not been validated for all mammalian and bacterial cell types and should only be used as a template. Please reach out to our support team if you have any questions. If you have previously used another flow cytometry method on your sample, you can use the same preparation protocol and then perform HCR™ Gold RNA Flow Cytometry using a protocol provided here.

Sample Preparation: Mammalian Cells in Suspension

User-Supplied Materials

Reagent†	Supplier	Comments
DPBS	Gibco	Avoid using DPBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.
0.25% Trypsin-EDTA	Any	—
UltraPure H_2O	Any	Type I water
16% Formaldehyde (FA), Methanol-free	Polysciences	—
4% Formaldehyde (FA) Solution	—	See Common Recipes
200-Proof Ethanol (EtOH)	Any	—
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

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

1. Aspirate growth media from culture plate and wash cells with DPBS.
2. Add 3 mL of trypsin per 10 cm plate and incubate in a 5% CO_2 incubator at 37 °C for 5 min.
3. Quench trypsin by adding 3 mL of growth media.
4. Transfer cells to a 15 mL conical tube and centrifuge for 5 min at 180 × g.
   NOTE: *Centrifugation should be as gentle as possible to pellet cells. All centrifugation steps are done at 180 × g.*
5. Aspirate supernatant and resuspend cells in 4% formaldehyde to reach approximately 10^6 cells/mL.
   CAUTION: *Use formaldehyde with extreme care as it is a hazardous material.*
6. Fix cells for at least 1 h at room temperature.
7. Centrifuge for 5 minutes and aspirate supernatant.
8. Wash cells 4 times with 1× PBST (use the same volume as formaldehyde solution). Pellet cells by centrifugation between washes.
9. Resuspend cells in ice-cold 70% ethanol (use the same volume as formaldehyde and PBST solutions).
10. Store cells at 4 °C overnight before use.
    NOTE: *Samples can be stored at -20 °C for up to a week. Longer storage time should be validated experimentally.*
11. Prior to RNA flow cytometry, transfer desired amount (0.5–1 × 10^6) of fixed cells into a 1.5 mL tube per sample.
12. Proceed to HCR™ Gold RNA Flow Cytometry assay.

Sample Preparation: Bacteria in Suspension

User-Supplied Materials

Reagent†	Supplier	Comments
LB Broth (Miller)	Any	—
UltraPure H_2O	Any	Type I water
16% Formaldehyde (FA), Methanol-free	Polysciences	—
4% Formaldehyde (FA) Solution	—	See Common Recipes
100% Methanol (MeOH)	Any	—
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

This protocol has been optimized for *E. coli* and should only be used as a template for other types of bacteria.

1. Grow *E. coli* from a streaked plate or a frozen glycerol stock in 2–3 mL of LB media overnight in a 37 °C shaker.
2. Dilute to make a 5 mL liquid culture with OD_{600} = 0.05.
3. Incubate in a 37 °C shaker until $OD_{600} \approx 0.5$ (exponential phase).
4. Aliquot 1 mL of cells and centrifuge for 10 min.
   NOTE: *Centrifugation should be as gentle as possible to pellet cells. All centrifugation steps are done at 4000 × g.*
5. Remove supernatant and resuspend cells in 750 µL of 1× PBS.
   NOTE: *Remove all solutions via pipetting throughout the protocol.*
6. Add 250 µL of 4% formaldehyde and incubate overnight at 4 °C.
   CAUTION: *Use formaldehyde with extreme care as it is a hazardous material.*
7. Centrifuge for 10 minutes and remove supernatant.
8. Resuspend cells in 150 µL of 1× PBS.
9. Add 850 µL of 100% MeOH and store cells at -20 °C before use.
   NOTE: *Samples can be stored at -20 °C for up to a week. Longer storage time should be validated experimentally.*
   NOTE: *Additional permeabilization (e.g., lysozyme) may be needed for gram-positive bacteria.*
10. Prior to RNA flow cytometry, transfer 150 µL of cells into a 1.5 mL Eppendorf tube for each sample.
11. Proceed to HCR™ Gold RNA Flow Cytometry assay.

Common Recipes

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

LB media
5 g of Novagen LB Broth Miller powder
Fill up to 200 mL with ultrapure H_2O
Autoclave at 121 °C for 20 min

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

Frequently Asked Questions (FAQ)

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

- We offer new users a complimentary HCR™ Gold RNA-FISH Starter Kit for performing 3-plex HCR™ Gold RNA-FISH. Please register for an account and fill out this questionnaire.

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

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, . . .).
- 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 experiment?

- Order one HCR™ Gold RNA-FISH kit for each target RNA:
  - HCR™ HiFi Probe (for use with a different amplifier for each target)
  - HCR™ HiFi Probe Hybridization Buffer
  - HCR™ HiFi Probe Wash Buffer
  - HCR™ Gold Amplifier (for example, 647 amplifier for target 1, 546 amplifier 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 RNA-FISH kit for target RNA1:
  - HCR™ HiFi Probe: for target RNA1 for use with amplifier X3
  - HCR™ HiFi Probe Hybridization Buffer (for use with all kits)
  - HCR™ HiFi Probe 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 RNA-FISH kit for target RNA2:
  - HCR™ HiFi Probe: for target RNA2 for use with amplifier X2
  - HCR™ Gold Amplifier: X2 with label 546
- HCR™ Gold RNA-FISH kit for target RNA3:
  - HCR™ HiFi Probe: for target RNA3 for use with amplifier X1
  - HCR™ Gold Amplifier: X1 with label 488

Can I combine HCR™ Gold RNA-FISH with traditional IF for simultaneous RNA and protein expression profiling?

- Yes, HCR™ Gold RNA-FISH is compatible with traditional immunofluorescence (IF). We recommend performing IF first followed by a 4% formaldehyde (FA) fixation before proceeding to HCR™ Gold RNA-FISH. All IF reagents should be RNase-free.

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).