HCR™ Pro RNA Flow Cytometry User Guide

This User Guide provides comprehensive instructions for performing the HCR™ Pro RNA Flow Cytometry assay. This assay leverages enzymatic signal amplification to enable extreme sensitivity RNA flow cytometry. Technical support: support@molecularinstruments.com

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Ordering for 1-Plex Experiment

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Example 1-Plex Experiment

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The HCR™ Pro RNA Flow Cytometry kit is used with an HRP fluorogenic substrate for RNA-FISH (e.g., any of a variety of recommended third-party fluorescent tyramides).

Safety Data Sheets (SDS)

- www.molecularinstruments.com/safety

Patents

- www.molecularinstruments.com/patents

HCR™ Pro RNA Flow Cytometry Storage Conditions and Shelf Life

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

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

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

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HCR™ Pro RNA Flow Cytometry Workflow

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

Pretreatment

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

Probe Hybridization

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

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

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

Amplification

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

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

9. **Catalytic Reporter Deposition:** Introduce an HRP fluorogenic substrate to perform RNA Flow Cytometry (e.g., any of a variety of recommended third-party fluorescent tyramides), leading to enzyme-mediated catalytic reporter deposition at the site of the target.

Flow Cytometry

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

HCR™ Pro 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.

Pretreatment

1. Pre-warm Pretreat to room temperature.
2. Pre-warm HCR™ HiFi Probe Wash Buffer to room temperature.
   NOTE: *HCR™ HiFi Probe Wash Buffer provided at 4×; dilute to 1× with UltraPure H_2O before use.*
3. Centrifuge cell sample for 5 min 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.*
4. Wash cells with 500 µL of 1× PBST. Centrifuge for 5 min and remove supernatant. Repeat this step.
5. Resuspend the cell pellet with 200 µL of Pretreat and incubate at room temperature for 10 min.
6. Centrifuge cell sample for 5 min and remove Pretreat.
7. Wash cells with 500 µL of HCR™ HiFi Probe Wash Buffer. Centrifuge for 5 min and remove supernatant. Repeat this step two additional times.

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. Resuspend the pellet with 100 µL of HCR™ HiFi Probe Hybridization Buffer and pre-hybridize for 30 min at 37 °C.
3. Prepare probe solution by adding 4 µL of each HCR™ HiFi Probe to 100 µL of HCR™ HiFi Probe Hybridization Buffer at 37 °C.
4. Add the probe solution directly to the sample to reach a final volume of 200 µL.
5. Incubate sample for >3 h at 37 °C.
   NOTE: *Incubation times can be adjusted for optimal results.*
6. Pre-heat HCR™ HiFi Probe Wash Buffer to 37 °C.
   NOTE: *HCR™ HiFi Probe Wash Buffer provided at 4×; dilute to 1× with UltraPure H_2O before use.*
7. Add 500 µL of HCR™ HiFi Probe Wash Buffer to the sample to dilute the probes.
8. Centrifuge for 5 min and remove the wash solution.
9. Resuspend the cell pellet with 500 µL of HCR™ HiFi Probe Wash Buffer at 37 °C.

10. Incubate for 10 min at 37 °C and remove the wash solution by centrifugation for 5 min.
11. Repeat steps 9 and 10 two additional times.

Amplification

HCR™ Pro Detect A

1. Pre-warm HCR™ Pro Detect A to room temperature.
2. Resuspend the cell pellet with 100 µL of HCR™ Pro Detect A.
3. Incubate sample for 30 min at room temperature.

HCR™ Pro Detect B

1. Pre-warm HCR™ Pro Detect B to room temperature.
2. Add 100 µL of HCR™ Pro Detect B directly to the sample to reach a final volume of 200 µL.
3. Incubate sample for >3 h at room temperature.
   NOTE: *Incubation times can be adjusted for optimal results.*
4. Pre-warm HCR™ Pro Amplifier Wash Buffer to room temperature.
   NOTE: *HCR™ Pro Amplifier Wash Buffer provided at 10×; dilute to 1× with UltraPure H_2O before use.*
5. Add 500 µL of HCR™ Pro Amplifier Wash Buffer to the sample to dilute the mixture.
6. Centrifuge for 5 min and remove the wash solution.
7. Resuspend the cell pellet with 500 µL of HCR™ Pro Amplifier Wash Buffer.
8. Without incubation, remove the wash solution by centrifugation for 5 min.
9. Repeat steps 7 and 8 three additional times.

HCR™ Pro Detect C

1. Pre-warm HCR™ Pro Detect C to room temperature.
2. Resuspend the cell pellet with 100 µL of HCR™ Pro Detect C.
3. Incubate sample for 15 min at room temperature.

HCR™ Pro Detect D

1. Pre-warm HCR™ Pro Detect D to room temperature.
2. Add 100 µL of HCR™ Pro Detect D directly to the sample to reach a final volume of 200 µL.
3. Incubate sample for 30 min at room temperature.
4. Pre-warm HCR™ Pro Amplifier Wash Buffer to room temperature.
   NOTE: *HCR™ Pro Amplifier Wash Buffer provided at 10×; dilute to 1× with UltraPure H_2O before use.*

5. Add 500 µL of HCR™ Pro Amplifier Wash Buffer to the sample to dilute the mixture.
6. Centrifuge for 5 min and remove the wash solution.
7. Resuspend the cell pellet with 500 µL of HCR™ Pro Amplifier Wash Buffer.
8. Without incubation, remove the wash solution by centrifugation for 5 min.
9. Repeat steps 7 and 8 two additional times.

Fluorescent Reporter Deposition

1. Prepare fluorescent tyramide solution following the manufacturer's instructions.
2. Add 200 µL of fluorescent tyramide solution.
3. Incubate according to the manufacturer's instructions.
4. Add 500 µL of HCR™ Pro Amplifier Wash Buffer to the sample to dilute the mixture.
   NOTE: *HCR™ Pro Amplifier Wash Buffer provided at 10×; dilute to 1× with UltraPure H_2O before use.*
5. Centrifuge for 5 min to remove the tyramide solution.
6. Resuspend the cell pellet with 500 µL of HCR™ Pro Amplifier Wash Buffer.
7. Without incubation, remove the wash solution by centrifugation for 5 min.
8. Repeat steps 6 and 7 two additional times.
9. Resuspend the cell pellet in the desired buffer and volume.
   NOTE: *If continuing with an HRP-based IF assay, add 100 µL of Post-Process solution to sample and incubate at 37 °C for 30 min in a humidified chamber. Following incubation, remove the Post-Process reagent by centrifugation for 5 min. You may stop the HCR™ Pro RNA-ISH assay at this point and proceed directly to your HRP-based IF workflow.*
10. Store sample at 4 °C and shield from light prior to flow cytometry.
11. 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™ Pro 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™ Pro 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™ Pro 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

Recommended Third-Party Fluorescent Tyramides

Fluorescent Tyramides	Incubation Time (min)	Recommended Starting Concentration	Supplier	Catalog Number
CF488A	8–24	5 µM	Biotium	92171
CF550R	8–24	5 µM	Biotium	96077
CF555	8–24	5 µM	Biotium	96021
CF583R	8–24	5 µM	Biotium	96085
CF594	8–24	5 µM	Biotium	92174
CF640R	8–24	5 µM	Biotium	92175
CF740	8–24	5 µM	Biotium	96124
Alexa Fluor 488 - Tyramide	8–24	1×	ThermoFisher	B40953
Alexa Fluor 546 - Tyramide	8–24	1×	ThermoFisher	B40954
Alexa Fluor 594 - Tyramide	8–24	1×	ThermoFisher	B40957
iFluor 488 Tyramide	8–24	1:600	AAT Bioquest	45100
iFluor 647 Tyramide	8–24	1:200	AAT Bioquest	45110

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).