HCR™ Gold Northern Blot User Guide

This User Guide enables multiplex HCR™ Gold Northern Blots for simultaneous quantification of RNA target size and abundance.

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HCR™ Gold RNA-FISH Kit Information

Ordering for Multiplex Experiment

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

Example 2-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™ 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 X1
  - HCR™ Gold Amplifier: X1 with label 488

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™ 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† / Equipment	Supplier	Comments
20× SSC	Any	—
UltraPure H_2O	Any	Type I water
Hybridization Bottle	VWR	—
Hybridization Oven	Thomas Scientific	—
UltraPure SDS Solution, 10%	Any	—
2× SSC, 0.1% SDS	—	See Common Recipes
0.2× SSC, 0.1% SDS	—	See Common Recipes

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

HCR™ Gold Northern Blot Workflow

Below is a general overview of the steps involved and their purposes in the HCR™ Gold Northern Blot assay.

Sample Preparation

1. **RNA Isolation:** Extract total RNA from cells or tissues using RNA purification techniques.
2. **RNA Denaturation & Gel Electrophoresis:** Heat the RNA sample in a denaturing reagent (e.g., formamide) to disrupt secondary structures. Run a denaturing agarose gel to separate RNA molecules by size.
3. **RNA Transfer:** Transfer RNA from the gel onto a membrane using capillary action or an electrophoretic transfer apparatus.
4. **Crosslinking:** Immobilize RNA on the membrane by crosslinking (heat, UV, or chemical fixation).

Probe Hybridization

5. **Pre-Hybridization:** Pre-condition the sample in HCR™ 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 in addition to signal amplification.
6. **Probe Hybridization:** Introduce HCR™ HiFi Probes complementary to the RNA targets of interest to localize initiation sites at cognate targets.
7. **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.

Scanning

11. Scan the HCR™ Gold Northern Blot using a fluorescent scanner.

HCR™ Gold Northern Blot Protocol

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

Sample Preparation

Samples should be prepared in the same manner as for traditional northern blot up to the probe hybridization step. Then proceed with the protocol described below.

Probe Hybridization

1. Let $\mathcal{V}$ denote the volume of buffer to be used for blots, calculated as:
   (a) 1 mL per 10 cm^2 of membrane
   (b) rounded up to the nearest 0.5 mL
   (c) no less than 1.5 mL

   For example, $\mathcal{V}$ = 3.5 mL for a membrane that is 32 cm^2 and $\mathcal{V}$ = 1.5 mL for a membrane that is 9 cm^2.
2. Pre-heat the HCR™ HiFi Probe Hybridization Buffer to 37 °C.
   CAUTION: *HCR™ HiFi Probe Hybridization Buffer contains formamide, a hazardous material.*
3. Pre-hybridize blot in ($\mathcal{V}$ – 0.5 mL) of HCR™ HiFi Probe Hybridization Buffer in a hybridization bottle for 30–60 min at 37 °C in a rolling hybridization incubator.
4. Prepare probe solution by adding for each mL of $\mathcal{V}$, 20 µL of each HCR™ HiFi Probe, to 500 µL of HCR™ HiFi Probe Hybridization Buffer at 37 °C.
5. Add the HCR™ HiFi Probe solution directly to the pre-hybridization solution and incubate blot overnight (>12 h) at 37 °C in a rolling hybridization incubator.
6. Remove excess probe by washing 4× using pre-heated 37 °C wash solutions (volume 2$\mathcal{V}$ per wash):
   (a) 2× low-stringency wash (2× SSC, 0.1% SDS; 5 min at 37 °C)
   (b) 2× high-stringency wash (0.2× SSC, 0.1% SDS; 15 min at 37 °C)

Amplification

1. Pre-heat HCR™ Gold Amplifier Buffer to 37 °C.
2. Pre-amplify blot in ($\mathcal{V}$ – 0.5 mL) of HCR™ Gold Amplifier Buffer in a hybridization bottle for 30–60 min at 37 °C in a rolling hybridization incubator.
3. Separately prepare hairpin h1 and 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). Snap cool 20 µL of hairpin h1 and 20 µL of hairpin h2 per mL of $\mathcal{V}$.
4. Prepare amplifier solution by adding all snap-cooled hairpins into a final volume of 0.5 mL HCR™ Gold Amplifier Buffer at 37 °C.

5. Add the amplifier solution directly to the pre-amplification solution and incubate blot for 4 h at 37 °C in the dark in a rolling hybridization incubator.
   NOTE: *Amplification time can be increased beyond 4 h if desired.*
   NOTE: *Amplification may alternatively be carried out at room temperature.*

6. Remove excess amplifier by washing in pre-heated HCR™ Gold Amplifier Wash Buffer (volume $2\mathcal{V}$ per wash): 2 × 15 min at 37 °C in the dark.
   NOTE: *If amplification is performed at room temperature, washes should also be performed at room temperature.*

Scanning

1. Scan the HCR™ Gold Northern Blot using a fluorescent scanner.

Sample Preparation Protocol: Northern Blot

This section provides an MI-validated sample preparation protocol for HCR™ Gold Northern Blots. Please note that this protocol has not been validated for all RNA sample variations and should only be used as a template. If you have previously used another northern blot method on your RNA sample, you can use the same preparation protocol and then perform the HCR™ Gold Northern Blot using the protocol provided above.

User-Supplied Materials

Reagent†	Supplier	Comments
NorthernMax™ Denaturing Gel Buffer (10×)	Thermo Fisher	—
NorthernMax™ Running Buffer (10×)	Thermo Fisher	—
UltraPure H_2O	Any	Type I water
Nylon Membrane, Positvely-Charged	Millipore Sigma	—
Whatman 3MM Chromatography Paper	Cytiva	—
Blotting Papers, Grade GB003	Cytiva	—
Formamide, Deionized	Any	—
20× SSC	Any	—
2× SSC	Any	See Common Recipes

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

Denaturing and Agarose Gel Electrophoresis

1. Prepare a 1% denaturing agarose gel.
   NOTE: *The gel should be ≤ 6 mm for efficient RNA transfer.*
   NOTE: *We recommend using NorthernMax™ Denaturing Gel Buffer for preparing denaturing agarose gels.*

2. Mix RNA samples in a 1:1 ratio with formamide and heat to 65 °C for 15 min prior to gel loading.
   CAUTION: *Use formamide with extreme care as it is a hazardous material.*

3. Run RNA samples (typically 1–20 µg) at 5V per cm in 1× NorthernMax™ Running Buffer until your targets of interest are well-resolved.
   NOTE: *The distance measured is the distance between the electrodes in the electrophoresis chamber.*
   NOTE: *For targets with low or unknown expression levels, we recommend starting with 10–20 µg of total RNA.*

RNA Transfer and Crosslinking

1. Wash the gel 4 × 5 min in UltraPure H_2O.

2. Wet membrane in UltraPure H_2O and equilibrate for 5 min in 20× SSC.
   NOTE: *A positively charged nylon membrane is recommended.*

3. Perform a capillary transfer of RNA onto the membrane in 20× SSC.
   NOTE: *We recommend using the TurboBlotter Rapid Downward Transfer System with chromatography and blotting papers suggested in the table above. Alternative transfer methods may be used.*

4. Wash the membrane for 5 min in 2× SSC.

5. Crosslink the RNA to the membrane by incubating at 80 °C for 2 h.
   NOTE: *Alternative RNA crosslinking methods may be used.*

6. Proceed to HCR™ Gold Northern Blot assay.

Common Recipes

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

2× SSC, 0.1% SDS
2× sodium chloride sodium citrate (SSC)
0.1% SDS

For 100 mL of solution
10 mL of 20× SSC
1 mL of 10% SDS
Fill up to 100 mL with UltraPure H_2O

0.2× SSC, 0.1% SDS
0.2× sodium chloride sodium citrate (SSC)
0.1% SDS

For 100 mL of solution
1 mL of 20× SSC
1 mL of 10% SDS
Fill up to 100 mL with UltraPure H_2O

Frequently Asked Questions (FAQ)

What comes in an HCR™ Gold RNA-FISH kit (for use performing HCR™ Gold Northern Blots)?

- HCR™ HiFi Probe
- HCR™ HiFi Probe Hybridization 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.
- 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.

What do I order for a multiplex HCR™ Gold Northern Blot experiment?

- Order one HCR™ Gold RNA-FISH kit for each target RNA (with the "Sample Type" set to "Northern Blot"):
  - HCR™ HiFi Probe (for use with a different amplifier for each target)
  - HCR™ HiFi Probe Hybridization Buffer
  - HCR™ Gold Amplifier (for example, amplifier X3 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 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™ 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

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).