# HCR™ northern blot (v3.0) protocol

**Technical Support**
support@molecularinstruments.com

**Safety Data Sheets (SDS)**
www.molecularinstruments.com/safety-v3

**Patents**
www.molecularinstruments.com/patents

**Ordering for Multiplex Experiment**
Order one HCR™ RNA-FISH (v3.0) kit per target RNA

**Example 2-Plex Experiment**

- HCR™ RNA-FISH (v3.0) kit for target mRNA1
  - HCR™ Probe (v3.0): target mRNA1 for use with amplifier B1
  - HCR™ Amplifier (v3.0): B1-647
  - HCR™ RNA-FISH Buffers (v3.0): HCR™ Probe Hybridization Buffer (v3.0), HCR™ Probe Wash Buffer (v3.0), HCR™ Amplifier Buffer (v3.0) (for use with all kits)
- HCR™ RNA-FISH (v3.0) kit for target mRNA2
  - HCR™ Probe (v3.0): target mRNA2 for use with amplifier B2
  - HCR™ Amplifier (v3.0): B2-488

**Storage conditions**

- Store HCR™ Probes (v3.0), HCR™ Amplifiers (v3.0), HCR™ Probe Hybridization Buffer (v3.0), and HCR™ Probe Wash Buffer (v3.0) at -20 °C.
- Store HCR™ Amplifier Buffer (v3.0) at 4 °C.
- On the bench top, keep stock solutions on ice.
- Make sure all solutions are well mixed before use.

## Denaturing 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: *formamide is a hazardous material.*

3. Run RNA samples (typically 1–20 $\mu$g) 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 $\mu$g 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. 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.*

## Multiplexed HCR™ northern blot (v3.0) protocol

### Detection stage

1. Let $V$ 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, $V = 3.5$ mL for a membrane that is 32 $cm^2$ and $V = 1.5$ mL for a membrane that is 9 $cm^2$.
2. Pre-heat HCR™ Probe Hybridization Buffer (v3.0) to 37 °C.
   CAUTION: *HCR™ Probe Hybridization Buffer (v3.0) contains formamide, a hazardous material.*
3. Pre-hybridize blot in $(V - 0.5$ mL$)$ of HCR™ Probe Hybridization Buffer (v3.0) in a hybridization bottle for 30–60 min at 37 °C in a rolling hybridization incubator.
4. Prepare probe solution by adding each HCR™ Probe (v3.0) into a total volume of 0.5 mL of HCR™ Probe Hybridization Buffer (v3.0) at 37 °C such that each probe will be at a final concentration of 5 nM in volume $V$.
5. Add the probe solution to the pre-hybridization solution and incubate blot overnight at 37 °C in a rolling hybridization incubator.
6. Remove excess probes by washing four times using pre-heated 37 °C wash solutions (volume $2V$ per wash) :
   (a) two low-stringency washes (2× SSC, 0.1% SDS; 5 min at 37 °C)
   (b) two high-stringency washes (0.2× SSC, 0.1% SDS; 15 min at 37 °C)

### Amplification stage

1. Pre-heat HCR™ Amplifier Buffer (v3.0) to 37 °C.
2. Pre-amplify blot in $(V - 0.5$ mL$)$ of HCR™ Amplifier Buffer (v3.0) in a hybridization bottle for 30–60 min at 37 °C in a rolling hybridization incubator.
3. Separately prepare hairpin h1 and hairpin h2 by snap cooling (heat at 95 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
   NOTE: *Hairpins h1 and h2 are provided in hairpin storage buffer ready for snap cooling. Snap cool an amount of each hairpin corresponding to 30 nM final concentration in a final volume $V$. h1 and h2 should be snap cooled in separate tubes.*
4. Prepare hairpin solution by adding all snap-cooled hairpins into a final volume of 0.5 mL HCR™ Amplifier Buffer (v3.0) at 37 °C.
5. Add the hairpin solution 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 hairpins by washing in pre-heated 5× SSCT (volume $2V$ per wash): 2 × 15 min at 37 °C in the dark.
   NOTE: *If amplification is performed at room temperature, hairpin washes should also be performed at room temperature.*

## Buffer recipes for sample preparation

**10% SDS**
10% SDS

For 100 mL of solution
10 g of SDS powder
Fill up to 100 mL with ultrapure $H_2O$
Heat solution to 68 °C to dissolve powder if necessary.

**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 $H_2O$

**5× SSCT**
5× sodium chloride sodium citrate (SSC)
0.1% Tween 20

For 40 mL of solution
10 mL of 20× SSC
400 $\mu$L of 10% Tween 20
Fill up to 40 mL with ultrapure $H_2O$

## Reagents and supplies

NorthernMax™ denaturing gel buffer (10X) (ThermoFisher Scientific, Cat. # AM8676)
NorthernMax™ (10X) running buffer (ThermoFisher Scientific, Cat. # AM8671)
Nylon membrane, positively-charged (Roche, Cat. # 11209272001)
3 mm Whatman chromatography paper (GE Healthcare Life Sciences, Cat. # 3030-6188)
Whatman TurboBlotter (Fisher Scientific, Cat. # 09-301-184)
Whatman gel blotting paper (GE Healthcare Life Sciences, Cat. # 10427805)
Whatman GB003 filter paper (GE Healthcare Life Sciences, Cat. # 10427812)
Hybridization bottle (Wheaton, Cat. # 805000 or 805021)
Rolling hybridization incubator (SciGene, Cat. # 1040-50-1)

## 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).