

## HCR northern blot protocol

### Technical support

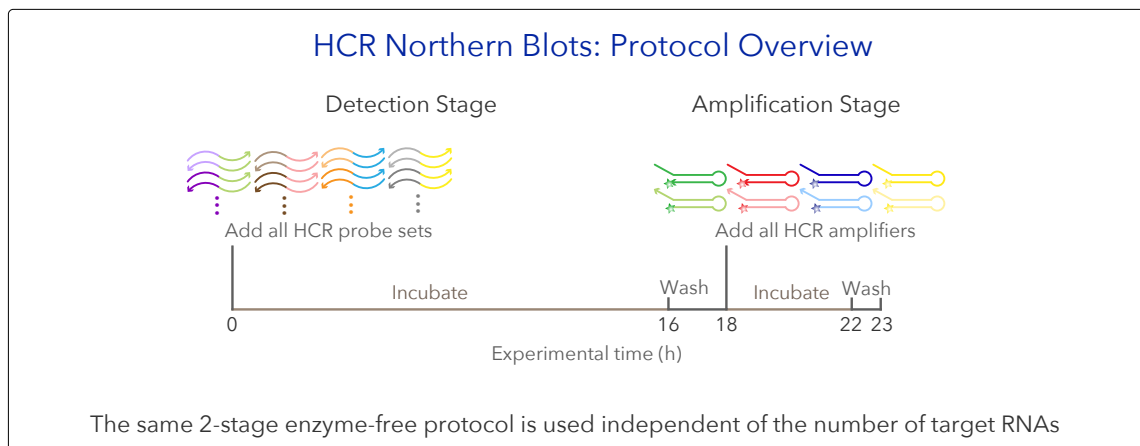
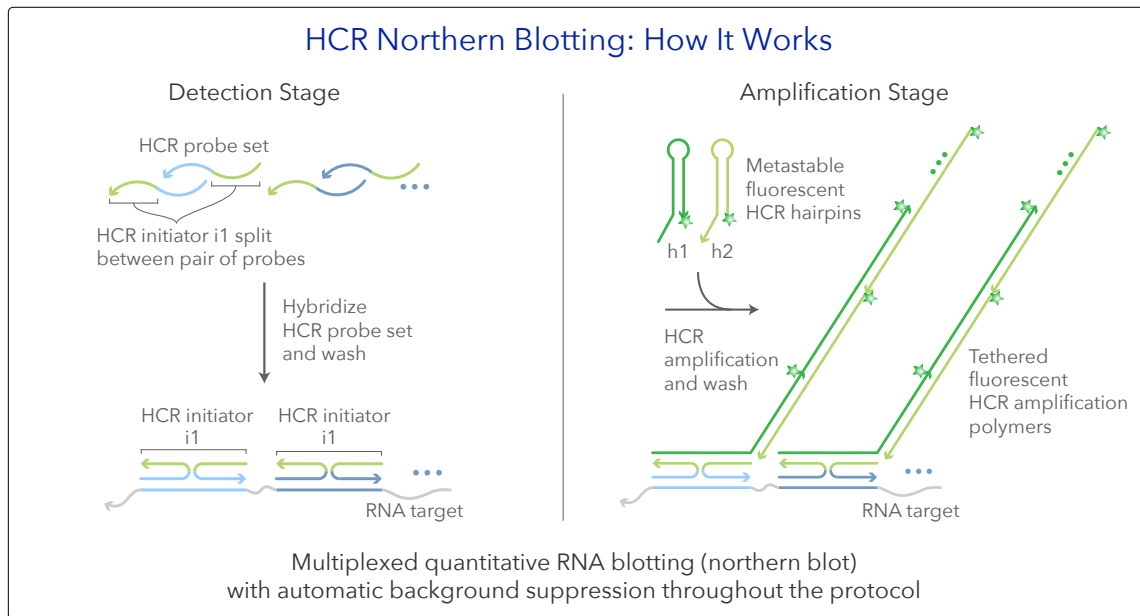
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## **HCR Northern Blots**

### *Multiplexed quantitative RNA blotting*

#### **Multiplexed Experiment**

- Order one HCR RNA-FISH bundle per target RNA

#### **Example 2-Plex Experiment**

- HCR bundle for target mRNA1
  - HCR split-initiator probe set: target mRNA1 for use with amplifier B1
  - HCR amplifier: B1-647
  - HCR RNA-FISH buffers: probe hybridization buffer, probe wash buffer, amplification buffer (for use with all bundles)
- HCR bundle for target mRNA2
  - HCR split-initiator probe set: target mRNA2 for use with amplifier B2
  - HCR amplifier: B2-488

#### **Storage conditions**

- Store HCR probe sets, HCR amplifiers, HCR probe hybridization buffer, and HCR probe wash buffer at -20 °C.
- Store HCR amplification buffer 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  $\leq 6$  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 $\times$  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  $\times$  5 min, in ultrapure H<sub>2</sub>O.
2. Wet membrane in ultrapure H<sub>2</sub>O and equilibrate for 5 min in 20 $\times$  SSC.  
*NOTE: A positively charged nylon membrane is recommended.*
3. Perform a capillary transfer of RNA onto the membrane in 20 $\times$  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 $\times$  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 protocol

### Detection stage

1. Let  $V$  denote the volume of buffer to be used for blots, calculated as:

- (a) 1 mL per 10 cm<sup>2</sup> 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<sup>2</sup> and  $V = 1.5$  mL for a membrane that is 9 cm<sup>2</sup>.

2. Pre-heat HCR probe hybridization buffer to 37 °C.

**CAUTION:** *HCR probe hybridization buffer contains formamide, a hazardous material.*

3. Pre-hybridize blot in ( $V - 0.5$  mL) of HCR 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 each probe set into a total volume of 0.5 mL of HCR probe hybridization buffer at 37 °C such that each probe set 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 ( $2 \times$  SSC, 0.1% SDS; 5 min at 37 °C)
- (b) two high-stringency washes ( $0.2 \times$  SSC, 0.1% SDS; 15 min at 37 °C)

### Amplification stage

1. Pre-heat HCR amplification buffer to 37 °C.

2. Pre-amplify blot in ( $V - 0.5$  mL) of HCR amplification 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 by snap cooling (heat at 95 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).

**NOTE:** *HCR 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 amplification buffer 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:** *HCR amplification may alternatively be carried out at room temperature.*

6. Remove excess hairpins by washing in pre-heated  $5 \times$  SSCT (volume  $2V$  per wash):  $2 \times 15$  min at 37 °C in the dark.

**NOTE:** *If HCR 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<sub>2</sub>O

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<sub>2</sub>O

### 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<sub>2</sub>O

### 5× SSCT

5× sodium chloride sodium citrate (SSC)

0.1% Tween 20

### For 40 mL of solution

10 mL of 20× SSC

400 μL of 10% Tween 20

Fill up to 40 mL with ultrapure H<sub>2</sub>O

## 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:

- **HCR IHC + HCR RNA-FISH**

[HCR IHC + HCR RNA-FISH](#) enables a unified approach to multiplexed, quantitative, high-resolution protein immunohistochemistry (IHC) and RNA fluorescence in situ hybridization (RNA-FISH), with quantitative 1-step enzyme-free HCR signal amplification performed for all protein and RNA targets simultaneously ([Schwarzkopf et al., 2021](#)).

- **HCR IHC**

[HCR IHC](#) enables multiplexed, quantitative, high-resolution protein immunohistochemistry (IHC) in highly autofluorescent samples (e.g., FFPE brain tissue sections) ([Schwarzkopf et al., 2021](#)).

- **HCR RNA-FISH (v3.0)**

Third-generation [HCR RNA-FISH \(v3.0\)](#) enables multiplexed, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) with [automatic background suppression throughout the protocol](#) for dramatically enhanced performance (signal-to-background, qHCR precision, dHCR fidelity) and ease-of-use (no probe set optimization for new targets and organisms) ([Choi et al., 2018](#)). Quantitative analysis modes:

- [qHCR RNA imaging](#): analog mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples.
- [dHCR RNA imaging](#): digital mRNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples.
- [qHCR RNA flow cytometry](#): analog mRNA relative quantitation for high-throughput expression profiling of mammalian cells and bacteria.

[Protocols for HCR RNA-FISH \(v3.0\)](#) in diverse organisms are adapted from the Zoo paper.

- **qHCR RNA imaging**

[qHCR RNA imaging](#) enables mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples (e.g., whole-mount vertebrate embryos). The [read-out/read-in analysis framework](#) enables bidirectional quantitative discovery in an anatomical context ([Trivedi et al., 2018](#)).

- **Zoo paper**

Protocols for multiplexed mRNA imaging in diverse sample types ([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

- **dHCR imaging**

[dHCR RNA imaging](#) enables 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](#)).

- **qHCR northern blots**

[qHCR northern blots](#) enable multiplexed quantification of RNA target size and abundance for up to 5 target RNAs ([Schwarzkopf & Pierce, 2016](#)).

- **HCR RNA-FISH (v2.0)**

Second-generation in situ HCR RNA-FISH technology (v2.0) using DNA HCR probes and DNA HCR probes and DNA HCR amplifiers: 10× increase in signal, 10× reduction in cost, dramatic increase in reagent durability ([Choi et al., 2014](#)).

- **HCR RNA-FISH (v1.0)**

First-generation HCR RNA-FISH technology (v1.0) using RNA HCR probes and RNA HCR amplifiers: multiplexed mRNA imaging in whole-mount vertebrate embryos with simultaneous signal amplification for up to 5 target mRNAs ([Choi et al., 2010](#)).

- **HCR mechanism**

The [hybridization chain reaction \(HCR\) mechanism](#) enables multiplexed, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings ([Dirks & Pierce, 2004](#)).



## HCR Technology References

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