Molecular Instruments
NORTHERN BLOT

HCR v3.0 multiplexed mRNA northern blot protocol

Technical support: support@molecularinstruments.com
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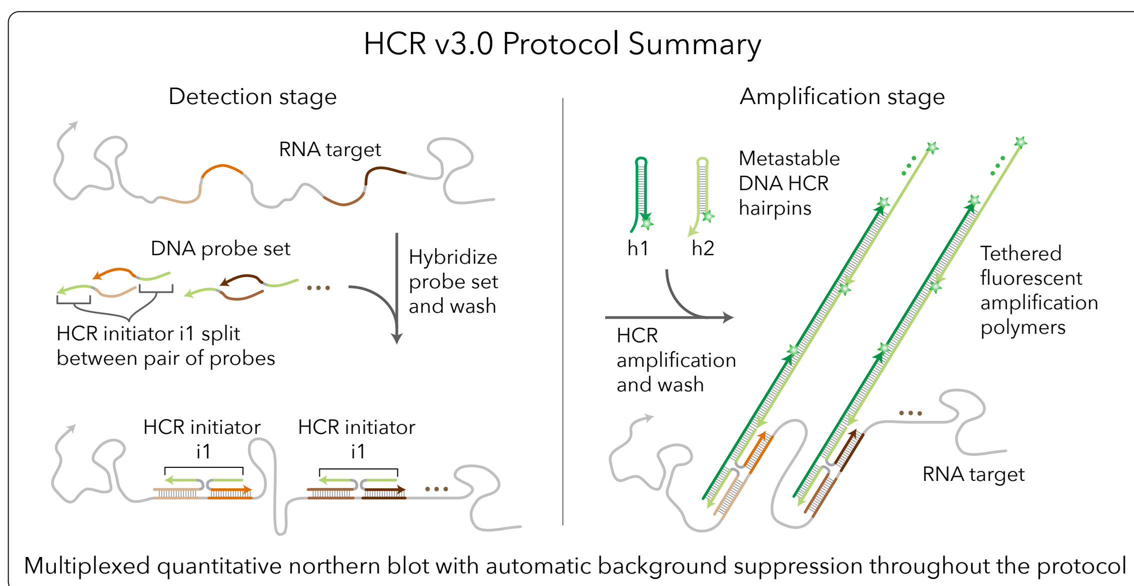
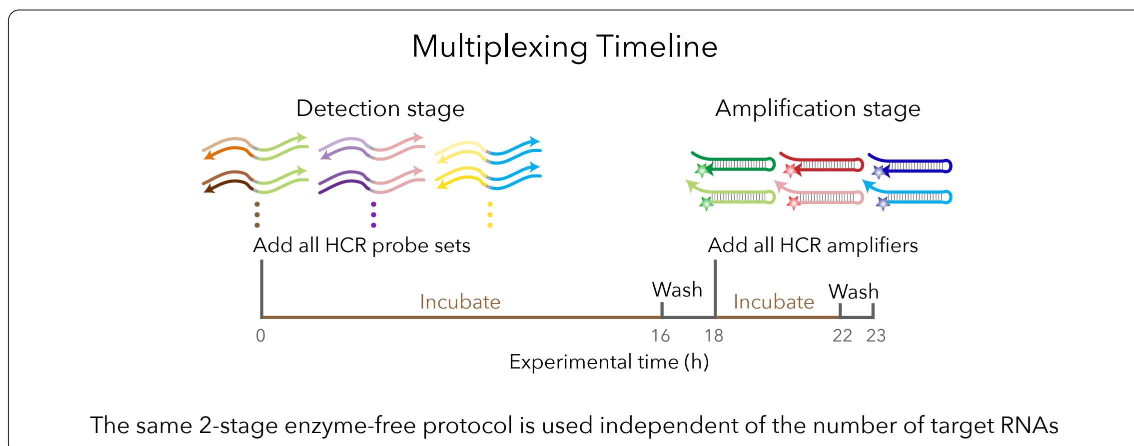
Storage conditions

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 ≤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 μg) at 55 V in 1× NorthernMax™ running buffer until your targets of interest are well-resolved.
   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. 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 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 to 60–68 °C for 1 h and maintain at 37 °C until use.
   CAUTION: *HCR probe hybridization buffer contains formamide, a hazardous material.*
3. Pre-hybridize blot in $(V - 0.5 \text{ mL})$ 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× 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 amplification buffer to 37 °C.
2. Pre-amplify blot in $(V - 0.5 \text{ mL})$ 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× SSCT (volume $2V$ per wash): 2 × 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

Buffer	Recipe
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 μL of 10% Tween 20 Fill up to 40 mL with ultrapure H_2O

Reagents and supplies

NorthernMax^{TM} denaturing gel buffer (10X) (ThermoFisher Scientific, Cat. # AM8676)
NorthernMax^{TM} (10X) running buffer (ThermoFisher Scientific, Cat. # AM8671)
Formamide (deionized) (Life Technologies, Cat. # AM9342)
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)
UltraPure Sodum Dodecyl Sulfate (SDS) (Life Technologies, Cat. # 15525-017)
20× SSC (sodium chloride sodium citrate; Invitrogen, Cat. # 15557-044)
50% Tween 20 (Invitrogen, Cat. # 00-3005)

Citation Notes

For citation, please select from the list below as appropriate for your application:

- **HCR v3.0**
  Automatic background suppression 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 imaging: analog mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples.

  qHCR flow cytometry: analog mRNA relative quantitation for high-throughput expression profiling of mammalian and bacterial cells.

  dHCR imaging: digital mRNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples.

  Protocols for v3.0 in diverse organisms are adapted from the Zoo paper.

- **qHCR imaging**
  mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos; read-out/read-in analysis framwork (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 suspension	generic sample on slide
whole-mount chicken embryos	whole-mount fruit fly embryos
whole-mount mouse embryos	whole-mount sea urchin embryos
whole-mount worm larvae	whole-mount zebrafish embryos and larvae

- **dHCR imaging**
  Single-molecule mRNA imaging in thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah *et al.*, 2016).

- **qHCR northern blot**
  Simultaneous quantification of RNA target size and abundance for up to 5 target RNAs (Schwarzkopf & Pierce, 2016).

- **HCR v2.0**
  2nd generation in situ HCR technology (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).

- **HCR v1.0**
  1st generation in situ HCR 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**
  Isothermal enzyme-free molecular signal amplification (Dirks & Pierce, 2004).

References

Choi, H. M. T., Chang, J. Y., Trinh, L. A., Padilla, J. E., Fraser, S. E., & Pierce, N. A. (2010). Programmable in situ amplification for multiplexed imaging of mRNA expression. *Nat Biotechnol*, **28**(11), 1208–12.

Choi, H. M. T., Beck, V. A., & Pierce, N. A. (2014). Next-generation in situ hybridization chain reaction: higher gain, lower cost, greater durability. *ACS Nano*, **8**(5), 4284–4294.

Choi, H. M. T., Calvert, C. R., Husain, N., Huss, D., Barsi, J. C., Deverman, B. E., Hunter, R. C., Kato, M., Lee, S. M., Abelin, A. C. T., Rosenthal, A. Z., Akbari, O. S., Li, Y., Hay, B. A., Sternberg, P. W., Patterson, P. H., Davidson, E. H., Mazmanian, S. K., Prober, D. A., van de Rijn, M., Leadbetter, J. R., Newman, D. K., Readhead, C., Bronner, M. E., Wold, B., Lansford, R., Sauka-Spengler, T., Fraser, S. E., & Pierce, N. A. (2016). Mapping a multiplexed zoo of mRNA expression. *Development*, **143**, 3632–3637.

Choi, H. M. T., Schwarzkopf, M., Fornace, M. E., Acharya, A., Artavanis, G., Stegmaier, J., Cunha, A., & Pierce, N. A. (2018). Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development*, **145**, dev165753.

Dirks, R. M., & Pierce, N. A. (2004). Triggered amplification by hybridization chain reaction. *Proc Natl Acad Sci USA*, **101**(43), 15275–15278.

Schwarzkopf, M., & Pierce, N. A. (2016). Multiplexed miRNA northern blots via hybridization chain reaction. *Nucleic Acids Res*, **44**(15), e129.

Shah, S., Lubeck, E., Schwarzkopf, M., He, T.-F., Greenbaum, A., Sohn, C. H., Lignell, A., Choi, H. M. T., Gradinaru, V., Pierce, N. A., & Cai, L. (2016). Single-molecule RNA detection at depth via hybridization chain reaction and tissue hydrogel embedding and clearing. *Development*, **143**, 2862–2867.

Trivedi, V., Choi, H. M. T., Fraser, S. E., & Pierce, N. A. (2018). Multidimensional quantitative analysis of mRNA expression within intact vertebrate embryos. *Development*, **145**, dev.156869.