Multiplexed miRNA northern blots via hybridization chain reaction

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ABSTRACT

Northern blots enable detection of a target RNA of interest in a biological sample using standard benchtop equipment. miRNAs are the most challenging targets as they must be detected with a single short nucleic acid probe. With existing approaches, it is cumbersome to perform multiplexed blots in which several RNAs are detected simultaneously, impeding the study of interacting regulatory elements. Here, we address this shortcoming by demonstrating multiplexed northern blotting based on the mechanism of hybridization chain reaction (HCR). With this approach, nucleic acid probes complementary to RNA targets trigger chain reactions in which fluorophore-labeled DNA hairpins self-assemble into tethered fluorescent amplification polymers. The programmability of HCR allows multiple amplifiers to operate simultaneously and independently within a blot, enabling straightforward multiplexing. We demonstrate simultaneous detection of three endogenous miRNAs in total RNA extracted from 293T and HeLa cells. For a given target, HCR signal scales linearly with target abundance, enabling relative and absolute quantitation. Using non-radioactive HCR, sensitive and selective miRNA detection is achieved using 2′OMe-RNA probes. The HCR northern blot protocol takes ~1.5 days independent of the number of target RNAs.

INTRODUCTION

To perform a northern blot analysis, the RNA in a sample is size-separated via denaturing gel electrophoresis, transferred and crosslinked to a membrane and hybridized using a nucleic acid probe complementary to a target RNA of interest. Signal is generated either radioactively using a 32P-labeled probe (1–4), or non-radioactively via catalytic deposition of reporter molecules (5–7). The location of the signal on the blot characterizes target size and the intensity of the signal characterizes target abundance. The ability to characterize target size is a key advantage of northern blots relative to alternative approaches that are more sensitive and quantitative (real-time polymerase chain reaction (PCR)) or higher-throughput (microarrays) (8,9).

For a target RNA of interest, northern blots enable convenient comparison of relative target abundance across multiple samples within a single blot (8,9). Unfortunately, multiplexed blots, in which multiple target RNAs are detected in the same blot, require serial probing and/or serial signal amplification, leading to sample degradation and cumbersome protocols lasting several days (10,11). Here, we overcome this challenge by drawing on principles from the emerging discipline of dynamic nucleic acid nanotechnology, employing programmable signal amplifiers based on the mechanism of hybridization chain reaction (HCR; Figure 1).

An HCR amplifier consists of two DNA hairpins (H1 and H2) that coexist metastably in the absence of a cognate DNA initiator sequence (I1; Figure 1A) (12). The initiator triggers a chain reaction in which fluorophore-labeled H1 and H2 hairpins sequentially nucleate and open to assemble into a long nicked double-stranded amplification polymer (12). HCR is programmable, providing the basis for straightforward multiplexing using orthogonal amplifiers that operate independently and carry spectrally distinct fluorophores (13,14). Here, we provide a protocol for performing multiplexed HCR northern blots that is independent of the number of target RNAs: in the detection stage, all probes are hybridized in parallel; in the amplification stage, all HCR amplifiers operate in parallel (Figure 1B and C). The resulting amplification polymers are tethered to their initiating probes, localizing the signal at the site of the detected target within the blot.

The most challenging targets for northern blot analyses are miRNAs and other classes of small regulatory RNAs (15) that must be detected with a single short probe. Over the last decade, northern blot protocols have been optimized using N-Ethyl-N’-(3-dimethylaminopropyl)carbodiimide (EDC) crosslinking (11,16), locked nucleic acid (LNA) probes (2,3) and catalytic deposition of reporter molecules (6) to enable robust non-radioactive detection of endogenous miRNAs in total

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Figure 1. Multiplexed HCR northern blots. (A) HCR mechanism (12). A DNA initiator sequence (I1) triggers self-assembly of metastable DNA hairpins (H1 and H2) into a fluorescent amplification polymer via a cascade of alternating H1 and H2 polymerization steps. Blue stars denote fluorophores. (B) Multiplexed detection and amplification. Detection stage: probes hybridize to RNA targets and unused probes are washed from the sample. Amplification stage: initiators trigger self-assembly of tethered fluorescent HCR amplification polymers, and unused hairpins are washed from the sample. Probes for different target RNAs carry orthogonal initiators that trigger orthogonal HCR amplifiers labeled by spectrally distinct fluorophores. (C) Experimental timeline.

RNA (7). Here, we focus on this most challenging class of target RNAs to demonstrate multiplexed HCR northern blots.

MATERIALS AND METHODS

Oligonucleotides

2′OMe-RNA probes, DNA probes and DNA HCR amplifiers were purchased from Molecular Instruments (www.molecularinstruments.org). LNA probes used for the sensitivity/selectivity comparisons of Supplementary Sections S5 and 6 were synthesized by Exiqon using catalog detection sequences with a DNA HCR initiator at each end. Except for these sensitivity/selectivity comparisons, probes for detection of miRNA targets and miRNA markers were 2′OMe-RNA with DNA HCR initiators. Probes for detection of RNU48 and U6 targets and the low range ssRNA ladder were DNA with DNA HCR initiators. Target and probe sequences are provided in Supplementary Section S1. The low range ssRNA ladder and microRNA marker were purchased from New England Biolabs (catalog #N0364 and #N2102). Synthetic miRNA targets used for sensitivity and selectivity studies were purchased 5′-phosphorylated and HPLC-purified from Integrated DNA Technologies (IDT). Poly-ATGC DNA strands (a 16-nt oligo comprising four repeats and a 32-nt oligo comprising eight repeats) were purchased from IDT (standard desalting) to serve as a background for sensitivity studies. Stock samples of targets, probes and background strands were resuspended in molecular biology grade water (Corning, catalog #46-000-CM) in RNA/DNA LoBind microcentrifuge tubes (Eppendorf, catalog #022431021) and concentrations were determined by measuring absorption at 260 nm. Experimental samples were prepared in PCR tubes (GeneMate, catalog #T-3035-2).

Tissue culture

293T and HeLa cell lines were purchased from the American Type Culture Collection (ATCC, catalog #CRL-3216 and #CCL-2, respectively). 293T cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, catalog #11995-065) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, catalog #16140-071). HeLa cells were grown in Eagle’s minimum essential medium (ATCC, catalog #30-2003) supplemented with 10% FBS. Both cell lines were maintained at 37°C in 5% CO₂.

Total RNA extraction

Cells were washed with Dulbecco’s phosphate-buffered saline (DPBS) (Life Technologies, catalog #14190-144) followed by incubation in Trypsin-ethylendiaminetetraacetic acid (Trypsin-EDTA) (0.25%) for 5 min (Life Technologies, catalog #25200-072) at 37°C in 5% CO₂. The trypsin was quenched by the addition of an equal volume of growth media. Cells were pelleted at 300 rcf for 4 min, resuspended in growth media and pelleted again. Cells were disrupted by the addition of TRIzol Reagent (Life Technologies, catalog #1596-026) followed by RNA purification with Direct-zol (Zymo Research, catalog #R2050) according to the manufacturer’s instructions (omitting the optional DNase I digestion step).

Multiplexed miRNA HCR northern blot protocol

Denaturing polyacrylamide gel electrophoresis. Pre-run a 15% denaturing polyacrylamide gel at 400 V for 30–90 min in 1× Tris-Borate-EDTA (TBE) (10× TBE: Corning, catalog #46-011-CM). Mix RNA samples in a 1:1 ratio with formamide and heat to 65°C for 15 min prior to gel loading (formamide, deionized: Life Technologies, catalog #10900-014).
onto the membrane in 0.5 ml of buffer to be used for blots, calculated as: (i) 1 ml per 10 cm² of membrane; (ii) rounded up to the nearest 0.5 ml; (iii) no less than 1.5 ml. For example, V = 3.5 ml for a membrane that is 32 cm² and V = 1.5 ml for a membrane that is 9 cm². Pre-heat hybridization buffer (HB; ULTRAhyb-Oligo, Life Technologies, catalog #AM8663) to 60–68 °C in a rolling hybridization incubator. Prepare probe solution by adding each probe into a final volume of 0.5 ml of HB at 37 °C until use. Pre-hybridize blot for 4 h at 37 °C in a rolling hybridization incubator. Remove excess hairpins by washing twice using pre-heated 37 °C wash solutions with volume 5× SSCT for 15 min at 37 °C in the dark (per wash). Wash reagents: (5× SSCT: 5× SSC, 0.1% Tween 20, 50% Tween 20: Invitrogen, catalog # AM9342).

Run RNA (typically 1–20 µg) at 300 V for 25–30 min in 1× TBE. For targets with low or unknown expression levels we recommend starting with 10–20 µg of total RNA.

**RNA transfer and crosslinking.** Wet membrane in ultrapure H₂O and equilibrate for 5 min in 0.5× TBE (nylon membrane, positively-charged: Roche, catalog #11209272001). Layer three sheets of chromatography paper (3MM Whatman chromatography paper; GE Healthcare Life Sciences, catalog #3030-6188) pre-soaked in 0.5× TBE, then add the gel, followed by the membrane and three additional sheets of chromatography paper pre-soaked in 0.5× TBE. Ensure there are no air bubbles between the layers. Perform a semi-dry transfer of RNA onto the membrane in 0.5× TBE at 1 mA per cm² of gel for 45 min using electroblotting system (Owl HEP Series Semidry Electroblotting system: Thermo Scientific, catalog #H780-01). Prepare fresh EDC crosslinking solution: (i) to 10 ml of ultrapure H₂O add 122.5 µl 1-Methylimidazole (MP Biomedicals, catalog #151655); (ii) adjust the pH of the solution to 8.0 using hydrochloric acid, 1N (Amresco, catalog #E447); (iii) add 0.373 g of EDC hydrochloride (Sigma-Aldrich, catalog #E7750) and adjust the solution volume to 12 ml using ultrapure H₂O. In an open-faced container, saturate one sheet of chromatography paper in EDC crosslinking solution, place the membrane on top of the saturated paper with the RNA side facing up, wrap the container in cling film and incubate for 2 h at 60 °C. Wash the membrane in ultrapure H₂O to remove excess crosslinking solution and air-dry on top of gel blotting paper (Whatman gel blotting paper: GE Healthcare Life Sciences, catalog #10427805).

**Multiplexed probe hybridization.** Let V denote the volume of buffer to be used for blots, calculated as: (i) 1 ml per 10 cm² of membrane; (ii) rounded up to the nearest 0.5 ml; (iii) no less than 1.5 ml. For example, V = 3.5 ml for a membrane that is 32 cm² and V = 1.5 ml for a membrane that is 9 cm². Pre-heat hybridization buffer (HB; ULTRAhyb-Oligo, Life Technologies, catalog #AM8663) to 60–68 °C for 1 h and maintain at 37 °C until use. Pre-hybridize blot in (V − 0.5 ml) of HB in a hybridization bottle (Wheaton, catalog #805000 or #805021) for 30–60 min at 37 °C in a rolling hybridization incubator (SciGene, catalog #1040-50-1). Prepare probe solution by adding each probe into a total volume of 0.5 ml of HB at 37 °C such that each probe will be at a final concentration of 5 nM in volume V. Add the probe solution to the pre-hybridization solution and incubate blot overnight at 37 °C in a rolling hybridization incubator. Remove excess probes by washing four times using pre-heated 37 °C wash solutions with volume 2V per wash: (i) two low-stringency washes (2× saline sodium citrate (SSC), 0.1% sodium dodecyl sulphate (SDS); 5 min at 37 °C); (ii) two high-stringency washes (0.2× SSC, 0.1% SDS; 15 min at 37 °C). Wash reagents: (20× SSC: Invitrogen, catalog #15557-044), (SDS: Life Technologies, catalog #15525-017).

**Multiplexed HCR amplification.** Pre-heat amplification buffer (AB: Molecular Instruments) to 37 °C. Pre-amplify blot in (V − 0.5 ml) of AB in a hybridization bottle for 30–60 min at 37 °C in a rolling hybridization incubator. Snap-cool each amplifier hairpin (an amount corresponding to 30 nM final concentration in a final volume V); heat at 95 °C for 90 s and cool to room temperature in the dark for 30 min. Prepare amplification solution by adding all snap-cooled hairpins into a final volume of 0.5 ml AB at 37 °C. Add the amplification solution to the pre-amplification solution and incubate blot for 4 h at 37 °C in the dark in a rolling hybridization incubator. Remove excess hairpins by washing twice using pre-heated 37 °C wash solutions with volume 2V per wash: 5× SSCT for 15 min at 37 °C in the dark (per wash). Wash reagents: (5× SSCT: 5× SSC, 0.1% Tween 20), (50% Tween 20: Invitrogen, catalog # 00-3005).

**Fluorescent scanning**
Membranes were imaged on an FLA-5100 imaging system (Fuji Photo Film) with excitation laser sources and emission filters as follows: Alexa 488 (473 nm laser with 530 ± 10 nm bandpass filter), Alexa 546 (532 nm laser with 570 ± 10 nm bandpass filter), Alexa 647 (635 nm laser with 665 nm long-pass filter).

**Relative and absolute quantitation**
Blots used for relative quantitation studies (Figures 3 and 4) were performed three times (Supplementary Sections S2.1 and 3.1). Blots used for absolute quantitation studies (Figure 5) were performed twice (Supplementary Section S4). The fluorescence intensity profile for each band was calculated using Multi Gauge software (Fuji Photo Film). For each band, signal is quantified using a Matlab script to integrate the total fluorescence in the profile and subtract the estimated background, obtained by fitting a line to the intensity values in the last 1 mm at either end of the quantified window. For relative quantitation of different amounts of the same target (e.g., Figures 3 and 4), relative signal is calculated by normalizing to the band with the maximum signal for each target type and band intensity profiles are displayed with intensity values normalized so that the peak value is unity for the brightest band of each target type, with all peak values centered at zero on the horizontal axis. To perform relative quantitation for a cognate target and various off-targets (e.g., the selectivity studies of Figure 7 and

![Figure 2. Multiplexed detection of three endogenous target RNAs via HCR northern blot. Targets: U6, RNU48, miR-18a in 5µg of total RNA extracted from either 293T or HeLa cells.](https://academic.oup.com/nar/article-abstract/44/15/e129/2457701)
Supplementary Figure S14), relative signal is calculated by normalizing to the cognate target band, which is also used for normalized display of band intensity profiles (Supplementary Figures S15–17). Based on repeated quantification of a single blot using different box sizes, the uncertainty in quantifying bands within a blot is estimated to be $\sim 3\%$.

**Sensitivity studies**

Blots used for miRNA sensitivity studies (Figure 6) were performed three times using 2'OMe-RNA probes at 37°C (Supplementary Figure S11). Comparison blots using 2'OMe-RNA or LNA probes at 60°C were performed twice (Supplementary Figures S12 and 13) using the identical protocol except that the probe pre-hybridization, hybridization and wash steps were performed at 60°C instead of 37°C. For sensitivity studies, synthetic miRNA targets were serially diluted (200–12.5 amol) and introduced into a mixture of DNA background strands (2.5 μg of 16-nt poly-ACGT and 2.5 μg of 32-nt poly-ACGT). Background strands were included to minimize target adsorption to tubes at low target concentrations.

**Selectivity studies**

Blots used for miRNA selectivity studies (Figure 7) were performed twice using 2'OMe-RNA probes at 37°C (Supplementary Figure S15). Comparison blots using 2'OMe-RNA or LNA probes at 60°C were also performed twice (Supplementary Figures S16 and 17) using the identical protocol except that the probe pre-hybridization, hybridization and wash steps were performed at 60°C instead of 37°C. For selectivity studies, each sample was 10 fmol of a 5'-phosphorylated synthetic RNA target.

**RESULTS**

**Multiplexing**

Multiplexed HCR northern blotting is demonstrated in Figure 2 for three endogenous target RNAs (U6, RNU48, miR-18a) of different sizes (106, 63, 23 nt) in total RNA extracted from either of two cell lines (293T or HeLa). Signal amplification was performed simultaneously for all three targets using three orthogonal HCR amplifiers carrying spectrally distinct fluorophores. A fourth orthogonal HCR amplifier carrying the same fluorophore as the amplifier for U6 was used to generate signal for the RNA markers. Expression of miR-18a is low in HeLa cells relative to that in 293T cells, in qualitative agreement with the data for 293 and HeLa variants in the miRNA expression atlas (17).

**Relative quantitation**

To test whether HCR northern blots enable quantitative comparisons between samples, Figure 3 examines the same three targets over a range of total RNA quantities (10–0.625 μg).
μg) extracted from 293T cells. For each target, the signal scales linearly with target abundance, demonstrating that HCR northern blots are suitable for relative quantitation between samples within a blot.

miRNA multiplexing and relative quantitation

The multiplexed northern blot of Figure 4A demonstrates simultaneous examination of three endogenous miRNAs (miR-16, miR-18a, miR-30a) in total RNA extracted from either 293T or HeLa cells. For multiplexed miRNA blots, the target bands are all roughly co-localized on the blot. Hence, there is the potential that spectral crosstalk or steric inhibition between HCR amplifiers could interfere with relative quantitation. However, control experiments reveal no measurable spectral crosstalk (Supplementary Section S3.2) or steric inhibition (Supplementary Section S3.3) for these endogenous miRNAs. The relative target abundance in 293T and HeLa total RNA is displayed in Figure 4B for each of the three endogenous miRNAs.

miRNA absolute quantitation

If desired, HCR northern blots can also be used for absolute quantitation of endogenous RNA targets. Some lanes within a blot are allocated to synthetic samples (each with a known abundance of a target RNA of interest) and others are allocated to biological samples containing unknown quantities of endogenous target. The measured band intensities from the dilution series are used to create a standard curve, enabling deduction of endogenous target quantities via comparison of measured band intensities from the biological samples. Figure 5 demonstrates absolute quantitation of miR-16 in total RNA extracted from either 293T or HeLa cells.
trally distinct HCR amplifiers are valuable for confirming
multiple channels using two or more probes that activate specific targets, signal can be increased as desired using a probe set containing multiple probes that recognize different subsequences along the target (all carrying initiators for the same HCR amplifier) (13,14). Multiplexed redundant detection experiments, in which a target molecule is detected in multiple channels using two or more probe sets that activate spectrally distinct HCR amplifiers are valuable for confirming target identity (13,14) and also provide an attractive avenue for selective detection of mRNA splice variants.

In contrast to existing northern blot techniques, HCR northern blots enable straightforward multiplexing, with all targets detected in parallel, and all signal amplification performed in parallel. The same ~1.5-day protocol is used independently of the number of target RNAs. For a given target RNA, HCR signal scales linearly with target abundance, enabling relative quantitation between samples within a blot or absolute quantitation via comparison to a standard curve generated using samples of known quantity.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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**Conflict of interest statement.** The authors declare competing financial interests in the form of US and EPO patents and pending patents.

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