Multiplexed miRNA northern blots via hybridization chain reaction

Maayan Schwarzkopf1 and Niles A. Pierce1,2,*

1Division of Biology & Biological Engineering, California Institute of Technology, Pasadena, CA 91125, USA and 2Division of Engineering & Applied Science, California Institute of Technology, Pasadena, CA 91125, USA

ABSTRACT

Northern blots enable detection of a target RNA of interest in a biological sample using standard bench-top 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
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-ethylenediaminetetraacetic 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 #15596-026) 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
onto the membrane in 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 2$V'$ 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).

Figure 2. Multiplexed detection of three endogenous target RNAs via HCR northern blot. Targets: U6, RNU48, miR-18a in 50–68 nt.

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 2$V'$ 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).

Relative and absolute quantitation

Blots used for relative quantitation studies (Figures 3 and 4) were performed three times (Supplementary Sections S2.1 and S3.1). 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 3. Relative quantitation of RNA via HCR northern blot. (A) Multiplexed detection of three endogenous target RNAs (U6, RNU48, miR-18a) in total RNA extracted from 293T cells. Compare between lanes for a given target. (B) Normalized band intensity profiles for each target. (C) Linear regression analysis for each target. See Supplementary Section S2 for additional data.

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 ∼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...
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.

Figure 5. miRNA absolute quantitation via HCR northern blot. Standard curve samples: synthetic 5′-phosphorylated miR-16. Biological samples: 10 μg of total RNA extracted from either 293T or HeLa cells. (A) HCR northern blot for miR-16. (B) Normalized band intensity profiles. (C) Absolute quantitation using a standard curve. The 293T and HeLa band intensities enable deduction of absolute quantities from the standard curve (black line). (D) Absolute target abundance in 293T and HeLa total RNA (symbols denote N = 2 replicate blots). See Supplementary Section S4 for additional data.

miRNA sensitivity and selectivity

To characterize the sensitivity of HCR northern blots for miRNA detection, we performed blots on serial dilutions of synthetic miR-16, miR-18a, and miR-30a, revealing a detection limit of ~25 amol for these miRNAs (Figure 6). To characterize selectivity for the same three miRNAs, we assembled panels of the most closely related human miRNAs supplemented by other synthetic off-targets as needed. Using 2′OMe-RNA probes hybridized at 37°C, the median discrimination ratio (complement yield/mismatch yield) for a variety of sequences is ~2 for two mismatches, ~5 for three mismatches and >10 for four mismatches or more (Figure 7).

DISCUSSION

Once target RNAs have been crosslinked in place within a band on a membrane, the experimental situation is similar to that of an in situ hybridization experiment with target RNAs crosslinked in place within a cell, tissue section or whole-mount embryo. The present work leverages this similarity by adapting the multiplexed signal amplification capabilities of in situ HCR (13,14) to the northern blot setting. Because HCR initiator sequences are independent of target RNA sequences, previously validated sets of orthogonal HCR amplifiers (14) can be used to perform multiplexed northern blots without modification.

Each probe carries a DNA HCR initiator at each end, enabling growth of two tethered fluorescent HCR amplification polymers per probe. We employ 50-nt DNA probes
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Figure 7. miRNA selectivity for HCR northern blots using 2′OMe-RNA probes at 37°C. (A) miR-16 and related off-targets. (B) miR-18a and related off-targets. (C) miR-30a and related off-targets. Off-target sequences depict mismatches in orange and wobble pairs in blue. Each sample is 10 fmol of a 5′-phosphorylated synthetic RNA target. Normalized signal (symbols denote N = 2 replicate blots). See Supplementary Section S6 for additional data, including comparisons to 2′OMe-RNA probes at 60°C and LNA probes at 60°C.

for targets ≥50 nt (RNU48 and U6) to minimize cost while striking a balance between affinity and selectivity. For miRNA targets (~23 nt), we employ 2′OMe-RNA probes (again with DNA HCR initiators) to enhance affinity and selectivity while striking a balance on cost. 2′OMe-RNA probes are ~3× more expensive than DNA probes, but ~15× less expensive than (proprietary) LNA probes, which have previously been used to optimize the performance of miRNA northern blots (2,3,7). Using non-radioactive HCR signal amplification and 2′OMe-RNA probes, we observe miRNA sensitivity and selectivity comparable to previous radioactive and non-radioactive miRNA northern blot methods employing more costly LNA probes (2,3,7). For blots containing multiple targets of varying length, the present results demonstrate that 50-nt DNA probes and 23-nt 2′OMe-RNA probes can be used concurrently in the same blot.

Based on these results, and our experience mapping mRNA targets using in situ HCR within vertebrate embryos (14), we recommend using the same 50-nt DNA probes for HCR northern blots with mRNAs and rRNAs. For long 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 independent 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.

REFERENCES


