Published in final edited form as:

Analyst. 2018 February 26; 143(5): 1210–1216. doi:10.1039/c7an02045g.

Combining asymmetric PCR-based enzymatic amplification with silicon photonic microring resonators for the detection of lncRNAs from low input human RNA samples

Maria C. Cardenosa-Rubio^a, Richard M. Graybill^{a,b}, and Ryan C. Bailey^{a,*}

^aDepartment of Chemistry, University of Michigan, 930 N. University Ave., Ann Arbor, Michigan 48104

^bDepartment of Chemistry, University of Illinois at Urbana-Champaign, 600 S. Mathews Ave., Urbana, Illinois 61801

Abstract

A method for quantifying biologically relevant long-non-coding RNAs by combining nucleic acid amplification via asymmetric polymerase chain reaction (PCR) with label-free PCR product detection using silicon photonic microring resonator arrays is described. This approach eliminates the need for fluorophores, which presents a limit for spectral multiplexing in conventional qPCR methods, and rather offers potential for much higher levels of plexity by spatially arraying capture probes. Here, we demonstrate the potential of this technique to detect two differentially expressed lncRNA transcripts and an internal control mRNA transcript in different commercial human tissue specimens, as well as in a glioblastoma cell line using only nanogram input amounts of total RNA. The obtained results were validated using single-plex RT-qPCR and found to be in good agreement, demonstrating the potential of this technique for lncRNA quantification applications.

1. Introduction

In recent years, increased attention has been given to multiplexed biomolecular analysis because of its potential to revolutionize areas of human health, such as disease diagnosis and therapeutic selection. The discovery of potential biomarkers for different diseases has been one of the main drivers in the development of multiplexed diagnostic analysis. Among the different types of biomarkers, RNA molecules have risen in importance thanks to the use of next generation sequencing and the resulting insights in cell signaling regulation.¹ Furthermore, accumulating reports noting the differential expression of non-coding RNAs (ncRNAs) in disease suggest that they may also emerge as diagnostic biomarkers for a number of diseases.²

Though having profound regulatory effects at the levels of transcription and translation, ncRNAs do not encode for proteins. ncRNAs are commonly divided into two major groups

*Corresponding author: ryancb@umich.edu (R.C. Bailey).

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here].
See DOI: 10.1039/x0xx00000x

based on their length: small non coding RNAs, which include Piwi-interacting RNAs, small-interfering RNAs and microRNAs; and long non-coding RNAs, lncRNAs, which are defined as those longer than 200 nucleotides.³ Since the discovery of the first well-studied lncRNA, H19,⁴ many additional lncRNAs have been identified that affect diverse biological functions.^{5–7} For example, in cancer lncRNAs can support both tumor suppressive and oncogenic functions.^{8,9}

It has been suggested that multiplexed lncRNA-based diagnostics revealing differential, disease-specific expression in cancers such as glioma might provide valuable information regarding subtype and malignancy in these tumors.^{10,11} One study, based on in silico analysis of data from The Cancer Genome Atlas, identified a signature of six lncRNAs that might be predictive of survival in patients with glioblastoma. Another report utilized microarray data to subtype and grade of glioma based on lncRNA signatures. Importantly, this study showed that from an initial large pool of potential lncRNA biomarkers (~2,000 potential lncRNA), around 25-50 targets held diagnostic significance. This example, which is supported by other biomarkers studies, places an impetus on developing moderately multiplexed approaches to detecting targeted panels of lncRNAs for clinical diagnostic applications.

To date, the most common approaches to detecting lncRNA expression have been based upon RT-qPCR, hybridization assays such as microarrays, and RNA sequencing.^{12,13} While these techniques are generally robust, sensitive, and, in the case of RNA sequencing, allow for the discovery of new lncRNAs, each has limitations for the realization of panel-based diagnostics in the clinical laboratory settings. RT-qPCR-based approaches typically only detect a single target per sample. Microarrays detect many targets, but are laborious and often only qualitative. RNA sequencing has become commonplace for lncRNA discovery and expression profiling; however, it is still prohibitively expensive for studies looking at large numbers of clinical samples, and also requires extensive bioinformatic data analysis. Therefore, there is an opportunity for the emergence of new biosensing technologies to fill this void by offering moderately multiplexed, targeted lncRNA detection capabilities that are quantitative, robust, and cost-effective.

In response to this challenge, we have developed a silicon photonic microring resonator technology that is well-suited to the multiplexed detection of targeted biomarker panels. The specifics of this technology have been previously discussed generally,^{14,15} and more relevant to this work, for label-free RNA analysis.¹⁶ The sensing principle is based on the change of refractive index near the surface of the rings, which occurs when biomolecules selectively bind to receptors covalently bound to the microring surface. The label-free operation of this technology provides advantages over those that rely upon fluorescent or enzymatic tags to generate the detectable signal. Another advantage of this technology is the ability to create spatially-arrayed, high density sensor arrays, which intrinsically affords the potential for multiplexed detection. The promise of these sensors for multiplexed analysis has been previously demonstrated for RNA targets;^{16–19} however, the limits of detection for these approaches were insufficient for many clinical applications where sample input is restricted.

In this manuscript, we report an approach to lncRNA analysis that leverages asymmetric PCR (aPCR) to selectively produce single stranded DNA (ssDNA) products upstream of label-free, hybridization-based detection using microring resonator arrays (Figure 1). aPCR is based upon standard PCR; however, an excess of one primer results in the creation of single stranded products after consumption of all of the limiting primer.^{20,21} This combination allowed RNA quantification with a 1000-fold reduction in input RNA, compared to our previous report of mRNA detection using microring resonators.¹⁸ In addition to detecting two lncRNAs, we also included in the panel an internal control mRNA sequence. We demonstrated the applicability to detect these disease-relevant targets from both cultured cells and commercially-obtained human total RNA samples. We validated this approach by parallel analysis with RT-qPCR and also found that disease- and tissue-specific relative lncRNA expression profiles were consistent with literature precedent. Together, these comparisons support the promise of this combined aPCR-microring resonator approach for multiplexed lncRNA analysis in both clinical and basic research settings.

2. Materials and methods

2.1. Materials

All nucleic acid sequences (primers and capture probes) were purchased from Integrated DNA Technologies (IDT; Coralville, IA). The TaqMan[®] microRNA Reverse Transcription Kit, Platinum[®] Multiplex PCR Master Mix, SYBR[®] Select Master Mix, 3-aminopropyltriethoxysilane (APTES) and bis(sulfosuccinimidyl)suberate (BS3) were purchased from ThermoFisher. All buffer dilutions, DNA primer reconstitution, and DNA primer dilutions were prepared in nuclease-free Ultrapure distilled water (Invitrogen). Phosphate-buffered saline (PBS) was obtained from Lonza and was used in the reconstitution of the oligonucleotide capture probes. A high stringency hybridization buffer was made in 50 mL batches containing 15 mL of formamide (Fisher), 1 mL 10% sodium dodecyl sulfate (Fisher), 10 mL 20× saline-sodium phosphate buffer (Invitrogen), 6 mL 0.25 M ethylenediaminetetraacetic acid (Invitrogen) and 2.5 mL 50× Denhardt's solution (Invitrogen).

2.2. RNA sample preparation

Total RNA was extracted from GBM6 cell lines lysates with a miRNeasy[®] Mini kit (Qiagen) using manufacture's protocol. After extraction, total RNA was assessed for purity and quantity using a Nanodrop UV-vis spectrometer and stored at -80°C until further use. Brain and Lung Reference total RNA samples were obtained from ThermoFisher and stored at -80°C until further use.

2.3. Amplification primers and capture probe design

Specific primers and capture probes for the amplification and detection of the human lncRNAs MALAT1 and KIAA0495, and the internal control β -actin, were designed using sequence annotations from Genbank (NCBI) and the Primer-Blast platform (NCBI). Secondary structures were visualized using the Dinamelt web server.²² Sequences of the primers and capture probes are listed in Table 1.

2.4. Silicon Photonic Microring Resonator Instrumentation

Microring sensor arrays and sensor scanner equipment were purchased from Genalyte, Inc. (San Diego, CA), and the detection instrumentation and method has been described previously.^{14,15} Sensor arrays were fabricated on silicon-on-insulator wafers by photolithography and standard etching techniques. After etching to define the microrings, a polymer cladding was deposited across the entire substrate and then selectively etched to expose active sensor rings. Four microrings remaining occluded and served as controls for thermal drift. The final sensor chips were diced to 6 mm × 4 mm and each contain 132 individually-addressable microring resonator sensors. For sensing experiments, the chips are loaded into a fluidic cartridge with a Mylar gasket and Teflon lid directing solution flow across the array in two separate channels. There are 64 active sensors in each channel. Fluid flow is controlled by integrated syringe pumps under software control.

2.5. Surface functionalization of the microring resonators

Sensors were first silanized with a 5% APTES solution in acetone for 4 min followed by successive two-minute rinses in acetone and isopropanol. These steps were completed with continuous shaking. Chips were then rinsed with water and dried under a stream of nitrogen. Next, 20 µL of a freshly prepared BS3 solution (2.85 mg/mL in 2 mM acetic acid) was pipetted onto the microring array and allowed to react for 3 minutes, followed by drying under a stream of nitrogen. The final step of functionalization involved spotting approximately 260 nL of 200 µM solutions containing 5'-amino functionalized DNA captures probes onto discrete microring sensors. The chips were then incubated overnight in a humidity chamber.

2.6. Reverse transcription–asymmetric PCR

Reverse transcription reactions were performed using the TaqMan microRNA Reverse Transcription Kit. Each 15 µL reaction volume contained 4.16 µL of nuclease free water, 1.5 µL of 10× RT buffer, 1 µL of Multiscribe™ RT enzyme (50 U/µL), 0.19 µL of RNase inhibitor (20 U/µL), 0.15 µL dNTP mix (100 mM), 5 µL of RNA sample (40 ng RNA total), and 3 µL of reverse transcription primer. The concentration of the reverse primer was 200 µM when only one transcript was reversed-transcribed and 66 µM when three transcripts were reverse transcribed. The thermal profile was completed following the manufacturer's suggested protocol: 65° C (5 min), 4° C (2 min), 42° C (30 min), and 85° C (5 min).

Asymmetric PCR was performed using the Platinum® Multiplex PCR Master Mix. Each 50 µL reaction volume was composed of 14 µL nuclease free water, 25 µL of Platinum® Multiplex PCR Master Mix, 5 µL of each primer, and 1 µL of the reversed transcription product. The concentration of the forward primer (limiting primer) was 2 µM while the concentration of the reverse primer (excess primer) was 200 µM. The reactions were incubated at 95° C for 2 min, followed by cycles of 95° C for 30 s, 58° C for 45 s, and 72° C for 30 s. To profile the amplification process, PCR samples were taken from the thermocycler at different cycle numbers (i.e. every two cycles or every five cycles).

2.7. Sensor fluidics and sample introduction

PCR samples (50 μ L) were diluted in 350 μ L of the hybridization buffer described in the Materials section. Hybridization of the amplified products was carried out at room temperature by flowing solutions across the chip surface at a rate of 20 μ L/min for 13 min. After the hybridization of each cycle sample, the chip was rinsed with the hybridization buffer for 2 min before introduction of the next sample of successively higher cycle number. The full hybridization assay protocol is provided in Table S1.

2.8. Quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed using a 7900HT Fast Real-Time PCR system (Thermo Fisher) using the RT protocol described above. Each reaction contained 5.6 μ L of nuclease free water, 10 μ L of SYBR[®] Select Master Mix, 2 μ L of each primer (2 μ M) and 0.4 μ L of the reverse transcription product. The reaction was initiated at 95° C for 2 min, followed by 40 cycles that included 95° C for 15 s followed by 58° C for 1 min. Threshold cycle (Ct) was automatically determined by the provided software.

2.9. Data analysis

The microring response was analyzed using OriginPro8 (OriginLab, Northampton, MA). The net shift signal from specific rings was corrected for temperature and instrument drift by subtracting the signal of thermal control rings from the active rings functionalized with DNA capture probes. The data for every target in each sample was detected and averaged using between 8 and 16 replicate microrings on a single chip.

3. Results and discussion

In this study, we used asymmetric PCR to amplify two long non-coding RNAs and quantify expression levels within three different RNA samples using a label-free microring resonator detection platform. The schematic of the assay is shown in Figure 1. In this assay, lncRNAs from different RNA samples were reverse transcribed using the target specific primers listed in Table 1. After reverse transcription, asymmetric PCR was used to selectively produce ssDNA at cycle numbers that are proportional to the initial concentration of the target within the sample of interest. In asymmetric PCR one of the primers is in a limiting concentration so that when all of the limiting primer is consumed, exponential amplification transitions to linear amplification creating ssDNA from the excess primer.^{20,21} The resulting ssDNA product can then be detected by hybridization to complementary capture probes covalently attached to the surface of microring resonators.

With this assay, we aimed to mimic the quantification process of qPCR, so we collected asymmetric PCR samples after varying numbers of thermal cycles. In the case of conventional qPCR, double stranded DNA is detected by the addition of SYBR dyes[®] and fluorescence intensity increases upon double stranded DNA (dsDNA) accumulation. The fluorescent intensity is plotted against increasing PCR cycle number and expression profiles can be calculated based on the cycle at which fluorescent signal begins. With the aPCR-microring system, we quantitate ssDNA accumulation instead of dsDNA. Resonance wavelength shifts increase upon hybridization of increasing concentrations of generated

ssDNA, which occurs only after the transition from exponential dsDNA generation to linear amplification upon limiting primer consumption (Figure 1B). As a result, quantitation occurs at higher cycle numbers than traditional qPCR.

An important step in the optimization of this approach was the design of primer pairs and the resulting PCR products to avoid targeting regions of extensive secondary structure, which would preclude primer or capture probe hybridization. Primer-BLAST²³ was used to analyze secondary structures of lncRNAs to identify regions optimal for primer targeting. Following primer design, the secondary structure of the resulting amplicon was modeled using the Dinamelt web server²² to ensure that has minimal secondary structure at sequence regions targeted by surface capture probes. Examples of amplicon secondary structure analysis and effects of optimization are provided in the Supplemental Information (Figures S1 and S2). In particular, Figure S2 shows that the hybridization response for the optimized β -actin amplicon was improved through this secondary structure-based refinement approach; however, this generated amplicon still was restricted by secondary structure, which likely explain the relatively low hybridization response, though larger signal in gel analysis. It is also worth noting that the amplicon for KIAA was the largest in terms of size, which likely explain the fact that it displays the largest hybridization response for this optimized condition calibration. Following design and optimization, all of the primer pairs were validated using gel electrophoresis to ensure that they selectively produce a single band of appropriate size (Figure S3).

With primer pairs for the lncRNA targets, we next proceeded to assess the specificity of detection to capture probes arrayed on the sensor chip surface. Four capture probes were spotted onto microrings in each channel of the sensor chip as indicated in Figure S4. Next, RT-aPCR was separately performed for each target using the healthy brain RNA reference sample. An initial input of 40 ng RNA was used for the RT, followed by 45 aPCR amplification cycles. Each aliquot of the sample, amplified using a single target-specific primer pair, was flowed separately across the functionalized sensor array. As shown in Figure 2, large positive resonance shifts were observed upon hybridization to ssDNA amplicons to sequence-specific capture probes with minimal non-specific binding.

After verification of aPCR primer sets and sequence specificity for hybridization-based RNA detection, an approach for determining relative target expression was developed. As opposed to smaller targets, such as microRNAs, synthetic target probes cannot be readily synthesized for lncRNAs and therefore we chose to use the commercially obtained healthy brain RNA sample as reference material. RNA targets were separately amplified with primer sets for defined numbers of cycles and then flowed across the sensor array sequentially with hybridization buffer steps in between. By introducing each amplicon-containing sample as a function of cycle number and increasing hybridization response was observed as each sample of increasing cycle number was flowed. Samples were analyzed every 2 cycles between 30 and 40. No detectable hybridization response was observed at cycle numbers below 30, and signal saturation was achieved beyond 40 cycles under the chosen sample dilution conditions. Samples were flowed across the sensor array for 13 mins allowing for hybridization. The chip was then washed for 2 minutes with hybridization buffer, after which the relative resonance wavelength shift was determined. Responses from control rings were

subtracted from the signal of target specific rings (Fig. 3A, red trace). After the buffer rinse, the next higher cycle number sample was flowed across the array. A representative real-time hybridization response for the detection of the lncRNA KIAA0495 is shown in Figure 3A with data representing the average 16 replicate microring measurements simultaneously made on a single chip.

Similar to traditional qPCR analysis, the resulting calculated net shift induced by ssDNA hybridization was plotted versus the PCR cycle number to enable relative quantitation, as shown for KIAA0495 in Figure 3B. In order to compare expression between samples, we determined a cycle threshold (C(t)) value, again analogous to qPCR.²⁴ To achieve this, the cycle number corresponding to 40% of the maximum resonance shift at high cycle numbers was extrapolated and assigned as the C(t) value from a given sample. Forty percent of the maximum signal was chosen because it was significantly above the noise of the baseline (signal obtained at lower cycles where no DNA is detectable) and also well below saturation.

Having established a method for C(t), which is related to the concentration of a given lncRNA within an unknown sample, we demonstrated the applicability of this combined approach to determine differential expression of these targets in multiple human-derived samples. Samples included two commercially available pooled healthy total RNA samples from brain and lung tissue, and total RNA extracted from a patient-derived xenograft glioblastoma cell line (GBM6). Given their roles in brain cancer, two lncRNAs of potential disease relevance were chosen. KIAA0495/PDAM has been shown to act as a tumor suppressor in oligodendroglioma.²⁵ MALAT1 (metastasis associated lung adenocarcinoma transcript 1), is a lncRNA that has been studied in different tumors.²⁶ MALAT1 was first studied in the context of NSCLC (non-small cell lung cancer cell) where it was found that higher expression of the lncRNA correlated with elevated metastasis.²⁷ In glioblastoma, however, MALAT1 can act as a tumor suppressor.²⁸ We also developed primer sets and a capture probe for β -actin, which was used as an input control to correct for any sample processing variability.²⁹

Hybridization response versus cycle number was measured for these three RNA targets in each sample as described above. Following control subtraction, real-time resonance wavelength shifts are shown in Figure S5. C(t) values (Table S2) were determined from the amplification curves presented in Figure 4 and were used to contextualize differences in RNA expression between samples. The internal control, β -actin, was used to normalize the results and correct for initial input of RNA and other variabilities. Expression differences between both lncRNA targets in healthy brain and lung samples were small, and consistent with RT-qPCR measurements, as shown in Figure 5A. Both MALAT1 and KIAA were found at slightly lower levels in lung tissue relative to brain, which is consistent with expression values available through the EMBL-EBI Expression Atlas³⁰ (KIAA is indexed as TP73-AS1). Comparison of the GBM6 cell line with healthy brain tissue was more interesting, as lncRNAs have been shown to be differentially expressed in cancer.³¹

Considering lncRNA expression in the GBM6 xenograft cell line, Figure 5B again shows consistency between the aPCR- microring and conventional RT-qPCR measurements. KIAA shows minimally higher expression in GBM6 cells compared to healthy brain tissue.

However, MALAT1 shows significantly lower expression in the GBM6 sample. This result is interesting given that this lncRNA has previously been found to be downregulated in glioblastoma samples.²⁸ Moreover, while the expression of MALAT1 in GBM 6 cells has not been previously reported, the downregulation of its tumor suppressive actions would be consistent with a malignant phenotype.

4. Conclusions

Given the continuing emergence of lncRNAs as important regulatory elements associated with many disease phenotypes, the development of analytical methods to characterize their expression from clinically-relevant samples is of high importance. We have demonstrated an integrated analysis approach for multiplexed detection of lncRNAs that leverages the ability for sequence-specific target amplification via asymmetric PCR and label-free, spatially-multiplexed hybridization-based detection using silicon photonic microring resonators. We report the design of specific primer sets for the detection of two lncRNAs and found that expression levels determined from healthy brain and lung tissue, as well as a patient-derived xenograft model of glioblastoma, were consistent with both conventional, single-plex RT-qPCR and literature precedent. We also designed primer sets for β -actin mRNA, which was used as a control to normalize for differences in input—a step important for applications on clinical samples. While this proof-of-principle study focused on a detailed discussion of the probe design and validation process for two targets, the multiplexing capacity of microring resonators makes this overall approach suitable for simultaneously probing many more lncRNAs from single samples in the future. Expanded lncRNA panels probed using this approach in the future might reveal new biomarker signatures as well as new fundamental insights into lncRNA actions in disease onset and progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge financial support from the National Cancer Institute of the National Institutes of Health through Grant R33CA1774. MCR acknowledges support from a la Caixa Banking Foundation Fellowship. RMG acknowledges support from the National Cancer Institute Alliance for Nanotechnology in Cancer “Midwest Cancer Nanotechnology Training Center” Grant R25CA154015A. We appreciate technical assistance from the University of Michigan DNA Sequencing Core. We would also like to thank Dr. Jann Sarkaria, Mark Schroeder, and Brett Carlson (Mayo Clinic) for providing the GBM6 patient-derived xenograft cells.

References

1. Byron SA, Van Keuren-Jensen KR, Engelthaler DM, Carpten JD, Craig DW. *Nat Rev Genet.* 2016; 17:257–271. [PubMed: 26996076]
2. Qi P, Du X. *Mod Pathol.* 2013; 26:155–165. [PubMed: 22996375]
3. Mattick JS, Makunin IV. *Hum Mol Genet.* 2006; 15:R17–R29. [PubMed: 16651366]
4. Bartolomei MS, Zemel S, Tilghman SM. *Nature.* 1991; 351:153–155. [PubMed: 1709450]
5. Baker M. *Nat Methods.* 2011; 8:379–383.
6. Fatica A, Bozzoni I. *Nat Rev Genet.* 2013; 15:7–21. [PubMed: 24296535]
7. Rinn JL, Chang HY. *Annu Rev Biochem.* 2012; 81:145–166. [PubMed: 22663078]

8. Gibb EA, Brown CJ, Lam WL. Mol Cancer. 2011; 10:38. [PubMed: 21489289]
9. Prensner JR, Chinnaiyan AM. Cancer Discov. 2011; 1:391–407. [PubMed: 22096659]
10. Zhang X, Sun S, Pu JKS, Tsang ACO, Lee D, Man VOY, Lui WM, Wong STS, Leung GKK. Neurobiol Dis. 2012; 48:1–8. [PubMed: 22709987]
11. Zhang XQ, Sun S, Lam KF, Kiang KMY, Pu JKS, Ho ASW, Lui WM, Fung CF, Wong TS, Leung GKK. Neurobiol Dis. 2013; 58:123–31. [PubMed: 23726844]
12. Graybill RM, Bailey RC. Anal Chem. 2016; 88:431–450. [PubMed: 26654257]
13. Pritchard CC, Cheng HH, Tewari M. Nat Rev Genet. 2012; 13:358–369. [PubMed: 22510765]
14. Iqbal M, Gleeson MA, Spaugh B, Tybor F, Gunn WG, Hochberg M, Baehr-Jones T, Bailey RC, Gunn LC. IEEE J Sel Top Quantum Electron. 2010; 16:654–661.
15. Washburn AL, Gunn LC, Bailey RC. Anal Chem. 2009; 81:9499–9506. [PubMed: 19848413]
16. Qavi AJ, Bailey RC. Angew Chemie Int Ed. 2010; 49:4608–4611.
17. Graybill RM, Para CS, Bailey RC. Anal Chem. 2016; 88:10347–10351. [PubMed: 27726377]
18. Kindt JT, Bailey RC. Anal Chem. 2012; 84:8067–74. [PubMed: 22913333]
19. Scheler O, Kindt JT, Qavi AJ, Kaplinski L, Glynn B, Barry T, Kurg A, Bailey RC. Biosens Bioelectron. 2012; 36:56–61. [PubMed: 22541813]
20. Gyllenstein UB, Erlich HA. Proc Natl Acad Sci U S A. 1988; 85:7652–6. [PubMed: 3174659]
21. Sanchez JA, Pierce KE, Rice JE, Wangh LJ. Proc Natl Acad Sci. 2004; 101:1933–1938. [PubMed: 14769930]
22. Markham NR, Zuker M. Nucleic Acids Res. 2005; 33:W577–W581. [PubMed: 15980540]
23. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. BMC Bioinformatics. 2012; 13:134. [PubMed: 22708584]
24. Schmittgen TD, Livak KJ. Nat Protoc. 2008; 3:1101–8. [PubMed: 18546601]
25. Pang JCS, Li KKW, Lau KM, Ng YL, Wong J, Chung NYF, Li HM, Chui YL, Lui VWY, Chen Z, Chan DTM, Poon WS, Wang Y, Mao Y, Zhou L, Ng HK. Brain Pathol. 2010; 20:1021–1032. [PubMed: 20477830]
26. Wu Y, Huang C, Meng X, Li J. Curr Pharm Des. 2015; 21:5017–5028. [PubMed: 26205289]
27. Ji P, Diederichs S, Wang W, Böing S, Metzger R, Schneider PM, Tidow N, Brandt B, Buerger H, Bulk E, Thomas M, Berdel WE, Serve H, Müller-Tidow C. Oncogene. 2003; 22:8031–8041. [PubMed: 12970751]
28. Han Y, Wu Z, Wu T, Huang Y, Cheng Z, Li X, Sun T, Xie X, Zhou Y, Du Z. Cell Death Dis. 2016; 7:e2123. [PubMed: 26938295]
29. Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E. J Biotechnol. 1999; 75:291–5. [PubMed: 10617337]
30. Petryszak R, Keays M, Tang YA, Fonseca NA, Barrera E, Burdett T, Füllgrabe A, Fuentes AMP, Jupp S, Koskinen S, Mannion O, Huerta L, Megy K, Snow C, Williams E, Barzine M, Hastings E, Weisser H, Wright J, Jaiswal P, Huber W, Choudhary J, Parkinson HE, Brazma A. Nucleic Acids Res. 2016; 44:D746–D752. [PubMed: 26481351]
31. Kiang K, Zhang XQ, Leung G. Cancers (Basel). 2015; 7:1406–1424. [PubMed: 26230711]

Figure 1. Schematic outline of the overall assay for lncRNA detection including (A) reverse transcription, (B) asymmetric PCR, and (C) label-free, hybridization-based detection with microring resonator arrays.

Figure 2.

Verification of capture probe specificity. Each target was amplified individually in a separate volume of sample and then flowed across a sensor array containing capture probes for all targets. Large, specific hybridization responses were observed at appropriately-functionalized microring sensors for (A) KIAA0495, (B) MALAT1, and (C) β -actin. Buffer was initially flowed across the sensor array before the amplicon solutions were introduced at $t=5$ minutes. The solution was changed back to buffer after hybridization ($t=22$ minutes). Resonance shifts localized only to buffer/sample changes were due to differences in bulk solution refractive index.

Figure 3.

(A) Detection of the KIAA0495 aPCR product from healthy brain total RNA at different cycle numbers. The amplicon solutions from each cycle number were sequentially flowed across the sensor array separated by brief returns to hybridization buffer. Real-time resonance wavelength shifts are shown for microrings functionalized with target-specific (red) probes. Increasing hybridization responses are clearly observed with increasing cycle numbers. Error bars represent $\pm$ s.d. from $n=16$ replicate sensors. (B) Normalized hybridization response for KIAA0495 from healthy brain reference RNA as a function of cycle number. A $C(t)$ value was assigned as the cycle number corresponding to 40% of the maximum hybridization response.

Figure 4.

Amplification curves for the lncRNAs and internal control target measured (A) Healthy brain total RNA, (B) Healthy Lung total RNA, and (C) total RNA extracted from GBM6 cells, a glioblastoma patient-derived xenograft sample. Error bars represent $\pm$ s.d. from n=8-16 replicate measurements on the same chip.

Figure 5.

Comparison of RNA target fold changes using the combined aPCR-microring resonator approach relative to conventional, single-plex RT-qPCR. Fold changes were calculated by normalizing to a β -actin internal control. (A) Comparison of lncRNA expression in GBM6 using healthy brain tissue as reference. KIAA showed slightly increased expression in the GBM6 sample, relative to healthy brain, but MALAT showed reduced expression in the xenograft cell line. (B) Comparison of KIAA and MALAT1 expression in healthy lung using brain tissue as reference. Both KIAA and MALAT1 have slightly lower expression in lung tissue, relative to brain.

Table 1

Primers (RP, reverse primer; FP, forward primer) and capture probes (CP) used in the experiments for reverse transcription (RT), asymmetric PCR, and RT-qPCR. Melting temperatures for primers were calculated using the Primer-Blast platform.

Targeted transcript		Sequence (5′ → 3′)	Tm (° C)	Amplicon length
KIAA0495	RP (RT primer)	GCTGCTTGCTGTACGTGGTG	62.18	178 nt
	FP	CGTGGCTGACACAAACTTGC	60.59	
	CP	/5AmMC12/GCTGCTTGCTGTACGTGGTG		
MALAT1	RP (RT primer)	GTGATGAAGGTAGCAGGCGG	60.81	150 nt
	FP	ACATATTGCCGACCTCACGG	60.18	
	CP	/5AmMC12/GTGATGAAGGTAGCAGGCGG		
β-actin	RP (RT primer)	CATTCCAAATATGAGATGCGTTGT	58.18	103 nt
	FP	TGTGGACTTGGGAGAGGACT	59.81	
	CP	/5AmMC12/CATTCCAAATATGAGATGCGTTGT		
Off Target Control	CP	/5AmMC12/CTACAAGTGCCTTCACTGCAGT		