analytical chemistry

Article

pubs.acs.org/ac

DNAzyme-Based Rolling-Circle Amplification DNA Machine for Ultrasensitive Analysis of MicroRNA in *Drosophila* Larva

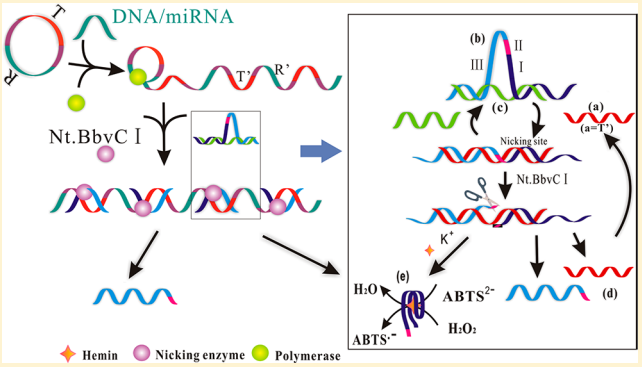
Yanqin Wen,[†] Yan Xu,[†] Xiuhai Mao,[†] Yingliang Wei,[‡] Haiyun Song,*[,‡] Nan Chen,[†] Qing Huang,[†] Chunhai Fan,[†] and Di Li*[,†]

[†]Laboratory of Physical Biology, Shanghai Institute of Applied Physics, Chinese Academy of Sciences, Shanghai 201800, China

[‡]Key Laboratory of Systems Biology, CAS, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

Supporting Information

ABSTRACT: We present a highly sensitive colorimetric method for microRNA (miRNA) detection. This method is based on a rolling-circle amplification (RCA) DNA machine, which integrates RCA, nicking enzyme signal amplification and DNAzyme signal amplification. The DNA machine is triggered by the hybridization of target miRNA with a rational designed padlock DNA template and activated by RCA. The resulting RCA product then autonomously replicates a multiple machinery cutter cycle and generates accumulated amount of products. Specifically, the DNA product in the present work is designed as a horseradish peroxidase (HRP)-mimicking DNAzyme, which could that catalyze a colorimetric reaction and generate colored product. Through these cascade amplifications, microRNA (miRNA) as low as 2 aM could be detected. As an example of in vivo application, miRNA from single *Drosophila* larva was successfully analyzed. *Drosophila* is a model organism that provides a powerful genetic tool to study gene functions. Study of *Drosophila* miRNAs has brought us knowledge of its biogenesis and biological functions. The analysis of miRNA typically requires a pretreatment process of extracting total RNAs from target cells, followed by quantitative analysis of target miRNA in total RNA samples, which nevertheless suffers from laborious total RNA extraction and time-consuming processes and poor limit of detection. Meanwhile, the tiny size of *Drosophila* makes it difficult to accurately measure trivial changes of its cellular miRNA levels. The ability to detect ultralow concentration of miRNA of the proposed method enables the analysis the expression of mir-1 in single *Drosophila* larva. We thus expect that the strategy may open new avenues for in situ miRNA analysis in single cell or living animals.



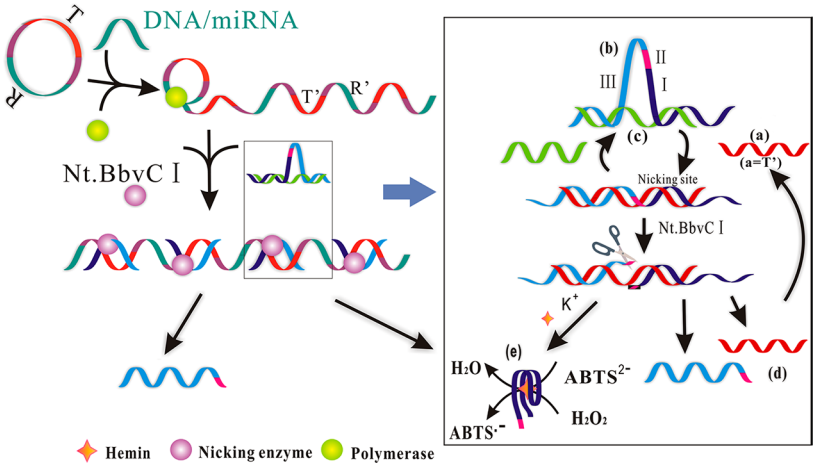
MicroRNAs (miRNAs) are a class of short (21−24 bases), noncoding RNAs that post-transcriptionally regulate downstream mRNA.[1] Accumulated evidence have shown that mis-regulated expression of miRNAs is closely correlated with cancer development and progression.[2−4] Particularly, the discovery of circulating miRNAs in human and model animals suggests that they may function as secreted signaling molecules to regulate remote recipient tissues and as potential biomarkers.[5,6] Hence, detection of miRNAs has intrigued great interest and shown high potential for clinical diagnostics.[7−9] *Drosophila* is a model organism that provides a powerful genetic tool to study gene functions. Study of *Drosophila* miRNAs has brought us knowledge of its biogenesis and biological functions, including their roles in cell growth, cell death, animal development, energy metabolism and stress response, etc.[10−12] However, the tiny size of *Drosophila* makes it difficult to accurately measure trivial changes of its cellular miRNA levels. Here, we report an ultrasensitive miRNA detection method based on a DNAzyme-based rolling-circle amplification (RCA) DNA machine, which provides a limit of detection (LOD) of 2 attomolar (aM) that enables analysis of miRNA in single *Drosophila* larva.

The analysis of miRNA typically requires a pretreatment process of extracting total RNA from target cell, followed by quantitative analysis of target miRNA in total RNA samples. Northern blot is the gold standard for the analysis of miRNA expression, which nevertheless suffers from laborious and time-consuming processes and poor LOD.[13] Several new strategies, including real time quantitative polymerase chain reaction (RT-qPCR),[14,15] rolling circle amplification[16−22] and high throughput microarrays,[23,24] have been developed for sensitive analysis of miRNA. However, the extremely short length of miRNAs complicated the design of PCR primers. In order to circumvent this difficulty, several signal amplification strategies have been developed, such as invasive signal amplification,[25,26] strand displacement amplification,[27] cascade enzymatic signal ampli-

Received: March 2, 2012
Accepted: August 28, 2012
Published: August 28, 2012

   dx.doi.org/10.1021/ac300616z | *Anal. Chem.* 2012, 84, 7664−7669

Scheme 1. Schematic Illustration the Principle of the RCA-Based DNA Machine for miRNA Detection[a]



[a]Briefly, hybridization of target miRNA (or DNA) with the circular DNA template activates RCA. As a result, the target miRNA (or DNA) is replicated to a long ss-DNA with periodic repeated sequence. Then each repeated sequence (T′ region) in the long ss-DNA further initiates an independent NESA amplification and generates HRP-mimicking DNAzyme products.

fication (CESA),[28] loop-mediated isothermal amplification (LAMP),[29] beacon assisted detection amplification (BAD-AMP),[30,31] Exonuclease III-aided signal amplification,[32] exponential isothermal amplification,[33] and nicking endonuclease signal amplification (NESA).[34−37] The essence of signal amplification is to amplify a target nucleic acid-specific signal, e.g. a DNA strand generated by specific hybridization with target nucleic acid, to detectable levels. Nevertheless, LODs of these amplification methods are typically in the range of 100 fM to 1 fM (~6×10^5 to 6×10^7 molecules/mL), which do not support the detection of miRNAs in few *Drosophila* larvae (~600 molecules/mL).

In the present work, we propose a machine-like amplification strategy for ultrasensitive analysis of miRNA. DNA machine or DNA nanodevice,[38,39] is a synthetic molecular machine that is constructed with DNA, which has found applications ranging from static supramolecular nanostructure[40,41] to movable mechanical nanodevices.[42,43] In this work, our amplification DNA machine is triggered by the hybridization of target miRNA with a rational designed DNA template, and then autonomously replicates a multiple machinery cutter that produce accumulated amount of products. Specifically, the DNA machine product in the present work is designed as a horseradish peroxidase (HRP)-mimicking DNAzyme, which, upon stacking with hemin, could catalyze the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{2-}$), by H_2O_2 to a colored product $ABTS^{-\bullet}$ as readout.[44] Through this multiple amplifications, target microRNA as low as 2 aM could be detected. The proposed method could successfully detect trace amount of miRNA extracted from single *Drosophila* larva.

EXPERIMENTAL SECTION

Materials. Nucleic acids (DNA and miRNA) were synthesized by TaKaRa Biotechnology Co. (Dalian, China) and purified by HPLC. The sequences of nucleic acids employed in this study were shown in Supporting Information Table S1. Total RNAs extracted from normal and cancer cells were purchased from Ambion with a stock solution of 1 $\mu g/\mu L$. Total RNA from 8 L3 stage wandering *Drosophila* larva was extracted with standard Trizol reagent. Nt.BbvCI was purchased from New England Biolabs (Beijing). Phi29 ($\phi 29$) DNA polymerase and buffer were purchased from fermentas.

miRNA Assay. The miRNA assay was initiated by hybridizing of circular DNA template (100 pM) with different concentrations of target miRNA. Then 20 unit of $\phi 29$ polymerase and 2.5 mM of dNTPs were incubated with the resulting mixture and incubated at 37 °C for 3 h in a buffer solution containing 33 mM Tris-Ac, 10 mM $MgCl_2$, 66 mM KAc, 1% Tween 20, 1 mM DTT (pH 7.9). Then the resulting mixture was heated to 80 °C for another 5 min to inactivate the RCA process. Then 20 μM of (**b**)/(**c**) duplex was added to the RCA product. The NESA was performed by incubating the resulting mixture with 10 unit of Nt. BbvC I at 37 °C for another 3 h in NEB buffer 2 (10 mM Tris-Cl, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT, pH 7.9) . Next, 20 mM of K^+ was added to facilitate the formation of G-quaduplex. The colorimetric assay was performed by adding 20 μM of hemin, 2 mM of $ABTS^{2-}$ and 50 mM of H_2O_2 to the mixture. The absorption of $ABTS^{-\bullet}$ at 416 nm was recorded as readout. All reactions were processing in a 0.2 mL PCR tube and the total volume of the reaction is 0.1 mL.

Clinical Relevant Human Cancer Total RNA and *Drosophila* Assay. The stock total RNA samples (1 $\mu g/\mu L$) from different human normal and cancer cells were diluted to 10 $ng/\mu L$. The content of let-7d in these cells was then analyzed with the proposed miRNA assay. Total RNA was extracted from 8 L3 stage wandering *Drosophila* larva using standard Trizol reagent and then diluted 10 and 100 times for further analysis. Exogenous let-7d (10 fM) was added to the *Drosophila* samples as an internal reference.

Instruments. Absorbance change of $ABTS^{2-}$ was recorded by Hitachi U-3010 UV−vis spectrophotometer.

RESULTS AND DISCUSSION

Principle of the RCA-Based Multiple Amplifications DNA Machine. The detailed principle of the proposed amplification machine is outlined in Scheme 1.We designed a circular DNA template with two functional regions (R and T). Region R (green) is a recognition region that is complementary to the target nucleic acid. Region T (red), embedded a (5′-*CCTCAGC*-3′) sequence, is a trigger region to initiate the

dx.doi.org/10.1021/ac300616z | *Anal. Chem.* 2012, 84, 7664−7669

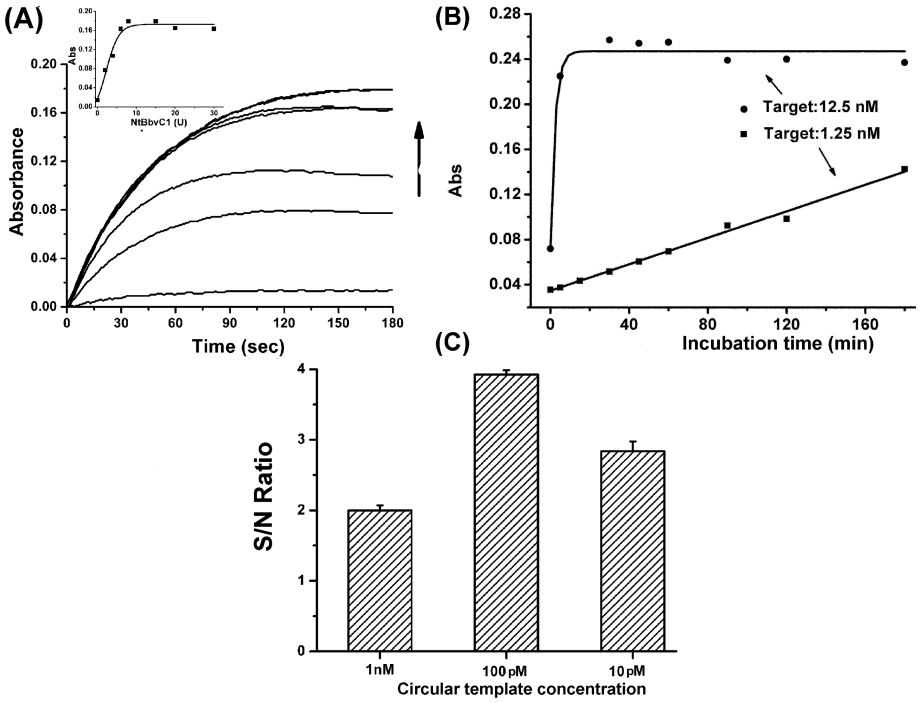


Figure 1. (A) The absorbance change of $ABTS^{2-}$ upon challenging (**b**)/(**c**) duplex (125 nM) with (**1**) (12.5 nM) and different amounts of Nt.BbvC I. From bottom to top, the amount of added Nt.BbvC I was 0 unit, 2 unit, 4 unit, 6 unit, 8 unit, 15 unit and 30 unit, respectively. Inset is the derived dose-dependent curve. (B) The incubation time-dependent cleavage of (**b**)/(**c**) duplex (12.5 nM) for high (12.5 nM) and low (1.25 nM) concentration of (**a**). (C) Comparison of signal-to-noise ratio of 10 fM of DNA (**a**) upon challenging the DNA machine with different concentration of circular template. Error bars are the standard deviations of measurements taken from three independent experiments.

amplification machine. Upon hybridization of region R with target nucleic acid, the amplification machine is activated by replication the circular DNA in the presence of ϕ29 DNA polymerase and dNTPs. As a result, target miRNA is replicated to a long single stranded (ss-) DNA with periodic repeated complementary parts of region R and T (named as R′ and T′). In principle, each repeated T′ region in this long ss-DNA could further initiate an independent step of NESA amplification. In NESA, signal amplification is achieved by a target-dependent cleavage of a signal DNA by nicking enzymes. Of note, nicking enzyme is a special family of restriction endonuclease, which recognizes a specific sequence along double-strand DNA and hydrolyzes only one specific strand instead of both strands, leaving a nick in the DNA strand. In the present study, nicking enzyme, Nt. BbvC I, which recognizes (5′-CCTCAGC-3′/3′-GGAGTCG-5′) and specifically cleaves the upper strand, is introduced to "recycle" the signal DNA. NESA, however, requires that the target should contain the recognition sequence of a nicking enzyme; thereby not appreciate for the analysis of random sequences. Herein, we integrated RCA with NESA, which not only provides a cascaded sequence and signal amplification, but also successfully surmounts the specific-sequence requirement of NESA.

Detailed principle of the NESA step is illustrated as the inset of Scheme 1. A DNA duplex (**b**/**c**) is added to initiate the NESA process. The DNA duplex is consisted of two ss-DNA (**b** and **c**). DNA (**b**) is composed of three functional regions. Region I (purple) is a G-quadruplex that forms the HRP-mimicking DNAzyme in the presence of hemin. Region II (pink, 5′-*CCTCAGC*-3′) is the core of NESA, which, upon hybridization with its complementary strand, forms the recognition site for nicking enzyme Nt. BbvC I. Region III (blue) is fully complementary with T′ region (or a ss-DNA named as (**a**)). DNA (**c**) is a "blocker" DNA that is partially complementary (7 bases) to region I and partially complementary (10 bases) to region III that minimizes the formation of free G-quadruplex structure. These two complementary parts coordinately stabilize this (**b**)/(**c**) duplex. If one hybridization end is separated, another end would not form stable DNA duplex at room temperature.[45] Region II is designed in the "pocket" of the (**b**)/(**c**) duplex and remained in single strand form; thereby could not be recognized and cleaved by Nt. BbvC I. Upon mixing this (**b**)/(**c**) duplex with the RCA products that contained replicated T′ region (or (**a**)), since T′ is fully complementary to region III in (**b**), hybridization of T′ with (**b**) would result both the release of (**c**) and formation of Nt. BbvC I recognition site. Consequently, (**a**)/(**b**) duplex is cleaved by Nt. BbvC I, resulting three free ss-DNA (**a**, **d**, and **e**). The fragment (**e**) contains region I and another 5 bases of region II that is remained after nicking, which forms G-quadruplex HRP-mimicking DNAzyme upon stacking with hemin, while the fragment (**d**) containing region III and another 2 bases in region II that is remained after nicking, is the "waste product". The released (**a**) could further react with another (**b**)/(**c**) duplex. Thus, one target nucleic acid could be first amplified to hundreds to thousands of repeated (**a**) by RCA. Then, each individual (**a**) could, in principle, initiate a Nt. BbvC I-induced strand-scission cycle that produces accumulated amounts of DNAzyme products. Next each DNAzyme product enzymatically catalyzes a colorimetric reaction. Finally, through these multiple amplifications, one target nucleic acid could generate increased detectable readout.

Optimizing Parameters to Improve the Assay Performance. The concentration of circular template, the amount of Nt. BbvC I and the nicking time play important role in the sensing performance. Therefore, we employed a ss-DNA (**a**),

dx.doi.org/10.1021/ac300616z | *Anal. Chem.* 2012, 84, 7664−7669

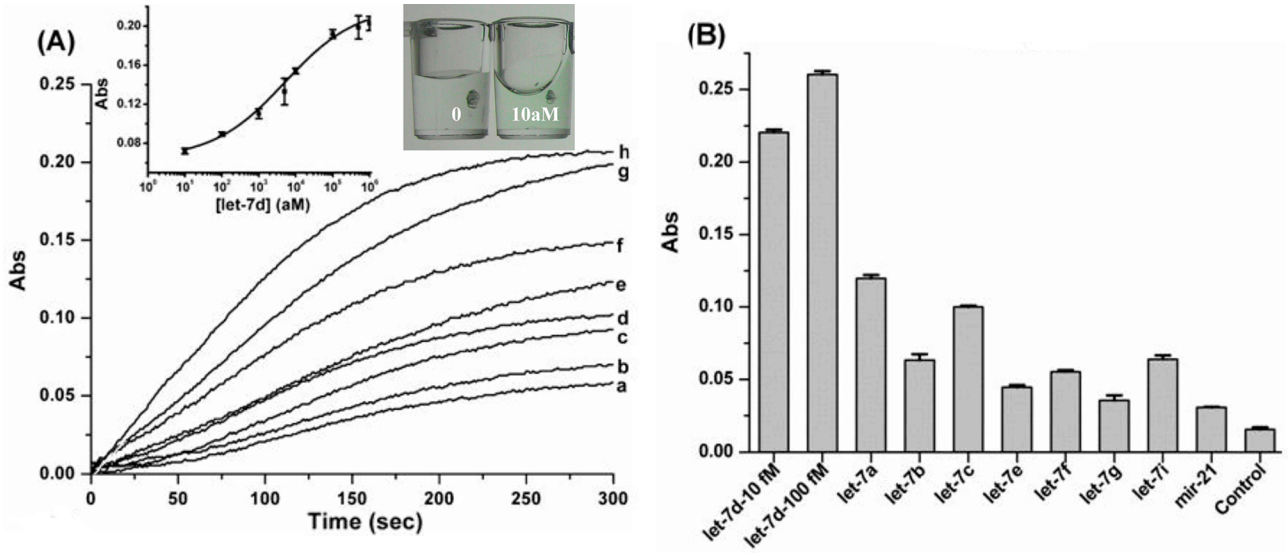


Figure 2. (A) Time-dependent absorbance changes of $ABTS^{\bullet-}$ upon challenging our machine with different concentrations of let-7d. (a–h): 0 M, 1×10^{-17} M, 1×10^{-16} M, 1×10^{-15} M, 5×10^{-15} M, 1×10^{-14} M, 1×10^{-13} M, 1×10^{-12} M. Inset is the derived calibration curve. Errorbars are the standard deviations of measurements taken from three independent experiments. (B) The selectivity of this assay toward let-7d (10 fM, 100 fM) over other miRNAs of let-7 family (100 fM) and a noncognate miRNA mir-21 (100 fM). The inset photos in Figure 2A shows the visual color changes of $ABTS^{2-}$ upon the oxidation of DNAzyme synthesized by the machine in the presence (10 aM) and absence of let-7d.

Table 1. Comparison of LOD of Different Signal Amplification-Based Nucleic Acid Assays

method	analyte	LOD	readout	reference
NESA	DNA	1 pM	fluorescence	35
RCA-assisted NESA	DNA	100 fM	fluorescence	34
nicking endonuclease assisted nanoparticle amplification (NEANA)	DNA	10 pM	color change	36
cascade enzymatic signal amplification (CESA)	DNA	1 fM	fluorescence	28
exonuclease III-assisted signal amplification	DNA	20 aM	fluorescence	32
beacon-assisted detection amplification (BAD-AMP)	DNA	88 fM	fluorescence	30, 31
Fok I-assisted signal amplification	DNA	10 fM	chemiluminescence	37
target-primed and branched RCA	miRNA let-7a	10 fM	fluorescence	18
exponential amplification reaction (EXPAR)	miRNA let-7a	0.1 aM	fluorescence	33
dumbbell probe-mediated RCA (D-RCA)	miRNA mir-21	1 fM	fluorescence	16
loop-mediated isothermal amplification (LAMP)	miRNA	1 pM	fluorescence	29
cascade RCA-NESA-DNAzyme amplification	DNA miRNA let-7d	2 aM	color change	present study

which has identical sequence with T′, to systematic investigated the several key parameters. This optimization was based on the following considerations: (1) optimized amount of nicking enzyme and nicking time that assures the sufficient cleavage of (**b**) in NESA; (2) optimized circular template concentration that favors the hybridization of target DNA with the circular template.

We first employed 12.5 nM of (**a**) to interrogate the NESA performance of (**b**)/(**c**) duplex (125 nM) in the presence of different amounts of Nt.BbvCI (Figure 1A). The absorbance change of $ABTS^{2-}$ increased dramatically with increasing amounts of Nt.BbvC I, indicating accumulated amounts of (**b**)/(**c**) duplex were cleaved and more DNAzyme products were generated. The absorbance change of $ABTS^{2-}$ leveled off above 10 unit Nt.BbvC I (Figure 1A, inset), indicating 10 unit of Nt.BbvC I could sufficiently cleave (**b**)/(**c**) duplex, therefore 10 unit of Nt.BbvC I was used in further optimizations. We then evaluated the nicking time to the NESA performance. We speculated that different concentrations of (**a**) might request different nicking time, thereby we chose low (1.25 nM) and high (12.5 nM) concentration of (**a**) to challenge the NESA assay in the presence of 10 unit of Nt.BbvC I. Indeed, we found the nicking time for the NESA process varies as different concentrations of (**a**) (Figure 1B). For higher concentration of (**a**) (12.5 nM, molar ratio of (**a**):(**b**)/(**c**) duplex is 1:1), the absorbance change leveled off after ca. 30 min of incubation, while for lower concentration of (**a**) (1.25 nM, molar ratio of (**a**): (**b**)/(**c**) duplex is 1:10), the absorbance change of $ABTS^{2-}$ still increased after 180 min of incubation, suggesting the hybridization and strand displacement of low concentration of (**a**) with (**b**)/(**c**) was kinetically slow. Since RCA might generate less amount of (**a**) for low concentration target, we thus chose 180 min (3 h) as the optimized incubation time.

With these two optimized NESA parameters, we further evaluated the concentration of circular template with 10 fM of target DNA (Figure 1C). We found that 100 pM of circular template possessed the highest signal-to-noise (S/N) ratio, while low concentration (10 pM) and high (1 nM) concentrations of circular template results poorer S/N ratio. Therefore we chose 100 pM of circular template in this study.

Sensing Performance of the Proposed DNA Machine for Synthetic miRNA let-7d. Under these optimized conditions, we first challenged this machine with DNA target and achieved excellent LOD (2 aM, Supporting Information

dx.doi.org/10.1021/ac300616z | *Anal. Chem.* 2012, 84, 7664–7669

Figure S1). We thus extended this strategy to the detection of synthesized miRNAs. Herein we employed synthetic miRNA let-7d, a member of let-7 miRNA family, to demonstrate our strategy for miRNA detection. The let-7 family is among the first two known human miRNAs, and highly conserved across species in sequence and function.[46] We designed a circular DNA template embedding a recognition region for let-7d. The proposed strategy reveals excellent response toward let-7d (Figure 2A). Higher concentration of let-7d results intensified color change and the LOD was calculated as 2.0 aM ($>3\sigma$) from the derived calibration curve (Figure 2A, inset). Table 1 summarizes the detection limits of some recent reported signal amplification nucleic acids assays. This comparison clearly highlighted the remarkable enhanced LOD of our strategy. Meanwhile, readout of the present method is color change, which could be easily recorded by UV/vis spectroscopy or even roughly determined by naked eyes (the inset digital images in Figure 2A inset show the color changes of $ABTS^{2-}$ induced by 0 and 10 aM of let-7d, respectively), revealing instrumental advantages over other assays that rely on fluorescent or chemiluminescent signals. The introduction of a RCA process prior to NESA plays a critical role in the present assay. In addition to its sequence amplification, the circular template could be designed to recognize any target of interest, thereby the sequence requirement of nicking enzyme recognition site in target nucleic acids is eliminated and the resulting RCA-NESA strategy could be applied for ultrasensitive detection of nucleic acids of random sequences. Moreover, the circular DNA template could embed multiple recognition regions that are complementary with different targets; therefore simultaneously detection of multiple analytes by using several nicking enzymes and different molecular beacons as readouts is envisioned.

Sequence-specificity in miRNA detection is of higher importance since miRNA families (e.g., let-7 family) often possess closely related sequences with high homology (a few bases difference). We challenged our assay with let-7d (10 fM) and other members of the let-7 family, let-7a, let-7b, let-7c, let-7e, let-7f, let-7 g, let-7i, and a noncognate miRNA sequence has-miR-21 (all of 100 fM) (Figure 2B). Clearly, let 7d could be easily differentiated from other let-7 family members, revealing good specificity.

Sensing Performance of the Proposed DNA Machine for Clinically Relevant Samples. The validity of our machine was further evaluated with clinically relevant samples. The response of let-7d in 10 ng of total RNA samples extracted from human liver node and HepG2 cells (Hepatocellular carcinoma, human), human prostate and PC3 cells (cell lines of prostate cancer), human lymph node and Raji cells (cell lines of Human Burkitt's lymphoma) was compared in Supporting Information Figure S2. We found that the expression of let-7d varied in different healthy tissues. Its expression in liver node was lower than in prostate and lymph. Meanwhile, its expression in HepG2 cells, PC3 cells and Raji cells are ca. 1.3, 3.1, and 3.1 fold higher than the corresponding healthy tissues, which correlated well with previous reports showing that let-7 acts as a tumor suppressor[47,48] and its expression is down regulated in prostate,[49] liver,[50,51] and lymph[52] derived from the standard $2^{-\Delta\Delta C_T}$ method in real time-PCR (RT-PCR).

Having established the highly sensitive selective assay and clinical validity, we employed this method for miRNA analysis in single *Drosophila* larva. We designed a circular DNA template embedding a recognition region for *Drosophila* mir-1. Figure 3 compares the response of mir-1 in control (buffer) and total RNA extracted from 8 L3 stage wandering larvae. In *Drosophila*, there is only single let-7 gene, and it becomes expressed only at the end of L3 wandering instar stage and peaks in pupae during metamorphogenesis,[53,54] thereby exogenous let-7d was added to the sample as an internal reference to demonstrate the specificity of our proposed strategy. Clearly, this assay easily detected mir-1 from 8 larvae, and the addition of let-7d internal reference (10 fM) to mir-1, 10 times and 100 times diluted mir-1 samples results less than 10% absorbance changes, suggesting excellent selectivity. In addition, 10 times and 100 times diluted mir-1 samples could still be easily differentiated from control experiment, demonstrating the ability of our strategy to analysis the expression of mir-1 in single *Drosophila* larva.

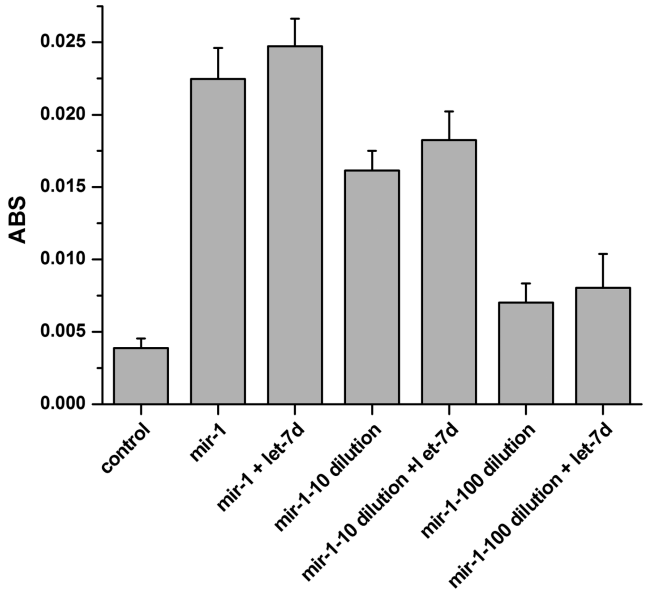


Figure 3. Response of miRNA mir-1 in total RNA extract from L3 stage wandering larva. Total RNA was extracted from 8 L3 stage wandering *Drosophila* larva using standard Trizol reagent and then diluted to 0 and 100 times for analysis. Exogenous let-7d (10 fM) was added to the sample as an internal reference.

CONCLUSIONS

In summary, we have developed a RCA-based DNA machine for ultrasensitive analysis of miRNA. The DNA machine integrated RCA, NESA and DNAzyme signal amplification, and a LOD as low as 2 aM was achieved. As an example of in vivo application, miRNA from single *Drosophila* larva was successfully analyzed. Our proposed amplification machine, triggered by target miRNA, could autonomously produce HRP-mimicking DNAzyme as products. The DNAzyme product catalyzes a colorimetric reaction, which not only further amplifies the analyte signal, but also avoids the introduction of any dye-labeled DNA that produces fluorescent readout. Meanwhile, this machine is operated isothermally, avoiding expensive PCR setup. We thus expect that the sensitive strategy might open new avenues for in situ miRNA analysis in single cell or living animals.

ASSOCIATED CONTENT

Supporting Information

Figure S1, Figure S2, and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

dx.doi.org/10.1021/ac300616z | *Anal. Chem.* 2012, 84, 7664−7669

AUTHOR INFORMATION

Corresponding Author

*E-mail: hysong@sibs.ac.cn, lidi@sinap.ac.cn. Fax: 86-21-39194702.

Author Contributions

The first two authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (21075128, 21073221, 21222508) and Chinese Academy of Sciences (KJCX2-EW-N03)

REFERENCES

(1) He, L.; Hannon, G. J. *Nat. Rev. Genet.* **2004**, *5*, 522−531.
(2) Calin, G. A.; Croce, C. M. *Nat. Rev. Cancer* **2006**, *6*, 857−866.
(3) Lu, J.; Getz, G.; Miska, E. A.; Alvarez-Saavedra, E.; Lamb, J.; Peck, D.; Sweet-Cordero, A.; Ebet, B. L.; Mak, R. H.; Ferrando, A. A.; Downing, J. R.; Jacks, T.; Horvitz, H. R.; Golub, T. R. *Nature* **2005**, *435*, 834−838.
(4) Kumar, M. S.; Lu, J.; Mercer, K. L.; Golub, T. R.; Jacks, T. *Nat. Genet.* **2007**, *39*, 673−677.
(5) Chen, X.; Ba, Y.; Ma, L. J.; Cai, X.; Yin, Y.; Wang, K. H.; Guo, J. G.; Zhang, Y. J.; Chen, J. N.; Guo, X.; Li, Q. B.; Li, X. Y.; Wang, W. J.; Zhang, Y.; Wang, J.; Jiang, X. Y.; Xiang, Y.; Xu, C.; Zheng, P. P.; Zhang, J. B.; Li, R. Q.; Zhang, H. J.; Shang, X. B.; Gong, T.; Ning, G.; Zen, K.; Zhang, J. F.; Zhang, C. Y. *Cell Res.* **2008**, *18*, 997−1006.
(6) Li, L. M.; Hu, Z. B.; Zhou, Z. X.; Chen, X.; Liu, F. Y.; Zhang, J. F.; Shen, H. B.; Zhang, C. Y.; Zen, K. *Cancer Res.* **2010**, *70*, 9798−9807.
(7) Hammond, S. M. *Nat. Methods* **2006**, *3*, 12−13.
(8) Cissell, K. A.; Shrestha, S.; Deo, S. K. *Anal. Chem.* **2007**, *79*, 4754−4761.
(9) Cissell, K. A.; Deo, S. K. *Anal. Bioanal. Chem.* **2009**, *394*, 1109−1116.
(10) Enright, A. J.; John, B.; Gaul, U.; Tuschl, T.; Sander, C.; Marks, D. S. *Genome Bio.* **2004**, *5*, R1.
(11) Hwang, H. W.; Mendell, J. T. *Br. J. Cancer* **2006**, *94*, 776−780.
(12) Jovanovic, M.; Hengartner, M. O. *Oncogene* **2006**, *25*, 6176−6187.
(13) Lagos-Quintana, M.; Rauhut, R.; Lendeckel, W.; Tuschl, T. *Science* **2001**, *294*, 853−858.
(14) Chen, C. F.; Ridzon, D. A.; Broomer, A. J.; Zhou, Z. H.; Lee, D. H.; Nguyen, J. T.; Barbisin, M.; Xu, N. L.; Mahuvakar, V. R.; Andersen, M. R.; Lao, K. Q.; Livak, K. J.; Guegler, K. J. *Nucleic Acids Res.* **2005**, *33*, e179.
(15) Benes, V.; Castoldi, M. *Methods* **2010**, *50*, 244−249.
(16) Zhou, Y. T.; Huang, Q.; Gao, J. M.; Lu, J. X.; Shen, X. Z.; Fan, C. H. *Nucleic Acids Res.* **2010**, *38*, e156.
(17) Zhao, W. A.; Ali, M. M.; Brook, M. A.; Li, Y. F. *Angew. Chem., Int. Ed.* **2008**, *47*, 6330−6337.
(18) Cheng, Y. Q.; Zhang, X.; Li, Z. P.; Jiao, X. X.; Wang, Y. C.; Zhang, Y. L. *Angew. Chem., Int. Ed.* **2009**, *48*, 3268−3272.
(19) Koster, D. M.; Haselbach, D.; Lehrach, H.; Seitz, H. *Mol. Biosyst.* **2011**, *7*, 2882−2889.
(20) Chapin, S. C.; Doyle, P. S. *Anal. Chem.* **2011**, *83*, 7179−7185.
(21) Bi, S.; Li, L.; Zhang, S. S. *Anal. Chem.* **2010**, *82*, 9447−9454.
(22) Xu, W.; Xie, X. J.; Li, D. W.; Yang, Z. Q.; Li, T. H.; Liu, X. G. *Small* **2012**, *8*, 1846−1850.
(23) Liu, C. G.; Calin, G. A.; Meloon, B.; Gamliel, N.; Sevignani, C.; Ferracin, M.; Dumitru, C. D.; Shimizu, M.; Zupo, S.; Dono, M.; Alder, H.; Bullrich, F.; Negrini, M.; Croce, C. M. *Proc. Natl. Acad. Sci.U.S.A.* **2004**, *101*, 9740−9744.
(24) Lee, J. M.; Jung, Y. W. *Angew. Chem., Int. Ed.* **2011**, *50*, 12487−12490.
(25) Eis, P. S.; Olson, M. C.; Takova, T.; Curtis, M. L.; Olson, S. M.; Vener, T. I.; Ip, H. S.; Vedvik, K. L.; Bartholomay, C. T.; Allawi, H. T.; Ma, W. P.; Hall, J. G.; Morin, M. D.; Rushmore, T. H.; Lyamichev, V. I.; Kwiatkowski, R. W. *Nat. Biotechnol.* **2001**, *19*, 673−676.
(26) Hall, J. G.; Eis, P. S.; Law, S. M.; Reynaldo, L. P.; Prudent, J. R.; Marshall, D. J.; Allawi, H. T.; Mast, A. L.; Dahlberg, J. E.; Kwiatkowski, R. W.; de Arruda, M.; Neri, B. P.; Lyamichev, V. I. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8272−8277.
(27) Weizmann, Y.; Beissenhirtz, M. K.; Cheglakov, Z.; Nowarski, R.; Kotler, M.; Willner, I. *Angew. Chem., Int. Ed.* **2006**, *45*, 7384−7388.
(28) Zou, B. J.; Ma, Y. J.; Wu, H. P.; Zhou, G. H. *Angew. Chem., Int. Ed.* **2011**, *50*, 7395−7398.
(29) Li, C. P.; Li, Z. P.; Jia, H. X.; Yan, J. L. *Chem. Commun.* **2011**, *47*, 2595−2597.
(30) Connolly, A. R.; Trau, M. *Angew. Chem., Int. Ed.* **2010**, *49*, 2720−2723.
(31) Connolly, A. R.; Trau, M. *Nat. Protoc.* **2011**, *6*, 772−778.
(32) Zuo, X. L.; Xia, F.; Xiao, Y.; Plaxco, K. W. *J. Am. Chem. Soc.* **2010**, *132*, 1816−1818.
(33) Jia, H. X.; Li, Z. P.; Liu, C. H.; Cheng, Y. Q. *Angew. Chem., Int. Ed.* **2010**, *49*, 5498−5501.
(34) Li, J. W. J.; Chu, Y. Z.; Lee, B. Y. H.; Xie, X. L. S. *Nucleic Acids Res.* **2008**, *36*, e36.
(35) Kiesling, T.; Cox, K.; Davidson, E. A.; Dretchen, K.; Grater, G.; Hibbard, S.; Lasken, R. S.; Leshin, J.; Skowronski, E.; Danielsen, M. *Nucleic Acids Res.* **2007**, *35*, e117.
(36) Xu, W.; Xue, X. J.; Li, T. H.; Zeng, H. Q.; Liu, X. G. *Angew. Chem., Int. Ed.* **2009**, *48*, 6849−6852.
(37) Weizmann, Y.; Cheglakov, Z.; Willner, I. *J. Am. Chem. Soc.* **2008**, *130*, 17224−17225.
(38) Seeman, N. C. *Annu. Rev. Biochem.* **2010**, *79*, 65−87.
(39) Bath, J.; Turberfield, A. J. *Nat. Nanotechnol.* **2007**, *2*, 275−284.
(40) Han, D. R.; Pal, S.; Liu, Y.; Yan, H. *Nat. Nanotechnol.* **2010**, *5*, 712−717.
(41) Li, D.; Song, S. P.; Fan, C. H. *Acc. Chem. Res.* **2010**, *43*, 631−641.
(42) Lund, K.; Manzo, A. J.; Dabby, N.; Michelotti, N.; Johnson-Buck, A.; Nangreave, J.; Taylor, S.; Pei, R. J.; Stojanovic, M. N.; Walter, N. G.; Winfree, E.; Yan, H. *Nature* **2010**, *465*, 206−210.
(43) Song, S. P.; Qin, Y.; He, Y.; Huang, Q.; Fan, C. H.; Chen, H. Y. *Chem. Soc. Rev.* **2010**, *39*, 4234−4243.
(44) Willner, I.; Shlyahovsky, B.; Zayats, M.; Willner, B. *Chem. Soc. Rev.* **2008**, *37*, 1153−1165.
(45) Li, D.; Shlyahovsky, B.; Elbaz, J.; Willner, I. *J. Am. Chem. Soc.* **2007**, *129*, 5804−5805.
(46) Roush, S.; Slack, F. J. *Trends Cell Biol.* **2008**, *18*, 505−516.
(47) Boyerinas, B.; Park, S. M.; Hau, A.; Murmann, A. E.; Peter, M. E. *Endocr.-Relat. Cancer* **2010**, *17*, F19−F36.
(48) Zhang, B. H.; Pan, X. P.; Cobb, G. P.; Anderson, T. A. *Dev. Biol.* **2007**, *302*, 1−12.
(49) Porkka, K. P.; Pfeiffer, M. J.; Waltering, K. K.; Vessella, R. L.; Tammela, T. L. J.; Visakorpi, T. *Cancer Res.* **2007**, *67*, 6130−6135.
(50) Jin, H. J.; Lv, S. Q.; Yang, J. H.; Wang, X. N.; Hu, H. Z.; Su, C. Q.; Zhou, C. L.; Li, J.; Huang, Y.; Li, L. F.; Liu, X. Y.; Wu, M. C.; Qian, Q. J. *PloS One* **2011**, *6*, 10.
(51) Tzur, G.; Israel, A.; Levy, A.; Benjamin, H.; Meiri, E.; Shufaro, Y.; Meir, K.; Khvalevsky, E.; Spector, Y.; Rojansky, N.; Bentwich, Z.; Reubinoff, B. E.; Galun, E. *PloS One* **2009**, *4*, 13.
(52) Yu, C. C.; Chen, Y. W.; Chiou, G. Y.; Tsai, L. L.; Huang, P. I.; Chang, C. Y.; Tseng, L. M.; Chiou, S. H.; Yen, S. H.; Chou, M. Y.; Chu, P. Y.; Lo, W. L. *Oral Oncol.* **2011**, *47*, 202−210.
(53) Pasquinelli, A. E.; Reinhart, B. J.; Slack, F. J.; Martindale, M. Q.; Kuroda, M. I.; Maller, B.; Hayward, D. C.; Ball, E. E.; Degnan, B.; Muller, P.; J., S.; Srinivasan, A.; Fishman, M.; Finnerty, J.; Corbo, J.; Levine, M.; Leahy, P.; Davidson, E.; Ruvkun, G. *Nature* **2000**, *408*, 86−89.
(54) Bashirullah, A.; Pasquinelli, A. E.; Kiger, A. A.; Perrimon, N.; Ruvkun, G.; Thummel, C. S. *Dev. Biol.* **2003**, *259*, 1−8.