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1 Exponential Strand-Displacement Amplification for Detection of microRNAs

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25ABSTRACT

26 MicroRNAs are promising targets for disease diagnosis. However, miRNA
 27detection requires rapid, sensitive and selective detection to be effective as a
 28diagnostic tool~~To take full advantage of the great potential of miRNAs as biomarkers~~
 29~~for disease diagnosis, simple, rapid and sensitive methods for miRNA detection~~
 30~~should be developed~~. Herein, a miRNA-initiated exponential strand-displacement
 31amplification (SDA) assay was reported. ~~With the Klenow fragment, and the nicking~~
 32~~enzyme *Nt.AlwI*, and two primers, the miRNA target can trigger triggered two cycles~~
 33~~of nicking, polymerization, and displacement reactions, using two strategically~~
 34~~designed primers~~. These reaction cycles amplified the target miRNA exponentially
 35and generated dsDNAs detectable with SYBR Green I in real-time PCR. As low as
 3616 zmol of the target miRNA was detected by this one-pot assay within 90 minutes,
 37and the dynamic range spanned over 9 orders of magnitude. ~~Negligible impact from~~
 38~~the complex biological matrix was observed on the amplification reaction, indicating~~
 39~~high potential of our assay to detect circulating miRNAs in biofluids~~the assay's ability
 40to directly detect miRNA levels in biofluids.

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46**Keywords:** microRNAs; Exponential strand-displacement amplification;
 47Homogeneous detection; Isothermal amplification□nicking enzyme

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49 MicroRNAs (miRNAs, ~ 22 nt) regulate gene expression by binding to target
 50mRNAs, which can either inhibit translation or induce degradation of target
 51transcripts. Such regulatory roles render miRNAs ~~capable of possible capability to~~
 52~~modulating~~ tumor development, progression and metastasis.¹⁻⁷ Compared to
 53proteins, miRNAs have simpler structure and less complex ~~post-synthetic~~
 54processing. These features make miRNAs promising biomarkers for cancer
 55screening. Sensitive detection of the expression levels of miRNAs in biological
 56samples or clinical specimens is thus of great importance to cancer diagnosis,
 57enabling timely disease intervention and enhance the survival rate of patients.⁸

58 The short length of miRNAs and their low abundance in biological samples
 59~~increase~~s the difficulty in miRNA detection.⁹ ~~Conventional methods, including~~
 60~~northern blotting, real-time PCR, and microarrays, possess limitations like such as~~
 61~~insufficient sensitivity, tedious sample preparation and analysis, long and tedious~~
 62~~process, requirement of sophisticated instruments, sophisticated instrumentation, and~~
 63~~high operation expenses, etc.~~⁷ In the past decade, many new methods have been
 64developed to improve miRNA detection. Among them, isothermal amplification of
 65miRNAs using a combination of DNA polymerases and processing enzymes have
 66attracted great attention, because of their high detection sensitivity, fast reaction
 67speed, and low operational cost.¹²⁻¹⁷ However, oligonucleotides with sophisticated
 68designs, like the dumbbell-shaped probe,¹⁴ circular template,¹³ hairpin structures,
 69fluorescently labeled strands,¹⁶ etc., are required. ~~Or, nanomaterials are employed to~~

70 ~~assist with signaling~~ [Nanomaterials may also be used to aid signaling.](#)¹⁵ Alternatively,
 71 isothermal amplifications originally designed for DNAs, such as the loop-mediated
 72 isothermal amplification (LAMP)¹⁸ and the exponential amplification reaction
 73 (EXPAR)¹⁹ can be applied to detect miRNAs with strategic modifications to the
 74 hybridization probes.

75 Herein, we reported a very simple and ultrasensitive detection method for
 76 miRNAs that was established upon the exponential strand-displacement amplification
 77 (SDA) originally designed for dsDNAs.²² Common strand-displacement reactions
 78 amplify target nucleic acids linearly,²³⁻²⁵ but exponential SDA overcome this limitation
 79 by employing four primers. Our present study demonstrated that, for detection of
 80 miRNAs, only two primers were needed to ~~achieve to same exponential amplification~~
 81 ~~effect by SDA,~~ [comparable exponential amplification to SDA,](#) which rendered sensitive
 82 detection of the target miRNA in complex biological matrix. Deliberate selection of
 83 the polymerase and the nicking enzyme enabled one-pot, rapid amplification under
 84 one mild reaction temperature, offering high potential for point-of-care diagnosis.

85 Scheme 1 illustrates the steps of the miRNA-initiated SDA. Exponential
 86 amplification is achieved with two repeated cycles of nicking, polymerization and
 87 displacement reactions, each assisted by one primer. Once the target miRNA
 88 hybridizes to the 3' end of Primer 1, the Klenow fragment polymerase (exo⁻) extends
 89 both Primer 1 and the miRNA, producing a chimeric double-stranded fragment.
 90 ~~Followed Next,~~ [Nt.AflwI](#) creates a strand break on Primer 1 at the site 4 random
 91 [nucleotides downstream of the recognition sequence](#). This break then primes the first

92 amplification cycle of displacement, nicking and polymerization, producing ssDNA
 93 T*. T* is complementary to the target miRNA. T* hybridizes with Primer 2 at its 3'
 94 end, and both are extended by the Klenow fragment. Primer 2 also includes the
 95 recognition sequence of *Nt.AlwI* at its 5' end, so that the second amplification cycle
 96 can be triggered. The displaced ssDNA, has the same sequence as that of the target
 97 miRNA but with all U nucleotides replaced by T. The two amplification cycles
 98 continue to produce T and T*, which hybridize and form dsDNAs easily detectable by
 99 SYBR Green I.

100 **The feasibility of our assay was demonstrated by testing the products of each**
 101 **reaction cycle by PAGE** (Figure 1). The reaction was initiated by the 22-nt miRNA
 102 *Let-7a* (sequence listed in Table 1 with other oligonucleotides used in this study).
 103 Lane 1 showed that *Let-7a* migrated slightly faster than the 20-bp band of the DNA
 104 ladder (Lane M), but its hybridization product with Primer 1 moved slower (Lane 2).
 105 With addition of the Klenow fragment (exo⁻), a chimeric DNA/RNA hybrid of 32 bp
 106 was formed (Lane 3), appearing close to the 40-bp band of the ladder. This band was
 107 much brighter than that in Lane 2, because successful extension of both the target
 108 miRNA and Primer 1 generated dsDNAs that gave out strong signal with SYBR
 109 Green I compared to ssDNAs. Once the nicking enzyme, *Nt.AlwI*, was added, the 32-
 110 bp fragment was cut, leaving a gap on this extension product (Lane 4). The Klenow
 111 fragment then grew a new strand to displace the original extension product T* off. In
 112 Lane 4, the lower band that migrated similarly to the 20-bp fragment of the ladder
 113 should be the hybridized product of T* and *Let-7a*, because a relatively high

114concentration of the target miRNA was used for visualization on the gel. At last, we
 115mixed T, Primer 1, Klenow, *Nt.AlwI*, and Primer 2 to trigger both amplification
 116cycles. Although ~~with~~ having a ten-fold lower target concentration, the exponential
 117amplification generated more of the 32-bp products than with the single cycle in Lane
 1184.

119 The Klenow fragment has no 3'→5' exonuclease activity, and cannot remove
 120non-templated 3'addition. It is possible that 1-3 nucleotides would be randomly
 121added to the end of the extension product from Primer 1,²⁸ and participate in
 122hybridization with Primer 2. This could be problematic because the amplification
 123cycle would be stopped if fully complementary binding with Primer 2 was not
 124formed. Since the random overhangs would hybridize with the 4 nucleotides between
 125the cleavage site and the recognition sequence for *Nt.AlwI*, we optimized their
 126sequences to obtain the best amplification effect. Based on the study conducted by
 127Garcia *et al.*,²⁸ addition of dATP was 5 times faster than other dNTPs. Indeed, a T
 128base upstream to the cleavage site in both primers increased the amplification rate
 129(Supporting Information Figure S1). ~~Though~~ However, increasing the number of T
 130base did not ~~further speed up the reaction~~ improve the rate further. Thus, 5'-CTCT-3'
 131and 5'-GTCT-3' were chosen to flank the recognition region and the cleave site on
 132Primer 1 and Primer 2, respectively, in the following studies. Additionally, the
 133concentrations of both enzymes used in our assay were optimized (Supporting
 134Information Figure S2).

135 With the appropriate primer design and optimized enzyme concentrations, we

136tested the sensitivity and specificity of the assay. Both enzymes and the two primers
 137were mixed with *Let-7a*, dNTPs, and SYBR Green I in 1×NEB buffer 2, yielding a
 138total reaction volume of 10 µL. The reaction temperature was set at 37 °C in the
 139CFX96™ Real-Time PCR detection system (Bio-Rad); and every reaction cycle
 140lasted one minute, which included a 30-s scan to record the increase of fluorescence
 141and 30 s interval time. Within 90 minutes, 1.0 pmol to 0.1 zmol *Let-7a* were detected
 142(Figure 2). The C_q values were linearly related to the negative logarithmic (lg) value
 143of the miRNA amount ($R^2=0.9865$). The limit of detection was calculated to be 16
 144zmol using the 3σ method.

145 To evaluate the specificity of our design, 0.1 pmol of selected members of the *Let-*
 1467 miRNA family (*Let-7a*, 7b, 7c, 7e and 7i; sequences in Table 1) at the amount of 0.1
 147pmol were detected by the primer pair designed for using the *Let-7a* primer pair.
 148Figure 3 showed that, the C_t value for *Let-7a* was significantly smaller than that for
 149*Let-7e*, -7b, and -7i, which were listed here in the order of increasing C_t values. The
 150sequence of *Let-7e* differs from *Let-7a* with only one U (in 7a)→ G (in 7e) mutation,
 151but the mutation locates close to the 3' end of Primer 2. Extension of Primer 2 was
 152thus disturbed, leading to low yield in the second amplification cycle. This also
 153proved that the overall amplification effect should rely on successful progression of
 154both cycles. However, it is difficult to distinguish between *Let-7a* and -7c, which also
 155differ in only one nucleotide. The mutated base located in the middle of the
 156hybridization region between primer 1 and *Let-7a*, which had low influence to the
 157extension of Primer 1.

158 It has been known that miRNAs can be released into the circulation system;²⁹⁻
 159³² and accumulating evidences also show that circulating miRNAs exhibit varied
 160patterns between cancer patients and healthy controls.³³⁻³⁵ Thus, it will be highly
 161useful for cancer diagnosis if our assay can be used to detect the levels of circulating
 162miRNAs in biofluids like serum. To demonstrate this, we spiked 0.1 fmol to 0.1 pmol
 163*Let-7a* into the commercial human serum (Lonza Biowhittaker human serum from
 164Fisher Scientific), in which the endogenous *Let-7a* was not detectable using the
 165Taqman® RT-PCR kit for miRNAs (Life Technologies, Inc.). The serum was diluted
 1662 times in the 10-μL reaction mixture. Using the same reaction conditions as
 167mentioned above, the Cq values increased linearly with the negative logarithmic value
 168of the amount of target miRNA, and the regression equation was found to be: Cq =
 169-130.1125 + 10.35186 (-lgA_{*Let-7a*}) (A_{*Let-7a*} was the concentration of *Let-7a*, R²=0.9681)
 170(see Figure S3 in the Supporting Information). This relationship was almost the same
 171as that for the standard curve obtained at the same time (Cq = -130.718 + 10.426 (-
 172lgA_{*Let-7a*}), R²=0.9901), proving that the reaction was not affected by the complex
 173components in serum. The method possesses good ability of anti-jamming in biofluids
 174like serum. Owing to the potential for the use of serum miRNAs as biomarkers of
 175disease and as targets of therapeutics, so our method is promising as a non-invasive,
 176highly sensitive, and accurate diagnosis for cancer.

177 In summary, the a one-pot, miRNA-assisted exponential SDA for quick and easy
 178detection of miRNAs in biological samples has been developed. It yielded low limit
 179of detection, has a large detection range, and provides good selectivity. Our method

180only requires two non-labeled primers with very simple design, yields exponential
 181amplification, and performs at a mild temperature of 37 °C. In contrast to previously
 182detecting microRNA methods, our method does not require any modified DNA, and can be ~~real-~~
 183~~timely~~ detected by in real time using SYBR Green I as the fluorescent dye. This greatly reduced
 184the detection cost. Moreover, the method posses good ability of anti-jamming iis resistant to
 185matrix interference, even in complex biofluids like such as serum. This simple and cost-effective
 186method has the potential to become an attractive tool for quantitative analysis of miRNAs
 187(biomarkers) in tissues or cells and gives valuable information for the fields of basic and clinic
 188research and diagnostics.

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195**Table 1. Sequences of DNA and RNA in experiment.**

miRNA	Sequence (5' to 3') ^a
<i>Let-7a</i>	UGAGGUAGUAGGUUGUAUAGUU
<i>Let-7b</i>	UGAGGUAGUAGGUUGUG <u>UG</u> GUU
<i>Let-7c</i>	UGAGGUAGUAGGUUGU <u>A</u> UGGUU
<i>Let-7e</i>	UGAGGUAG <u>G</u> AGGUUGUAUAGUU
<i>Let-7i</i>	UGAGGUAGUAG <u>U</u> UUGUG <u>CU</u> GUU
Primer 1	<u>CGGATC</u> CTCTTAACTATACAACC
Primer 2	<u>CGGATC</u> GTCTTTAGGTTAGTAGG
^a The portions underlined in primers indicate the recognition site of the nicking enzyme <i>Nt.AlaI</i> and the arrowheads (!) show the cutting site. The italic portions in Primer1 and 2 are the complementary sequence T and T*, respectively. The underlined and italic bases in the <i>Let</i> miRNAs are the bases different than those in <i>Let-7a</i> .	

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Scheme 1. Schematic illustration of exponential SDA initiated by miRNA *Let-7a*. The sequences of the target and primers are listed in Table 1.

Figure 1. The result of analyzing the amplification products by PAGE (17.5% polyacrylamide). **M:** 20-bp DNA ladder; **1:** 1.0×10^{-5} M Target; The product of amplification reaction in **2:** 10 μ L mixture containing 1.0×10^{-6} M Primer 1 and 1.0×10^{-6} M the target, 0.3 μ L 2.5 mM dNTPs, 1 $\times$ NEB buffer 2; **3:** the mixture in Lane 2 + 0.1 μ L Klenow fragment (exo⁻); **4:** the mixture in Lane 3 + 0.6 μ L *Nt.AlaI*; **5:** the mixture in Lane 4 but with a ten-fold lower target concentration + 1.0×10^{-6} M Primer

Figure 3. Real-time fluorescence curves for the exponential SDA amplification were triggered by 1.0×10^{-13} mol *Let-7a*, *Let-7b*, *Let-7c*, *Let-7e* and *Let-7i*, respectively. The other experimental conditions are the same as those for Figure 2.

Supporting Information

232 This material is available free of charge via the Internet at <http://pubs.acs.org>.

233REFERENCES

- 234(1) Chowdhury, D., Choi, Y.E. & Brault, M.E. *Nat. Rev. Mol. Cell Biol.* 2013, **14**, 181-189.
- 235(2) Wahlestedt, C. *Nat. Rev. Drug Discovery* 2013, **12**, 433-440.
- 236(3) Hauptman, N. & Glavac, D. *Int. J. Mol. Sci.* 2013, **14**, 4655-4669.
- 237(4) Kim, T. & Reitmair, A. *Int. J. Mol. Sci.* 2013, **14**, 4934-4968.
- 238(5) Nicoloso, M.S., Spizzo, R., Shimizu, M., Rossi, S. & Calin, G.A. *Nat. Rev. Cancer* 2009, **9**, 293-239302.
- 240(6) Volinia, S., *et al.* *Genome Res.* 2010, **20**, 589-599.
- 241(7) Ventura, A. & Jacks, T. *Cell* 2009, **136**, 586-591.
- 242(8) Wark, A.W., Lee, H.J. & Corn, R.M. *Angew Chem Int Ed* 2008, **47**, 644-652.
- 243(9) Valoczi, A., *et al.* *Nucleic Acids Res* 2004, **32**, e175.
- 244(10) Lagos-Quintana, M., Rauhut, R., Lendeckel, W. & Tuschl, T. *Science* 2001, **294**, 853-858.
- 245(11) Lee, I., *et al.* *Nucleic Acids Res* 2008, **36**, e27.
- 246(12) Duan, R., *et al.* *J. Am. Chem. Soc.* 2013, **135**, 4604-4607.
- 247(13) Yao, B., *et al.* *Rna* 2009, **15**, 1787-1794.
- 248(14) Bi, S., Cui, Y. & Li, L. *Anal. Chim. Acta* 2013, **760**, 69-74.
- 249(15) Dong, H., *et al.* *Anal. Chem.* 2012, **84**, 4587-4593 (2012).
- 250(16) Tian, T., *et al.* *Chem. Commun.* 2013, **49**, 75-77 (2013).
- 251(17) Wang, G.-l. & Zhang, C.-y. *Anal. Chem.* 2012, **84**, 7037-7042.
- 252(18) Kim, J. & Easley, C.J. *Bioanalysis* 2011, **3**, 227-239.
- 253(19) Van Ness, J., Van Ness, L.K. & Galas, D.J. *Proc. Natl. Acad. Sci. USA* 2003, **100**, 4504-4509.
- 254(20) Jia, H., Li, Z., Liu, C. & Cheng, Y. *Angew. Chem. Intl. Ed.* 2010, **49**, 5498-5501.
- 255(21) Li, C.P., Li, Z.P., Jia, H.X. & Yan, J.L. *Chem. Commun.* 2011, **47**, 2595-2597.
- 256(22) WALKER, G.T., LITTLE, M.C., NADEAU, J.G. & SHANK, D.D. *Proc. Natl. Acad. Sci. USA* 2571992, **89**, 392-396.
- 258(23) Ma, C., Wang, W., Yang, Q., Shi, C. & Cao, L. *Biosens Bioelectron* 2011, **26**, 3309-3312.
- 259(24) Ma, C., Zhao, C., Ge, Y. & Shi, C. *Clin. Chem* 2012, **58**, 384-390.
- 260(25) Shi, C., Zhao, C., Guo, Q. & Ma, C. *Chem Commun* 2011, **47**, 2895-2897.
- 261(26) Walker, G.T., *et al.* *Nucleic Acids Res.* 1992, **20**, 1691-1696.
- 262(27) Little, M.C., *et al.* *Clin. Chem.* 1999, **45**, 777-784.
- 263(28) Garcí a, P.B., Robledo, N.L. & Islas, Ä.L. *Biochemistry* 2004, **43**, 16515-16524.
- 264(29) Creemers, E.E., Tijssen, A.J. & Pinto, Y.M. *Circulation Res.* 2012, **110**, 483-495.
- 265(30) Lagana, A., *et al.* *RNA* 2013, **4**, 120.
- 266(31) Olivieri, F., Rippo, M.R., Procopio, A.D. & Fazioli, F. *Frontiers in Non-Coding RNA* 2013, **4**, 267121.
- 268(32) Rykova, E.Y., Laktionov, P.P. & Vlassov, V.V. *Nucleic Acids Mol. Biol.* 2010, **25**, 93-128 (2010).
- 269(33) Williams, Z., *et al.* *Proc. Natl. Acad. Sci. USA* 2013, **110**, 4255-4260.
- 270(34) Mitchell, P.S., *et al.* *Proc. Natl. Acad. Sci. USA* 2008, **105**, 10513-10518.
- 271(35) Russo, F., *et al.* *PLoS One* 2012, **7**, e47786.
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276TOC Figure

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