Exponential Strand-Displacement Amplification for Detection of microRNAs

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ABSTRACT

MicroRNAs are promising targets for disease diagnosis. However, miRNA detection requires rapid, sensitive and selective detection to be effective as a diagnostic tool~~To take full advantage of the great potential of miRNAs as biomarkers for disease diagnosis, simple, rapid and sensitive methods for miRNA detection should be developed~~. Herein, a miRNA-initiated exponential strand-displacement amplification (SDA) assay was reported. With the Klenow fragment~~, and the~~ nicking enzyme *Nt.AlwI*, and two primers, the miRNA target can trigger ~~triggered~~ two cycles of nicking, polymerization, and displacement reactions~~, using two strategically designed primers~~. These reaction cycles amplified the target miRNA exponentially and generated dsDNAs detectable with SYBR Green I in real-time PCR. As low as 16 zmol of the target miRNA was detected by this one-pot assay within 90 minutes, and the dynamic range spanned over 9 orders of magnitude. Negligible impact from the complex biological matrix was observed on the amplification reaction, indicating ~~high potential of our assay to detect circulating miRNAs in biofluids~~the assay's ability to directly detect miRNA levels in biofluids.

Keywords: microRNAs; Exponential strand-displacement amplification; Homogeneous detection; Isothermal amplification nicking enzyme

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

The short length of miRNAs and their low abundance in biological samples increases the difficulty in miRNA detection.[9] Conventional methods, including northern blotting, real-time PCR, and microarrays, possess limitations like such as insufficient sensitivity, tedious sample preparation and analysislong and tedious process, requirement of sophisticated instrumentssophisticated instrumentation, and high operation expenses, etc.[10] In the past decade, many new methods have been developed to improve miRNA detection. Among them, isothermal amplification of miRNAs using a combination of DNA polymerases and processing enzymes have attracted great attention, because of their high detection sensitivity, fast reaction speed, and low operational cost.[12-17] However, oligonucleotides with sophisticated designs, like the dumbbell-shaped probe,[14] circular template,[13] hairpin structures, fluorescently labeled strands,[16] etc., are required. Or, nanomaterials are employed to

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

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

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

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

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

concentration of the target miRNA was used for visualization on the gel. At last, we mixed T, Primer 1, Klenow, *Nt.AlwI*, and Primer 2 to trigger both amplification cycles. Although ~~with~~ having a ten-fold lower target concentration, the exponential amplification generated more of the 32-bp products than with the single cycle in Lane 4.

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

With the appropriate primer design and optimized enzyme concentrations, we

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

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

It has been known that miRNAs can be released into the circulation system;[29-32] and accumulating evidences also show that circulating miRNAs exhibit varied patterns between cancer patients and healthy controls.[33-35] Thus, it will be highly useful for cancer diagnosis if our assay can be used to detect the levels of circulating miRNAs in biofluids like serum. To demonstrate this, we spiked 0.1 fmol to 0.1 pmol *Let*-7a into ~~the~~ commercial human serum (Lonza Biowhittaker human serum from Fisher Scientific), in which ~~the~~ endogenous *Let*-7a was not detectable using the Taqman® RT-PCR kit ~~for miRNAs~~ (Life Technologies, Inc.). The serum was diluted 2 times in the 10-μL reaction mixture. Using the same reaction conditions as mentioned above, the Cq values increased linearly with the negative logarithmic value of the amount of target miRNA, and the regression equation was found to be: Cq = -130.1125 + 10.35186 ($-\lg A_{Let\text{-}7a}$) ($A_{Let\text{-}7a}$ was the concentration of *Let-7a*, R^2=0.9681) (see Figure S3 in the Supporting Information). This relationship was almost the same as that for the standard curve obtained at the same time (Cq = -130.718 + 10.426 ($-\lg A_{Let\text{-}7a}$), R^2=0.9901), proving that the reaction was not affected by the complex components in serum. ~~The method posses good ability of anti-jamming in biofluids like serum. Owing to the potential for the use of serum miRNAs as biomarkers of disease and as targets of therapeutics, so our method is promising as a non-invasive, highly sensitive, and accurate diagnosis for cancer~~.

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

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

ACKNOWLEDGMENT

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Table 1. Sequences of DNA and RNA in experiment.

miRNA	Sequence (5′ to 3′)[a]
Let-7a	UGAGGUAGUAGGUUGUAUAGUU
Let-7b	UGAGGUAGUAGGUUGU*G*U*G*GUU
Let-7c	UGAGGUAGUAGGUUGU*A*UGGUU
Let-7e	UGAGGUAG*G*AGGUUGUAUAGUU
Let-7i	UGAGGUAGUAG*U*UUGU*GCU*GUU
Primer 1	CGGATCCTCT↓*AACTATACAACC*
Primer 2	CGGATCGTCT↓*TGAGGTAGTAGG*

[a] The portions underlined in primers indicate the recognition site of the nicking enzyme *Nt.AlwI* 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 *Let* miRNAs are the bases different than those in *Let*-7a.

197**Scheme 1.** Schematic illustration of exponential SDA initiated by miRNA *Let*-7a. The

198sequences of the target and primers are listed in Table 1. □□

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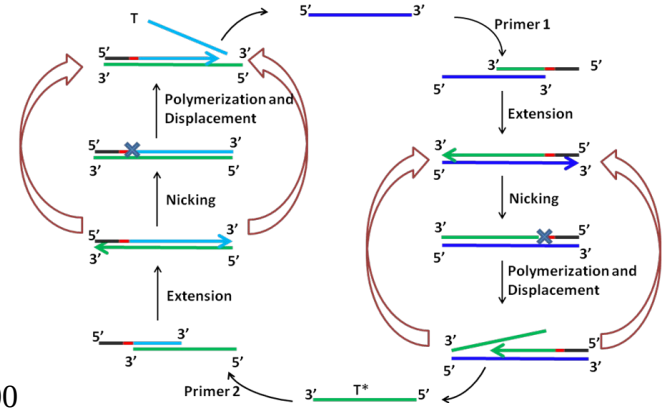

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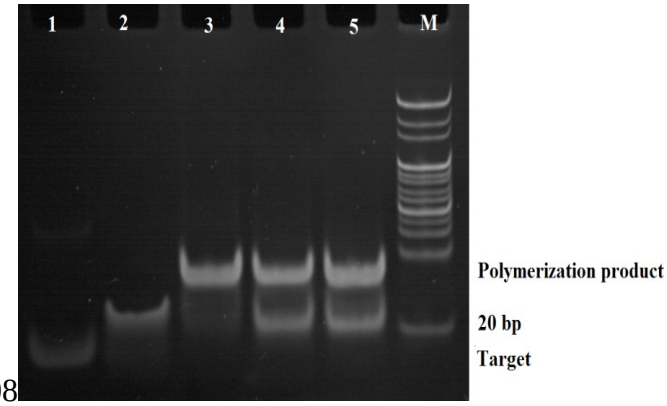
Figure 1. The result of analyzing the amplification products by PAGE (17.5% polyacrylamide). **M:** 20-bp DNA ladder; **1:** 1.0 × 10^{-5} M Target; The product of amplification reaction in **2:** 10 μL mixture containing 1.0 × 10^{-6} M Primer 1 and 1.0 × 10^{-6} M the target, 0.3 μL 2.5 mM dNTPs, 1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



209**Figure 2**. The linear relationship between Cq values and the negative logarithm (lg)

210values of the amount of *Let*-7a used to initiate the exponential SDA. Insert: The real-

211time fluorescence detection curves.

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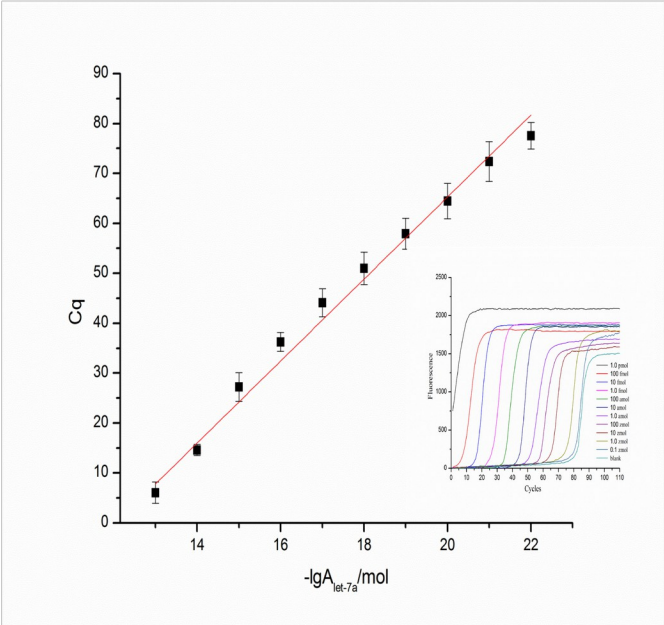
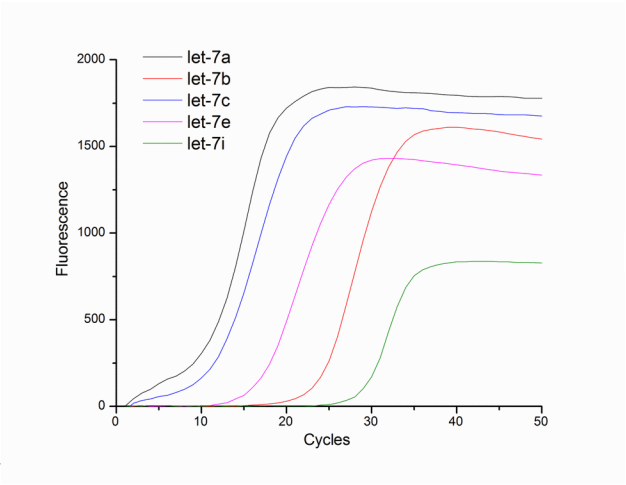


Figure 3. Real-time fluorescence curves for the exponential SDA amplification were triggered by 1.0×10^{-13} mol *Let*-7a, *Let*-7b, *Let*-7c, *Let*-7e and *Let*-7i, respectively. The other experimental conditions are the same as those for Figure 2.



Supporting Information

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

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276TOC Figure

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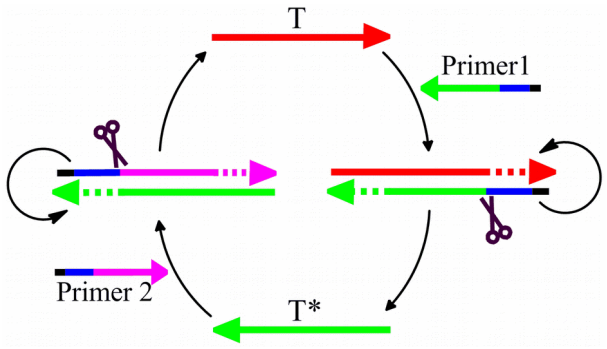

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