Biomolecule Detection

DOI: 10.1002/anie.201001375

Ultrasensitive Detection of microRNAs by Exponential Isothermal Amplification**

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MicroRNAs (miRNAs) are a class of endogenous, noncoding small RNA molecules (19–23 nucleotides (nt)). Through translational repression or target degradation by the formation of an RNA-induced silencing complex (RISC) with target messenger RNAs, miRNAs play important roles in a wide range of biological processes, including proliferation, development, metabolism, immunological response, tumorigenesis, and viral infection.[1] Recently, the biological functions of miRNAs have become an area of intense investigation. The detection of miRNAs is imperative for gaining a better understanding of the functions of these biomolecules and has great potential for the early diagnosis of human disease as well as the discovery of new drugs through the use of miRNAs as targets.[2]

Northern-blotting analysis is now considered the standard method for miRNA detection.[3] Microarrays are being used more and more for miRNA-expression analysis as a result of their high-throughput-screening capability.[4] However, the sensitivity and specificity of these methods are not satisfactory because of the small size, sequence similarity, and low abundance of miRNAs.[5,6] Various amplification strategies for miRNA analysis have been reported to improve the sensitivity and specificity of the approach, such as real-time PCR,[7] the modified invader assay,[8] ribozyme amplification,[9] nanoparticle amplification methods,[10] rolling circle amplification,[11] and conjugated-polymer-based methods.[12] Among these methods, real-time PCR is the most sensitive and practical. However, the short length of miRNAs makes the PCR design very sophisticated. Stem–loop DNA probes, LNA-modified DNA probes (LNA = locked nucleic acid), or doubly fluorescence labeled TaqMan probes have to be used, and these probes greatly increase the experimental cost and complexity. Therefore, a simple, low-cost, and highly sensitive method for miRNA detection is desirable.

In 2003, Galas and co-workers devised an exponential amplification reaction (EXPAR) for short oligonucleotides (called triggers) by a combination of polymerase strand extension and single-strand nicking.[13] The reaction provides 10^6–10^9-fold amplification under isothermal conditions within minutes. Compared with other amplification methods, the EXPAR method has the distinct advantages of its isothermal nature, high amplification efficiency, and rapid amplification kinetics. A potential disadvantage of the EXPAR method is the requirement for the generation of a trigger from genomic DNA or RNA. Although the EXPAR method has been applied to virus detection,[14] the generation of the trigger from a genomic target is limited to sequences within the genomic DNA that contain adjacent nicking-enzyme recognition sites. This limitation hampers the wide application of the EXPAR for nucleic acid detection.

Herein, we demonstrate that the EXPAR method is well-suited to the efficient amplification of small miRNAs. By means of the real-time fluorescence detection of EXPAR products, the formation of which is triggered by miRNAs, miRNAs can be detected in amounts as low as 0.1 zmol. Furthermore, the dynamic range is more than 10 orders of magnitude. The proposed method is one of the most sensitive for miRNA detection. Moreover, it does not require any modified DNA probes: use of the cyanine dye SYBR Green I, rather than a TaqMan probe, for the detection of EXPAR products considerably reduced the detection cost. The fast and isothermal EXPAR resulted in a simple and rapid assay procedure.

Our strategy for miRNA analysis on the basis of the EXPAR method is illustrated in Figure 1 a. The amplification

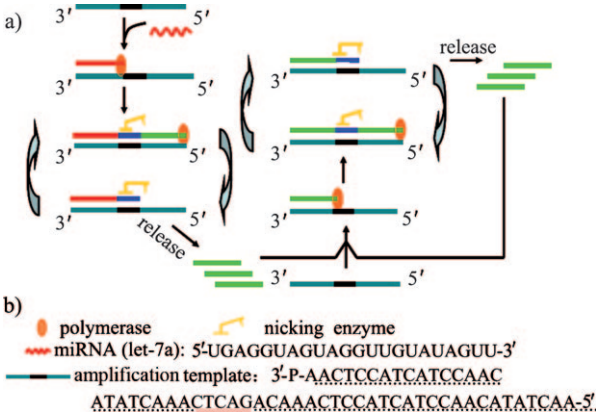

Figure 1. a) Schematic representation of the EXPAR with let-7a miRNA as the trigger. b) Sequences of let-7a miRNA and the amplification template. P indicates a phosphate group.

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[**] This project was supported by the National Natural Science Foundation of China (20925519, 20875021) and the National Science Foundation of Hebei Province (B2009001525).

Supporting information for this article (optimization of the amounts of the DNA polymerase, nicking enzyme, and amplification template used for the EXPAR reaction, and determination of the quantity of let-7a in the total RNA sample) is available on the WWW under http://dx.doi.org/10.1002/anie.201001375.

template contains two repeat sequences (indicated with dotted lines in Figure 1b), one at its 3′ terminus and one at its 5′ terminus, which are complementary to the target miRNA. The melting temperature (T_m) of this DNA sequence was determined to be 64.7 and 67.2 °C for DNA/DNA and DNA/RNA hybridization, respectively (see Figure S5 in the Supporting Information); the EXPAR is performed at 55 °C. Therefore, the target miRNA can hybridize with its complementary sequence at the 3′ terminus of the amplification template and then extend along the template in the presence of Vent (exo⁻) DNA polymerase and deoxyribonucleotide triphosphates (dNTPs) to form double-stranded (ds) DNA. The sequence 3′-CTCAG-5′ in the middle of the amplification template (underlined) is the recognition site of the nicking endonuclease Nt.BstNBI on the lower DNA strand. Therefore, the extension product contains the double-stranded nicking-enzyme recognition site in the middle of the dsDNA. The nicking enzyme recognizes the site and cleaves the upper DNA strand at a site four bases downstream. The cleaved DNA strand containing the recognition site will extend again, and the short single-stranded DNA will be displaced and released according to the strand-displacement activity of Vent DNA polymerase.[15] Thus, extension, cleavage, and strand displacement can be repeated continuously and result in the linear amplification of the target miRNA. The sequence of the released short DNA strands is the same as that of the miRNA target, except that the ribonucleotides and uridine in the miRNA are replaced with deoxyribonucleotides and thymine, respectively, in the DNA strand. Hybridization of these released DNA strands with other amplification templates and their extension on the template leads to exponential amplification. Finally, a large amount of dsDNA can be produced. SYBR Green I was utilized as the fluorescent dye for the real-time detection of the EXPAR products.

Recently, Tan et al. demonstrated that the sensitivity for nucleic acid detection based on the EXPAR method is mainly limited by nonspecific background amplification, which appears to involve the template and unprimed DNA polymerization arising from interactions between the single-stranded template and the DNA polymerase.[16] Nonspecific background amplification can be markedly reduced by physically separating the template and the polymerase until the final reaction temperature has been reached. Therefore, for miRNA detection, the amplification template and DNA polymerase for the EXPAR were prepared separately and mixed immediately before they were added to the real-time detection system (see the Experimental Section).

We found the optimum amounts of the DNA polymerase, nicking enzyme, and amplification template for the EXPAR to be 0.05 U μL^{-1}, 0.4 U μL^{-1}, and 0.1 μM, respectively, in a reaction volume of 10 μL (see the Supporting Information). Under the optimum conditions, the target miRNA, let-7a, could be detected quantitatively in the range from 0.1 zmol to 1.0 pmol by real-time measurement of the fluorescence intensity of the EXPAR products (Figure 2). For high accuracy and high resolution, the point of inflection (POI), which is defined as the time corresponding to the maximum slope in the fluorescence curve, was used for the quantitative detection of the miRNA target. The POI values are linearly

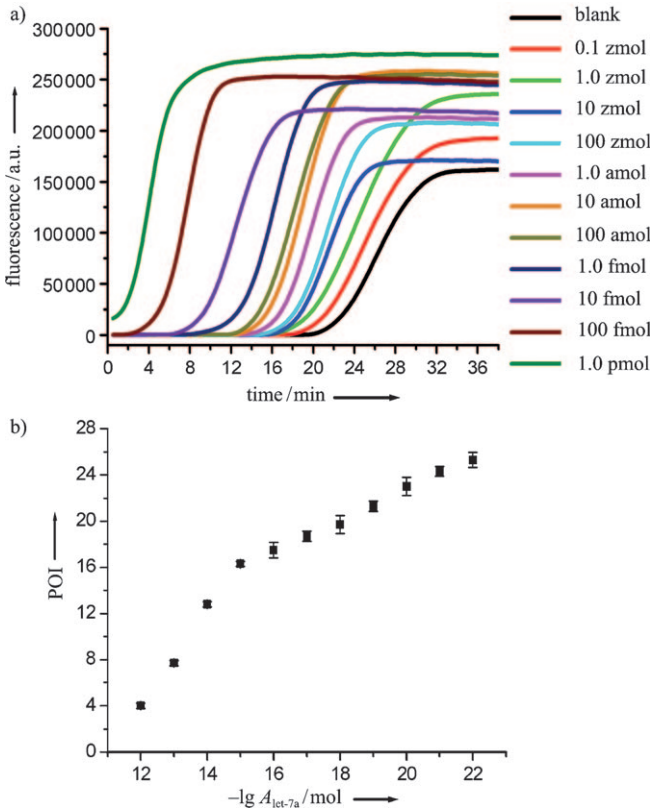

Figure 2. a) Real-time fluorescence curves for the EXPAR triggered by let-7a miRNA. b) Relationship between the POI value and the logarithm of the amount of let-7a miRNA. Final concentrations: [amplification template] = 0.1 μM, [each dNTP] = 250 μM, [Nt.BstNBI] = 0.4 U μL^{-1}, [Vent (exo⁻) DNA polymerase] = 0.05 U μL^{-1}, [ribonuclease (RNase) inhibitor] = 0.8 U μL^{-1}, [SYBR Green I] = 0.4 μg mL^{-1}.

dependent on the logarithm (lg) of the amount of target miRNA in the ranges 0.1 zmol–1.0 fmol and 1.0 fmol–1.0 pmol. The correlation equations are POI = −3.79–1.33 lgA_{miRNA} (mol) [correlation coefficient: $R = 0.9977$] and POI = −46.5–4.2 lgA_{miRNA} (mol) [$R = 0.9974$], respectively. Thus, the assay has a great dynamic range of more than 10 orders of magnitude.

To evaluate the specificity of the proposed miRNA assay, members of the let-7 miRNA family (let-7a–g and i) were selected as a model system because of their high sequence homology (Figure 3b). The real-time fluorescence signal produced by let-7a could be separated completely from those produced by other let-7 miRNAs (Figure 3a). Thus, the proposed miRNA assay with the EXPAR clearly discriminated all let-7a miRNA family members, even on the basis of a difference of only one base. The miRNA assay is based on the extension of the miRNA at the 3′ terminus to initiate the EXPAR. Relative to let-7a, the mismatched bases in let-7b, c, g, and i are located near their 3′ terminus, which results in efficient discrimination from let-7a. At the POI of the let-7a signal along the time axis, no fluorescence signal was yet observed for let-7b, c, g, or i. In let-7d, e, and f, the mismatched bases are distant from the 3′ terminus. Therefore, the signals produced by let-7d, e, and f are relatively similar to

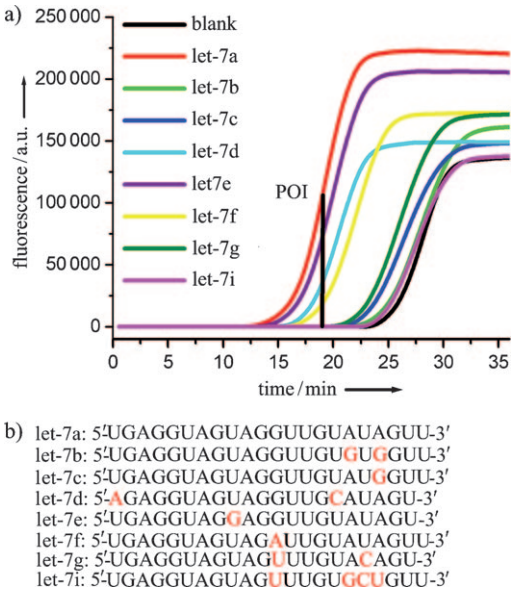

Figure 3. a) Real-time fluorescence curves for the EXPAR triggered by let-7a–g and let-7i (10 amol each). b) Sequences of let-7a–g and let-7i miRNA. The bases that differ from those in let-7a are marked in red. The experimental conditions are the same as those for Figure 2.

that produced by let-7a. Interference for the detection of the amount of let-7a by the signals produced by let-7d, e, and f was estimated to be 7.4, 17.8, and 0.6%, respectively (see the estimation in the Supporting Information).

The amount of let-7a miRNA in a human-brain total RNA sample was detected with the proposed miRNA assay by dilution of the total RNA sample. The well-defined signal of let-7a miRNA in the sample containing 10 pg of total RNA was detected (see Figure S4 in the Supporting Information). With a simultaneously constructed calibration curve (see Figure S4B in the Supporting Information), the amount of let-7a in the total RNA sample (10 pg) was estimated to be 0.28 zmol. The result was verified by the addition of synthetic let-7a miRNA (1 zmol) to the total RNA sample (10 pg): the average amount of let-7a determined for five repetitive measurements was 1.224 zmol. Therefore, the proposed method can be used to quantitatively detect as little as a zeptomole amount of an miRNA in a total RNA sample.

In summary, we have demonstrated that the EXPAR method can be applied to the ultrasensitive detection of miRNAs. By the real-time measurement of fluorescence intensity, the presence of as little as 0.1 zmol of an miRNA can be accurately determined. This amount corresponds to less than 100 copies of an miRNA molecule in a volume of 10 μL. The miRNA assay also exhibits a great dynamic range of over 10 orders of magnitude and high specificity to clearly discriminate a one-base difference in miRNA sequences. Moreover, the EXPAR reaction can be carried out under isothermal conditions within 30 minutes. In contrast to previously described methods, this method does not require the design of any modified DNA probes and can be performed by using SYBR Green I as the fluorescent dye, rather than a TaqMan probe. This simple, low-cost, and highly sensitive method should contribute significantly to future advances in research on the biological roles of miRNAs and applications in clinical diagnostics with miRNAs as targets.

Experimental Section

PAGE-purified DNA, HPLC-purified RNA, RNase inhibitor, and DEPC-treated water were obtained from TaKaRa Biotechnology Co. Ltd. (Dalian, China; DEPC = diethylpyrocarbonate). Vent (exo^-) DNA polymerase and the nicking endonuclease Nt.BstNBI were purchased from New England Biolabs. The human-brain total RNA sample (1 $\mu g\,\mu L^{-1}$) was purchased from Ambion (USA). SYBR Green I (20× stock solution in dimethyl sulfoxide, 20 $\mu g\,mL^{-1}$) was purchased from Xiamen Bio-Vision Biotechnology (Xiamen, China). All solutions for the EXPAR were prepared in DEPC-treated deionized water. The EXPAR and the real-time fluorescence measurements were performed with a 7300 Real-Time PCR System (Applied Biosystems, USA).

The reaction mixtures for the EXPAR were prepared separately on ice as part A and part B. Part A consisted of Nt.BstNBI buffer, the amplification template, dNTPs, RNase inhibitor, and the miRNA target; part B consisted of ThermoPol buffer, the nicking endonuclease Nt.BstNBI, Vent (exo^-) DNA polymerase, SYBR Green I, and DEPC-treated water. Parts A and B were mixed immediately before being placed in the Real-Time PCR System. The EXPAR was performed in a volume of 10 μL containing the amplification template (0.1 μM), dNTPs (250 μM), Nt.BstNBI (0.4 $U\,\mu L^{-1}$), Vent (exo^-) DNA polymerase (0.05 $U\,\mu L^{-1}$), RNase inhibitor (0.8 $U\,\mu L^{-1}$), SYBR Green I (0.4 $\mu g\,mL^{-1}$), 1× ThermoPol buffer (20 mM Tris–HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100; Tris = 2-amino-2-hydroxymethylpropane-1,3-diol), and 0.5× Nt.BstNBI buffer (25 mM Tris–HCl, pH 7.9, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol). The EXPAR was performed at 55 °C, and the real-time fluorescence intensity was monitored at intervals of 30 s.

Received: March 8, 2010
Revised: May 11, 2010
Published online: July 2, 2010

Keywords: analytical methods · exponential amplification reaction · fluorescence · microRNA · real-time detection

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