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One-Step, Multiplexed Fluorescence Detection of microRNAs Based on Duplex-Specific Nuclease Signal Amplification

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Supporting Information

ABSTRACT: Traditional molecular beacons, widely applied for detection of nucleic acids, have an intrinsic limitation on sensitivity, as one target molecule converts only one beacon molecule to its fluorescent form. Herein, we take advantage of the duplex-specific nuclease (DSN) to create a new signal-amplifying mechanism, duplex-specific nuclease signal amplification (DSNSA), to increase the detection sensitivity of molecular beacons (Taqman probes). DSN nuclease is employed to recycle the process of target-assisted digestion of Taqman probes, thus, resulting in a significant fluorescence signal amplification through which one target molecule cleaves thousands of probe molecules. We further demonstrate the efficiency of this DSNSA strategy for rapid direct quantification of multiple miRNAs in biological samples. Our experimental results showed a quantitative measurement of sequence-specific miRNAs with the detection limit in the femtomolar range, nearly 5 orders of magnitude lower than that of conventional molecular beacons. This amplification strategy also demonstrated a high selectivity for discriminating differences between miRNA family members. Considering the superior sensitivity and specificity, as well as the multiplex and simple-to-implement features, this method promises a great potential of becoming a routine tool for simultaneously quantitative analysis of multiple miRNAs in tissues or cells, and supplies valuable information for biomedical research and clinical early diagnosis.

MicroRNAs (miRNAs) are a group of small, noncoding RNAs with the length of 18−25 nucleotides (nt), processed from longer hairpin-shaped precursor miRNAs (~75 nt) by the Dicer enzyme. It is well-known that they play a critical role in physiologic and pathologic processes, including hematopoietic differentiation, cell cycle, regulation, and metabolism. More importantly, there is accumulating evidence that miRNAs expressions are frequently dysregulated in the development of a variety of cancers,[1,2] and their expression patterns in cancers appear to be tissue-specific.[3] miRNAs profiles are accessible from body fluids, such as blood, in a remarkably stable form that is protected from endogenous RNase activity.[4] In addition, recent studies have identified that a change in the cellular status is typically related to a simultaneous change in the level of several miRNAs. Owing to these properties, miRNAs have become an ideal class of biomarker candidates for clinical diagnosis. Thus, sensitive and selective detection of multiplex miRNAs in biological fluids is of great significance in understanding biological functions of miRNAs, early diagnosis of cancers, as well as discovery of new targets for drugs.

In contrast to nucleic acid detection, the unique characteristics of miRNAs, such as their short lengths, sequence homology among family members, and low abundance in total RNA samples, make them difficult to detect. Usually, the Northern blot technique is regarded as a standard method for miRNAs analysis in early miRNA profiling studies,[5,6] but it is unsatisfactory owing to its intrinsic drawbacks of low sensitivity, labor-intensive and time-consuming steps, and large amounts of sample required especially when monitoring the expression of low-abundance miRNAs. Various methods mainly based on sequence-biased amplification mechanism have been developed for miRNAs analysis, such as real-time PCR (RT-PCR) amplification,[7,8] rolling circle amplification (RCA),[9] and isothermal amplification.[10] Among these approaches, the most widely reported RT-PCR methods, such as stem-loop RT-PCR and polyA-tailed RT-PCR, have shown high sensitivity (~100 fM) and specificity, but they both need an essential step of reverse transcription, which undoubtedly increases the experimental cost and design complexity. RCA amplification is a simple enzymatic process to generate very long single-stranded DNA molecules with tandem repeats; however, it is very time-consuming. In Cheng et al.,[9] the entire experiment based on RCA was tedious and cost over 8 h of reaction time to achieve a detection limit of ~10 fM. Li and co-workers applied an exponential amplification reaction (EXPAR) method, first devised by Galas and co-workers,[11] to miRNAs detection with amazing detection limit of about 15 aM;[10] however, this method has a nonspecific background amplification problem, which is common in both thermal cycling and isothermal processing formats. These methods indeed have improved the sensitivity of miRNAs detection greatly; however, they are indirect and lack large-scale multiplexing capability, design simplicity, or scalability. Therefore, to develop simple, sensitive, direct, and nontarget-amplification methods for multiplexed miRNAs analysis is still a challenge.

Recently, instead of indirect detection that relies on target amplification, the direct detection based on nicking enzyme signal amplification (NESA) method has been widely used for sensitive DNA detection.[12−14] In NESA design, a probe and

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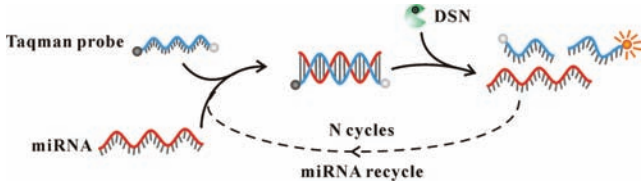
target DNA anneal to create a nicking endonuclease (NEase) restriction site that is recognized by sequence-specific NEase. NEase cleaves the probe in a DNA duplex into two pieces, leaving the target DNA intact. Then, the target DNA acts as a template for a fresh probe and the processes of hybridization, cleavage, and dissociation repeat, leading to signal amplification. However, NESA method requires the nicking enzyme recognition sequence to be present in the target DNA, which puts a limitation on its various applications, and shows the sensitivity of 10 pM, not sufficient for detecting ultralow DNA concentrations. On the other hand, NESA cannot be used for RNA detection. Herein, we take advantage of the duplex-specific nuclease (DSN) to create a new signal-amplifying mechanism, duplex-specific nuclease signal amplification (DSNSA), to increase the detection sensitivity of RNA. DSN enzyme is a nuclease isolated from hepatopancreas of the Kamchatka crab (*Paralithodes camtschaticus*).[15,16] It displays a strong preference for cleaving double-stranded DNA or DNA in DNA:RNA heteroduplexes, and is practically inactive toward single-stranded DNA, or single- or double-stranded RNA. Moreover, this enzyme shows a good capability to discriminate between perfectly and nonperfectly matched (up to one mismatch) short duplexes. DSN enzyme is widely applied in molecular biology field, including full-length cDNA library normalization,[17] subtraction,[18] quantitative telomeric overhang determination,[19] and genomic single-nucleotide polymorphism detection.[15] In this study, we report a one-step, direct method to quantitatively detect multiple miRNAs based on DSNSA method. As shown in Scheme 1, Taqman probe, as a signal

Scheme 1. Schematic Representation of miRNA Direct Detection Based on Duplex-Specific Nuclease Signal Amplification (DSNSA)



output, hybridizes to a target miRNA to form DNA:RNA heteroduplex. DSN enzyme (Evrogen, Moscow, Russia) is employed to recycle the process of target-assisted digestion of DNA Taqman probes, thus, resulting in a significant fluorescence signal amplification through which one target molecule cleaves thousands of probe molecules within 30 min. DSNSA method allows the direct detection of miRNAs in the femtomolar range, increasing the sensitivity by about 5 orders of magnitude, as compared to conventional hybridization assay. Moreover, it can be easily applied to all miRNAs, because DSN enzyme has no requirement for specific recognition sequence.

To demonstrate the working principle of DSNSA method, we first selected miR-141 as a model to optimize the experimental conditions. miR-141 is an epithelial-associated miRNA expressed in a wide range of common human cancers including breast, lung, colon, and prostate cancer. A 22-mer Taqman probe P-141 complementary to miR-141 was prepared (Table S1). We found the optimum amount of DSN enzyme was 0.1 U in 30 μL reaction volume (Figure S1) and the optimum temperature for its activity was 60 °C. Of note, DSN enzyme is stable at a wide range of pH (from 4 to 12) in the presence of 5 mM Mg^{2+}. At temperature of 80 °C, DSN enzyme retained only 10% activity. This sharp decrease in activity may be attributable, at least in part, to double-strand substrate denaturation. Therefore, we set 60 °C as the reaction temperature, because, aside for DSN's own temperature requirement, even more importantly, that higher temperature should facilitate faster hybridization and subsequent dissociation of miRNA.

Under the optimum conditions, we investigated the sensitivity of DSNSA method upon addition of different concentrations of miR-141. As shown in Figure 1A, a dramatic

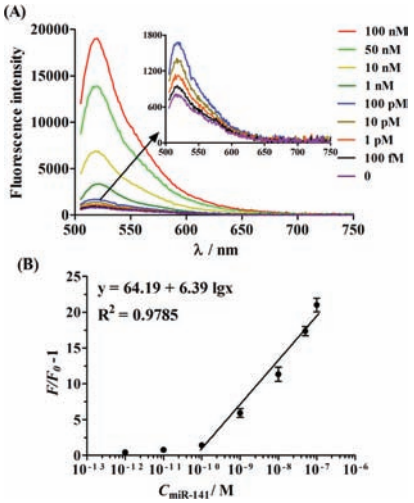


Figure 1. (A) Fluorescence emission spectra (excitation at 485 nm, emission at 518 nm) upon addition of miR-141 (0, 100 fM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, 50 nM, 100 nM). Inset: Fluorescence responses to miR-141 at low concentrations. (B) Scatter plot of $(F/F_0 - 1)$ as function of the concentrations of miR-141, where F_0 and F are the FAM fluorescence signals in the absence and the presence of miR-141, respectively.

increase in the fluorescence emission spectrum was observed with the increasing miR-141 concentrations from 0 to 100 nM. Figure 1B illustrates the changes in fluorescence intensity $(F/F_0 - 1)$ in responding to the different miR-141 concentrations. About 24-fold fluorescence enhancement is clearly observed at the concentration of 100 nM. The $(F/F_0 - 1)$ value is linearly dependent on the logarithm (lg) of miR-141 concentration in the ranges 100 pM−100 nM, with a correlation equation of $(F/F_0 - 1) = 64.19 + 6.39 \lg(\text{miRNA})$ ($R^2 = 0.9785$). The limit of detection based on 3σ method is approximately 100 fM, which has the similar performance as RT-PCR method (Figure S3). It is worthy to note that such a high sensitivity was achieved within 30 min, which is more than 2 orders of magnitude higher than that of basic NESA (10 pM, 120 min), and is comparable to the complicated extended NESA which is coupled with RCA assay.[13] The ultrahigh sensitivity of DSNSA is from the highly effective catalytic cleavage activity of DSN enzyme, resulting in an amplification effect of more than 10^5-fold within 30 min even if the amount of target miRNA is as low as 3 amol (100 fM in 30 μL).

A significant challenge for miRNAs analysis is the ability to distinguish miRNA family members, which is of great importance in better understanding the biological functions of individual miRNA. The miR-141 belongs to the miR-200 family,[20] which comprises five members miR-200a, miR-200b, miR-200c, miR-141, and miR-429. Three members of miR-200 family (miR-141, miR-200b, and miR-429), miR-21, and let-7d

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(Table S1) were used for the evaluation of sequence-specificity of DSNSA assay. miR-21 is reported as an antiapoptotic factor in cancer cells and shows overexpression in various cancers including breast, ovarian, lung, prostate, pancreas cancer, and glioblastomas.[21−23] let-7d is a member of let-7 miRNAs family, whose expression levels are closely associated with cell development and human cancers. Figure 2A exhibits the

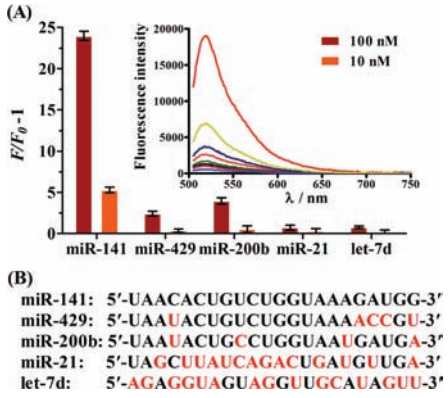

Figure 2. Specificity of DSNSA method. (A) Bars represent the fluorescence ratio ($F/F_0 - 1$) upon the different miRNAs targets. Inset: Fluorescence emission spectra toward the different miRNAs targets. (B) Sequences of miR-141, miR-429, miR-200b, miR-21, and let-7d. The bases that differ from those in miR-141 are marked in red.

comparison of fluorescence signals' response toward different miRNAs targets. As expected, it is clearly observed that the signal ($F/F_0 - 1$) from miR-141 was approximately 10-fold higher than that of miR-429 and miR-200b (Figure 2A). Moreover, there was nearly negligible fluorescence change in the presence of miR-21 and let-7d. These results suggest that the specificities of the DSNSA method are high enough to discriminate between the three miR-200 family members.

Some pathologies are typically associated with abnormal expressions of several miRNAs. The use of miRNA for clinical diagnostics requires simultaneously quantitative analysis of multiple miRNAs. Toward this goal, we selected miR-21 and let-7d together with miR-141 as targets to demonstrate the multiplexing capability of our method. We prepared seven samples via different combinations among these three miRNAs targets (Table S2). Three Taqman probes, P-141, P-21, and P-7d, which are perfectly complementary to miR-141, miR-21, and let-7d, were labeled with FAM, TAMRA, and Cy5, respectively (Table S1). A series of reaction mixtures containing 1× DSN buffer, 0.1 U DSN enzyme, 20 U RNase inhibitor, and three Taqman probes (each 100 nM) were added the different samples, respectively, and then analyzed. As shown in Figure 3, when only one type of miRNA was added, the solution only emitted the corresponding fluorescence spectrum of the respective Taqman probe. When all targets were present, the corresponding fluorescence emissions were observed in all three detection channels. The fluorescence signals corresponding to those Taqman probes were restored only when their cognate target miRNAs were present. The compositions and concentrations of miRNAs can be read directly from the fluorescence emission spectra at different channels. The signal response to specific miRNA was not affected by the presence of other miRNAs. Therefore, the DSNSA method is deemed suitable for multiplexed detection of miRNAs. Another control experiment was performed to study the digestion function of

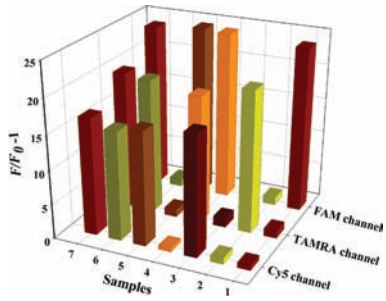

Figure 3. Multiplexing miRNAs detection. Three Taqman probes are hybridized with their perfectly complementary miRNAs targets. miR-141 (FAM, blue emission at 518 nm); miR-21 (TAMRA, orange emission at 580 nm); let-7d (Cy5, red emission at 663 nm).

DSN enzyme on single-strand Taqman probes. The fluorescence intensity increase was <15% in the presence of 0.1 U DSN enzyme (Figure S2).

It is well-known that cancers are often associated with multiple miRNA markers, which have proven valuable for the early phase diagnosis of cancers in asymptomatic individuals. We further investigated the feasibility of DSNSA method to multiplexed detection of miRNAs in a complex biological matrix. The cell lysate samples from six human cancer cell lines, including hepatocellular carcinoma cell lines (BEL-7404), breast cancer cell lines (MDA-MB231), prostate carcinoma cell lines (22Rv1 and DU 145), pancreatic cancer cell lines (AsPc-1), and cervical cancer cell lines (HeLa), were tested. As shown in Figure 4, the results clearly demonstrated the

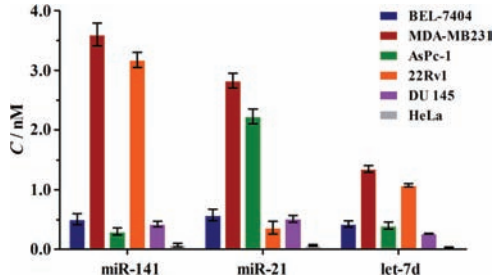

Figure 4. Cancer-derived miRNAs are detectable in cell lysate samples. Bars represent the concentrations of test miRNAs (miR-141, miR-21 and let-7d) in the different cancer cells including BEL-7404, MDA-MB231, AsPc-1, 22Rv1, DU 145, and HeLa cells.

concentration variation of the three miRNAs in different cancer cells. It was observed that miR-21 and let-7d had different expression levels in AsPC-1 cell, which is in good agreement with reported literature.[22] These results indicated that DSNSA method holds a great promise for practical applications in miRNA detection with great accuracy and reliability. Therefore, it not only shows the high sensitivity and selectivity in comparison with the traditional methods, but also provides the great potential in the early clinic diagnosis.

In summary, we take advantage of the duplex-specific nuclease to create a new signal-amplifying mechanism, duplex-specific nuclease signal amplification (DSNSA), and demonstrate its application for rapid ultrasensitive detection of miRNAs. This method is a single-step, direct miRNAs assay that requires no external modification on miRNA, while being quantitative and applicable to multiple miRNAs in the sample without involving complicated procedures or sophisticated instrumentations. It is very simple and fast, needing only mixing

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of Taqman probes, target miRNAs and DSN enzyme and incubating within 30 min, which is in the homogeneous solution, and not requiring separation and troublesome procedures. DSNSA method allows the detection of miRNA in the femtomolar range, nearly 5 orders of magnitude lower than that of the conventional molecular beacons method. Owing to DSN's one-base discrimination ability, it shows high selectivity for discriminating differences between miRNA family members. Moreover, multiplexed miRNAs detection can be easily realized by designing different fluorophore-labeled Taqman probes. More importantly, the duplex-specific nuclease signal amplification opens new opportunities for design of more novel sensing strategies and expansion of its application in different fields.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental method and data This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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