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A Simple and Highly Sensitive Fluorescence Assay for MicroRNAs

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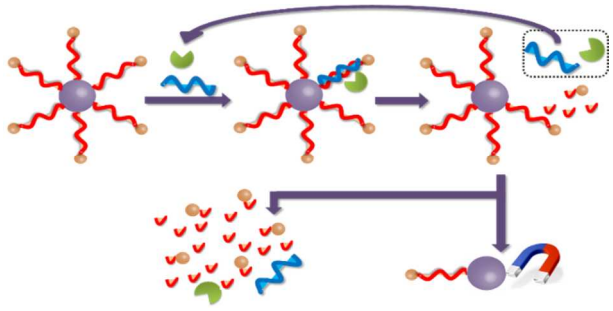

A simple and highly sensitive assay for miRNAs using magnetic bead-carried DNA probes coupled with DSN-facilitated isothermal target cycling.

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Herein, we reported a simple and highly sensitive fluorescence assay for the detection of microRNAs (miRNAs). The assay engaged a duplex-specific nuclease (DSN) to amplify the fluorescence signal and magnetic beads (MBs) to completely remove unreacted DNA detection probes. Briefly, fluorescein-capped DNA detection probes were first conjugated to the MBs. The use of the MBs produced a very low background since all unreacted DNA probes can be conveniently removed from the solution by a permanent magnet. During the assaying process, target miRNA strands hybridized with the DNA capture probes forming miRNA-DNA heteroduplexes. The DSN then selectively cleaved the DNA probes in the miRNA-DNA duplexes and release the target miRNA strands back to solution, thereby establishing a target recycling amplification mechanism – a cumulative signal amplification process. A much-amplified fluorescence signal was obtained in the presence of traces of the target miRNA. In addition, a negligible background was conveniently attained by the complete removal of the unreacted DNA detection probes so that minute change in the fluorescence signal can be unambiguously detected. The negligible background in association with the accumulative signal amplification significantly lowered the detection limit and broadened the dynamic range of the assay. Moreover, the high specificity of the DSN to perfectly matched duplexes endowed this assay a good selectivity when analyzing target miRNAs with high sequence similarities. Successful attempts were made in applying the proposed assay to detect let-7a in total RNA extracted from cultured cells.

Introduction

MicroRNAs (miRNAs) are a class of highly conserved,[1] non-coding, regulatory RNAs derived from an approximately 70 nucleotides[2] long hairpin stem-loop precursor known as pre-miRNAs. After the pre-miRNAs are cleaved by the ribonuclease Dicer, mature miRNAs with lengths of ~22 nucleotides are formed.[3, 4] The first miRNA, lin-4, was discovered in *C. elegans* by Victor and colleagues in 1993;[5] while it has not been regarded as a distinct class of biological regulator until the early 2000s when miRNAs were found playing a vital regulatory role in gene expression[4] and cell differentiation through their incorporation into the RNA-induced silencing complexes (RISC), which either inhibit the translation of imperfectly matched messenger RNAs (mRNAs) to protein or degrade the perfectly matched mRNAs.[4] A representative family of miRNAs is the lethal-7 (let-7), the first know human miRNA family that has ten mature sequences in humans, *i.e.* let-7a, 7b, 7c, 7d, 7e, 7f, 7g, 7i, mir98 and mir202.[6] They were firstly discovered in the nematode as crucial developmental regulators.[7] It has been observed that numerous diseases such as various types of cancer like lung cancer and breast cancer, metabolic syndromes like diabetes,[8] are closely associated with abnormal expression levels of miRNAs. Increasing evidence has suggested that miRNAs are potentially diagnostic as well as prognostic biomarkers. For example, several studies have indicated that the expression levels of let-7 miRNAs are frequently lower in tumor tissues than those in normal tissues.[9] Removal of let-7 binding site on mRNA causes overexpression of protein and produce tumor[10] since let-7 miRNAs acting as tumor suppressors can no longer function. As for the metabolic syndromes, an essential role of let-7 family plays is that it regulates glucose metabolism in multiple organs. Overexpression of let-7 in pancreas will lead to impaired glucose tolerance and reduced pancreatic insulin secretion, which may result in diabetes and ectopic fat deposition in the liver. Studies have indicated that let-7 inhibition not only prevents from diabetes and fatty liver but also provides a new therapy to reverse and cure them.[11]

Considering that the alteration of miRNA expression level is closely related to the diseases as well as the survival of patients,[12] the techniques that are capable of profiling the expressions of miRNAs are important and urgently demanded as early diagnosis could help to reduce the mortality rate of patients. Fortunately, coupled with their regulatory roles, miRNAs are an extremely stable form of nucleic acids which are immune to endogenous RNase degradation.[13] Besides, they can be easily obtained through body fluids such as blood and the levels of miRNAs in blood are stable, reproducible and

consistent among individuals of the same species,[14] hence making miRNAs ideal biomarkers for diagnosis and prognosis. However, miRNAs are generally in short length, low cellular abundance and there is an extremely high level of homology of down to one base difference within a miRNA family. These characteristics of miRNAs make their detection challenging as the detection method has to be both highly sensitive and selective. Currently, there are already several techniques for miRNA detection such as conventional Northern blotting,[15, 16] microarray,[17] rolling circle amplification (RCA),[18] and quantitative polymerase chain reaction (qPCR).[19] All these techniques have made great contributions to miRNA research in the early days and some are still popular tools in miRNA research. Nonetheless, each of them has its own limitations like tedious procedure, high cost, time consuming, or poor sensitivity and selectivity. For example, Northern blotting, whereby the detection of isolated RNAs allows the study of the level of gene expression in cells and also size determination, is the most established nucleic acid assay and still considered as the "gold standard" of miRNA expression analysis. However, its poor sensitivity in conjunction with its inherently tedious procedure and long assay time makes Northern blotting non-ideal for routine miRNA expression profiling.

More recently, several homogeneous amplification strategies for miRNAs that mainly rely on target recycling amplification to improve the sensitivities of miRNA assays have been developed.[20-25] Unlike heterogeneous assays which intrinsically suffer from poor reproducibility, the homogeneous miRNA assays are expected to greatly improve the reproducibility and simplify the assay procedure. Among them, the use of a duplex-specific nuclease (DSN) to amplify miRNA hybridization events appeared very attractive since the amplification process was carried out isothermally. As compared to other commonly used enzymatic amplification methods such as qPCR and ligase chain reaction (LCR)[26] utilizing polymerase and ligase respectively, the isothermal miRNA amplification realized by DSN completely removes the requirement of a thermal cycler. This greatly simplifies point-of-care diagnostics. It also implies that the assays are easier to use, cost effective and more tolerant to inhibitory components from crude samples. Ye's group[24] firstly utilized the DSN signal amplification strategy coupled with the Taqman probes to sensitively detect miRNAs. Soon after, this DSN amplification strategy has been further developed by other researchers. For instance, Lin *et al.*[27] employed backbone-modified molecular beacons for miRNA detection with reasonably good sensitivity and satisfactory selectivity. Despite achieving a sensitivity of 0.5 pM within 40 min and the advantages with the use of molecular beacons, it also has its limitations such as possible false positive signals due to the co-existing of the unhybridized molecular beacons. Moreover, the molecular beacons needed to be modified on their backbones, making the preparing steps more complex and costly. In another report, Deng and co-workers[28] proposed a dual-amplification strategy by making use of DNAzyme and achieved excellent sensitivity and selectivity. Unfortunately, dual amplification also means one more catalytic step before final detection, making it somehow cumbersome. Degliangeli and his colleagues[29] recently published a miRNA quantification method by using DNA-gold nanoparticle conjugates as detection probes. Nevertheless, the sensitivity of the assay – 5.0 pM after 5 h incubation – is not entirely satisfactory as compared to other miRNA assays. Meanwhile, a more sensitive miRNA assay was reported by Xi *et al.*[30] Through exploiting the differential affinity of WS_2 nanosheets toward short and long oligonucleotides and their efficient fluorescence-quenching ability, a miRNA assay was developed. The assay was highly sensitive and selective with a detection limit of 300 fM. Nonetheless, simple, rapid, low cost and ease-to-use miRNA assays with high sensitivity and selectivity suitable for uses at point-of-care are still in great demand. To significantly enhance the sensitivity and simplify the assay procedure, in this report, we proposed a simple, rapid, highly sensitive and selective homogeneous miRNA assay that couples magnetic beads (MBs) with the DSN for background reduction and signal amplification. By conveniently and completely removing unreacted detection probes, minute changes in fluorescence intensity of the analyzed solution could be unmistakably detected, thereby producing a detection limit of 60 fM and a very broad linear dynamic range from 100 fM to 2 nM.

Materials and Methods

Reagents and Apparatus

The DNA detection probes, synthetic miRNAs, and all other oligonucleotides were custom-made by Integrated DNA Technologies (Iowa, USA) and their respective sequences are listed in Table S-1. The DSN was purchased from Evrogen through Genomax (Singapore). Dynabeads (M-280 streptavidin-coated) were bought from Life Technologies (Singapore). Tris(hydroxymethyl) aminomethane (ACS grade) and magnesium chloride were purchased from Alfa Aesar (Lancashire, UK). Ethylenediaminetetraacetic acid (EDTA), disodium salt was obtained from 1st Base (Singapore). The composition of buffer solutions used in this work, including 1×, 2× binding and washing (B&W) buffer, and hybridization buffer, was listed in Table S-2. All solutions were prepared using nuclease-free ultrapure water (UP water) with an electrical resistance of 18.3 MΩ·cm. The pH values of all buffer solutions were adjusted with appropriate amounts of HCl or NaOH. Nuclease-free centrifuge tubes (0.65 mL and 2.0 mL) were purchased from Scientific Specialties Inc. (CA, USA). Microfuge tubes (0.2 mL) were from Theomo Fisher Scientific Inc. (DE, USA) and were certified free of DNase, RNase, human and genomic DNA contaminations. Fluorescence measurements were carried out on a Nanodrop 3300 (Theomo Fisher Scientific Inc., DE, USA). All fluorescence data were collected at 519 nm as the fluorescein-capped DNA detection probes have the maximum emission wavelength of 519 nm. The excitation wavelength for fluorescein was 495 nm.

Preparation and Quantification of DNA Detection Probe-MB Conjugates

The conjugation of the fluorescein-capped DNA detection probes to the streptavidin-coated MBs was performed according to the recommended procedure. The feeding ratio between the MBs and fluorescein-capped DNA detection probes was estimated based on the information provided in the user manual of Dynabeads M-280 streptavidin MBs. First, a desired volume of M-280 streptavidin MBs was placed into a centrifuge tube and the solvent was discarded with the help of a permanent magnet. After three careful washings with the 1×B&W buffer, the MBs were dispersed to the 2×B&W buffer and a required amount of dually tagged (biotin + fluorescein) DNA detection probes was added. The mixture was gently vortexed for 15 min at room temperature to ensure the completion of the interaction between biotin and streptavidin. The supernatant was then separated out; its fluorescent intensity

was determined and was used to estimate the coupling efficiency of the DNA detection probes to the MBs. Finally, the DNA detection probe-MB conjugates were washed three times with the hybridization buffer, dispersed in the hybridization buffer, and stored at 4 ºC for later use. It was estimated that ~4.1×10^5 DNA detection probes were coupled onto each MB, which occupied 19% of the total capacity of the MBs, leaving enough space for target miRNA hybridization and DSN cleavage. All the fluorescein-related steps were conducted in aluminum foil-wrapped centrifuge tubes to prevent from the exposure of the content to illumination which may adversely affect the fluorescence properties of the fluorescein tags.[31]

Optimization of Experimental Conditions and MiRNA Detection

All experimental conditions, including the dosage of the DSN, concentration of Mg^{2+}, pH of buffer solution, incubation temperature, and incubation time were optimized before the detection of the target miRNAs. The concentration of the DNA detection probes used in optimization experiments was 100 nM and the concentration of the target miRNA was 2.0 nM. For the target miRNA detection, 30 μL of 100 nM probes were used in the assay with varying concentration of the target miRNA. An appropriate amount of the DSN was added to each microfuge tube followed by vortexing to thoroughly mix the reaction mixture. After incubation, the DSN cleavage process was stopped when the MBs together with unreacted DNA detection probes were separated out by the permanent magnet. The supernatant then underwent fluorescence measurement. The selectivity of the assay was studied by comparing the responses of the target miRNA (let-7a) with both one and two-base mismatched miRNA in the let-7 family, namely let-7f and let-7d, respectively. The concentrations of let-7f, and let-7d were all kept at 2.0 nM and the comparison was conducted under optimized conditions.

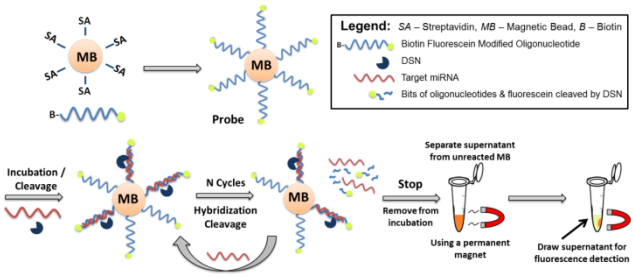
Analysis of MiRNA in Total RNA Extracted from Cultured Cells

Total RNA of cultured cells was extracted using a miRNeasy RNA extraction kit (Qiagen V.V., Hilden, Germany) according to the recommended procedure and miRNAs in the total RNA were enriched by using a clean-up kit. The yield, quality, and concentration of total RNA were routinely examined by UV-vis spectrophotometry. As a model miRNA, let-7a detection in the extracted total RNA was performed. Reference values of let-7a were obtained by qPCR.

Results and Discussion

Assay Principle

Among single-stranded DNAs, DNA homoduplexes, single-stranded RNAs, RNA homoduplexes, DNA-RNA heteroduplexes, the DSN displays a strong preference for cleaving DNA homoduplexes and DNA in DNA-RNA heteroduplexes. Moreover, the DSN is able to discriminate imperfectly matched duplexes from perfectly matched ones.[32] These unique attributes are the basis of the proposed miRNA assay. As illustrated in Scheme 1, when the target miRNA strands and the DNA detection probes form heteroduplexes, the DSN selectively cleaves the DNA detection probes while retains the target miRNA strands intact and release them back to solution for next cycle. The cleaved DNA detection probes together with their fluorescence tags are dispersed to the solution. A cumulative signal can, therefore, be obtained after numerous rounds of the hybridization and DSN cleavage cycles after a sufficiently long period of incubation. After the incubation, the unreacted DNA detection probes on the MBs are completely removed from the reaction mixture by using a permanent magnet. All the reaction mixture containing the cleaved DNA detection probes is pipetted out while holding the MBs at the bottom of the microfuge tube by the permanent magnet, thereby leaving only the cleaved DNA detection probes together with their fluorescein tags in the solution for detection. Hence, a negligible background is obtained and the fluorescence intensity of the solution is directly associated with the concentration of the target miRNA.



Scheme 1. Schematic illustration of the proposed miRNA assay.

Optimization

Contrary to the chemical coupling which may suffer from relatively large batch-to-batch variations in coupling efficiency, the dually tagged DNA detection probes offered a simple and highly efficient route to conjugate them onto the MBs through strong interaction between the biotin moieties on the DNA detection probes and the streptavidin moieties on the surface of the MBs. The dissociation constant of biotin-streptavidin conjugates K_d (4×10^{-14} M) is regarded as small enough to warrant robust attachment to the MBs and high stability of the attached DNA detection probes on the MB surface.[33] Figure S-1 indicated that during the first 15 min of incubation, the MBs were already completely coated with the DNA detection probes. Before conjugating BF oligonucleotides to the MBs, the original BF oligonucleotides solution has quite a high fluorescent intensity as the blue curve indicated; while after conjugation, the MBs with the conjugated BF oligonucleotides on their surface were separated out by a permanent magnet, and the orange curve indicated the fluorescent intensity of the solution containing the excess (free) BF oligonucleotides. In addition, the utilization of streptavidin molecules as anchoring sites ensures appropriate spacing of the DNA detection probes for easy access of the DSN.

To exploit the DSN cleavage process as a powerful amplification strategy, the cleavage of the DNA detection probes from the MBs should be exclusively associated with the concentration of the target miRNA or the fluorescence intensity must be solely controlled by the hybridization efficiency of the target miRNA. Therefore, to have the highest possible sensitivity and a straightforward correlation (linear dependence) between the analytical signal and the target miRNA concentration, the concentrations of the DSN should be kept sufficient in order to capitalize on its amplification power. The high concentrations of the DSN imply that the DSN cleavage proceeds much more rapidly than the miRNA hybridization process and the DNA detection probes are cleaved instantly as soon as they are hybridized with the target miRNA strands, thus

ensuring that the analytical signal is directly associated with the target miRNA concentration and its hybridization time. Insufficient amounts of the DSN will likely result in a non-linear behavior between the fluorescence intensity and the target miRNA concentration, whereas a high amount of the DSN will not only increase the detection cost, but also increase the possibility of false positive detection. To maximize the sensitivity of the assay while keeping its cost low, our attention had to be focused on the detection of traces of miRNAs where reasonable amounts of the DSN should be sufficient. Indeed, using let-7a as a model target miRNA, our experiments showed that the fluorescence intensity increases rapidly with the increase in the dosage of the DSN from 0.01 U to 0.30 U and approaches a steady-state in the presence of >0.40 U DSN (Figure S-2). It was found that 0.40 U DSN is sufficient for the detection of miRNAs down to femtomolar levels. Likewise, the concentration of the DNA detection probes should also be kept sufficient to make certain that the cleavage of the DNA detection probes from the MBs is solely controlled by the target miRNA hybridization process. Consequently, a considerably long period of hybridization, which favors the detection of miRNAs at ultralow levels, can be employed. Again, any notable depletion of the DNA detection probes will probably result in a non-linear dependence of the fluorescence intensity on the target miRNA concentration. Our experiments showed that 100 nM of the DNA detection probes is sufficient for the detection of target miRNA from femtomolars to nanomolars.

To take full advantage of the DSN cleavage process as a powerful amplification strategy for maximizing the assay sensitivity, besides the dosage of the DSN, all other experimental variables must be optimized. The concentration of Mg^{2+} is crucial to this assay because on one side, a certain amount of Mg^{2+} is necessary to sustain the activity of the DSN as well as the efficiency of the miRNA hybridization; while on the other side, the DSN is sensitive to the presence of high concentrations of salts. A 10-fold decrease of the DSN activity was reported in the presence of 0.20 M NaCl.[34] Therefore, an appropriate amount of Mg^{2+} should be present in the solution. From the experimental results shown in Figure 1, 25 mM of Mg^{2+} was found to be optimal for the assay to maintain efficient miRNA hybridization and maximal activity of the DSN.

The pH of the reaction medium is another important variable for the success of the assay. It has been observed that the activity of the DSN strongly depends on solution pH even though it has a relatively broad pH working range from 3.5 to 8.5 with the pH optimum at ~6.6. Extreme pH values below 3.0 or above 9.0 will completely inactive the DSN.[34] More importantly, the pH value of the buffer solution strongly affects the fluorescence of the fluorescein tags. Fluorescein is deprotonated in alkaline medium and protonated in acidic solution. In either case the conformation of fluorescein molecule is altered and subsequently the fluorescent efficiency (quantum yield) of fluorescein is significantly diminished. Taking these two aspects into consideration, it was found that pH 8.0 was optimal for the proposed assay, as reflected by the green curve in Figure 1.

In addition, the incubation temperature is expected to strongly affect both the hybridization efficiency and the DSN activity. Generally, the hybridization temperature should be 10 to 15 ºC lower than the melting temperature to have high hybridization efficiency while maintaining good selectivity. The estimated melting temperature of let-7a was ~55 ºC under the present experimental conditions. In principle, the assay should be performed at temperatures ≤45 ºC. However, the DSN prefers a higher temperature than 45 ºC to attain a better activity. The optimal temperature for the DSN to reach its highest activity is 60 ºC.[35] Temperature higher than 60 ºC adversely affects both the activity of the DSN and the hybridization efficiency. The blue curve in Figure 1 displayed the effect of the incubation temperature on the sensitivity of the assay. As seen in Figure 1, the highest sensitivity was achieved at 40 ºC.

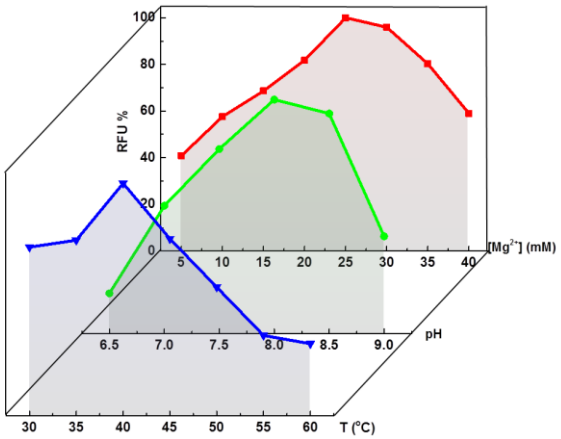


Figure 1. Optimization of the experimental conditions. 2.0 nM target miRNA let-7a, 100 nM probes, and 0.4 U DSN.

We also investigated the dependence of the fluorescence intensity of the assay on the DSN incubation time. As represented in Figure 2, the fluorescence intensity increased rapidly with the DSN incubation time in the first 60 min and then slowly leveled off with prolonged incubation. Our experiments indicated that a period of 2 h incubation is sufficient although longer incubation time, for example 240 min, could further increase the assay sensitivity by ~20%. Longer incubation time can be employed when even lower detection limit is required.

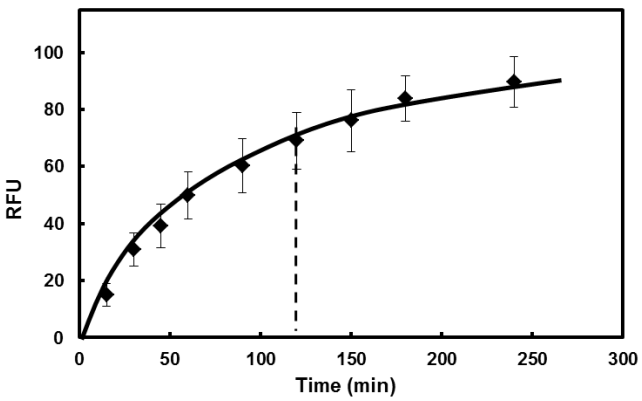


Figure 2. The dependence of the fluorescence signal on the DSN incubation time. 2.0 nM target miRNA let-7a, 100 nM probes, 40 ºC, 0.4 U DSN.

Analytical Performance

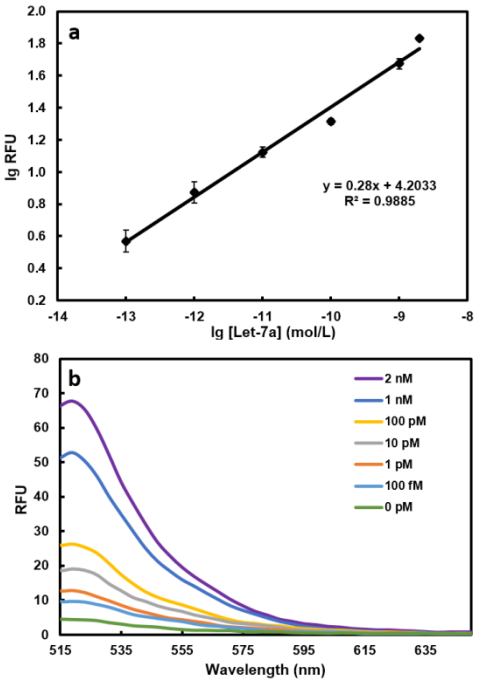


Figure 3. (a) The calibration curve of let-7a (100 nM probes, 0.4 U DSN and a period of 2 h incubation at 40 ºC) and (b) fluorescence spectra of varied concentrations of let-7a.

Utilizing the optimized experimental conditions, the correlation between the fluorescence intensity and the target miRNA concentration was investigated. As demonstrated in Figure 3, the calibration curve showed a linear dynamic range from 100 fM to 2.0 nM with a coefficient of 0.99. The relative standard deviation was less than 10% throughout the linear range from 100 fM to 2.0 nM. Figure 3b displayed the fluorescence spectra acquired when varied concentrations of the target miRNA let-7a used in the study. As can been seen from Figure 3b, the background signal of the assay, corresponding 0.0 nM of the target miRNA, was negligibly small, barely above zero. Obviously, the simple magnetic separation of the unreacted DNA detection probes from the reaction mixture was very efficient. The engagement of the MBs as the DNA detection probe carriers and the two-step procedure – amplification and magnetic separation – in this assay had a much lower non-target miRNA-related fluorescent responses simply due to the fact that practically all unreacted fluorescence tags are removed before fluorescence measurements, thus producing an extremely low background and providing substantial improvement in signal/noise ratio. The detection limit, estimated as three times of the standard deviation of the background, was 60 fM or 1.8 amol. In previous reports where the unreacted detection probes co-existed with cleaved ones, noticeable fluorescence responses were observed, evidently owing to the incomplete quenching the fluorescence tag. [24, 29, 30] Therefore, the separation of the unreacted DNA detection probes is of paramount importance in generating a sensitive fluorescence signal and in significantly reducing background fluorescence. The sensitivity obtained in this assay was much better than those of similar assays and comparable to that of qPCR miRNA assay. More importantly, the much reduced background fluorescence, only 1/10 – 1/100 of those observed in other fluorescence miRNA assays, produced an extremely wide linear calibration curve, extending from 100 fM to 2.0 nM. As comparing to qPCR miRNA assays, through the DSN-mediated target cycling, the amplification is accomplished isothermally at 40 ºC, suggesting that the proposed assay is easier to implement, more cost effective, and more tolerant of inhibitory components from crude samples than qPCR.

The selectivity of this assay was investigated through comparing the responses of the target miRNA let-7a to the one-base mismatched miRNA let-7f and two-base mismatched miRNA let-7d. As shown in Figure 4, both one-base mismatched let-7f and two-base mismatched let-7d were clearly discriminated from let-7a. The signal of the perfectly matched let-7a was 14 times higher than that of the one-base mismatched let-7f and 24 times higher than that of the two-base mismatched let-7d. The excellent selectivity is probably due partly to the mismatch discriminability of the DSN and partly to the fact that the DSN requires at least 15 perfectly paired bases in the DNA-RNA heteroduplex for cleavage. In our assay, the one-base mismatch of let-7f is in the center of the total 22 bases. Thus, only 10 or 11 perfectly matched bases are there in the DNA-RNA duplex, which effectively decreases the efficiency of the DSN cleavage.

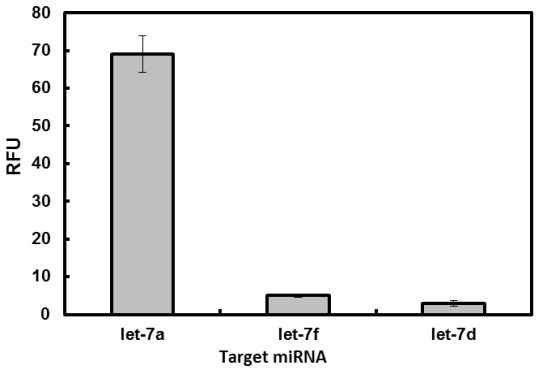


Figure 4. Responses of the let-7a assay to 2.0 nM of let-7a, let-7f, and let-7d. Experimental conditions are as for Figure 3.

Sample Analysis

Table 1. Analysis of let-7a in total RNA samples extracted from cultured cells

	Lung cancer cells (10^6 copy μg^{-1})	HeLa cells (10^6 copy μg^{-1})	Normal cells (10^6 copy μg^{-1})
This method	1.6±0.30	3.8±0.80	5.3±0.92
qPCR	1.8±0.32	3.3±0.68	5.6±0.80

To evaluate its practicability, the proposed assay was applied to analyze let-7a in three total RNA samples extracted from cultured cells. The results were summarized in Table 1. As seen in Table 1, the expression levels of let-7a obtained by the proposed assay agreed well with those obtained by qPCR. The relative errors associated with the detections were found to be less than 20%. As compared to PCR-based assays, the isothermal amplification via the DSN facilitated target cycling together with its excellent selectivity is very valuable since the freedom from thermal cycling would greatly improve the

adaptability of the assay for direct miRNA expression profiling with minimal requirement of sample preparation.

Conclusions

In conclusion, we have developed a simple and highly sensitive assay for miRNAs by using MB-carried DNA detection probes coupled with DSN-facilitated isothermal target cycling amplification. The accumulative nature of the target cycling amplification offered a powerful and yet flexible means to significantly improve the sensitivity of the assay. The isothermal amplification strategy greatly improved the suitability in direct profiling miRNA expressions with minimal or no sample pretreatments. The MBs ensured a complete removal of possible interference from unreacted DNA detection probes before fluorescence measurements, thus producing an ultralow fluorescence background and an extended dynamic range. The simplicity, high sensitivity, and the freedom from thermal amplification and miRNA tagging are some of the interesting features for the development of a simple, robust, low cost, and highly sensitive miRNA expression profiling tool uses at point-of-care.

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Notes and references

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