Published in final edited form as:

Anal Biochem. 2009 August 15; 391(2): 85–90. doi:10.1016/j.ab.2009.05.010.

Efficiency and specificity of microRNA-primed nucleotide analog incorporation by various DNA polymerases

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Abstract

MicroRNAs as endogenous regulators of gene expression have spurred a surge of interest for their quantification and expression analysis. High-sensitivity and specificity microRNA detection techniques such as Real-Time PCR and recently introduced bioluminescent microRNA detection require systematic study of DNA polymerases for use with microRNAs. In this study, a variety of DNA polymerases have been studied to assess their capabilities of using microRNA as a primer and incorporating 2'-deoxyadenosine-5'-O-(1-thiotriphosphate) as a dATP alternative during DNA strand extension. Five DNA polymerases were investigated including the mesophilic DNA polymerase I large Klenow fragment, 3'→5' exo⁻ Klenow DNA polymerase, thermophilic *Bst* DNA polymerase large fragment, TerminatorTM DNA polymerase, and *Taq* DNA polymerase. The experimental results show that except for *Taq* DNA polymerase, the polymerases can utilize microRNA as a primer and have both common and divergent properties of the nucleotide analog incorporation and microRNA discrimination. DNA polymerase I large Klenow fragment showed no detectable polymerization product with the thio-modified dATP as a substrate. Thermophilic *Bst* DNA polymerase had the highest specificity for microRNA recognition on a DNA template. The study provides a novel method for microRNA detection without reverse transcription to cDNA, which is faster, simpler, and less prone to biases and errors.

INTRODUCTION

MicroRNAs (miRNAs), a large class of 18–25-nt non-coding RNAs found in nematodes, plants, insects and mammals, are known to act as post-transcriptional modulators of gene expression by hybridizing to the complementary target mRNA transcripts and suppressing the protein production by preventing the mRNA translation and/or catalyzing mRNA degradation. In invertebrates, miRNAs regulate developmental timing, neuronal differentiation, cell proliferation, programmed cell death, and fat metabolism. It was reported that miRNAs play an important role in T cell responsiveness to antigen stimulation and mediate autoimmune disease pathology (1,2). The important role played by miRNAs in developmental and other cellular processes has stimulated a significant interest in studying miRNA expression patterns. miRNA quantification methods have been developed including real-time PCR (3,4) and microarrays for high-throughput miRNA profiling (5,6).

DNA polymerases are responsible for the accurate genome replication and maintenance. Structural and kinetic analyses of various DNA polymerases have provided solid ground for understanding their characteristics of nucleotide selection and incorporation (7). However, there are no previous systematic studies in which DNA polymerases have used miRNA as a primer for strand synthesis. In this paper, the miRNA was used as a primer to hybridize with a long complementary oligonucleotide template. The hybridized duplex was subsequently

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extended during the DNA polymerase reaction. The incorporation of dATP and its analog 2'-deoxyadenosine-5'-O-(1-thiotriphosphate) (dATPaS) by mesophilic DNA polymerases (DNA polymerase I, 3'→5' exo⁻ Klenow DNA polymerase) and thermophilic DNA polymerases (*Bst* DNA polymerase large fragment, TerminatorTM DNA polymerase, and *Taq* DNA polymerase) was investigated. Nucleotide analogs, such as ddNTP, acyNTP, dATPaS, and dATPβS et al., play an important role in identifying the dNTP recognition determinants in the polymerase reaction and elucidating the reaction mechanisms (7–10). In addition, incorporation of radioisotope, biotin, aminoallyl, digoxigenin, and fluorophore modified dNTPs has been applied to DNA sequencing, mRNA gene expression analysis, and detection of single nucleotide polymorphisms (11–14). The dNTP analog dATPaS results from the modification of the triphosphate moiety at the α position in dNTP molecules. The dATPaS has been widely used for radiolabeling (i.e., dATPa³⁵S) and as a substitute for dATP in DNA pyrosequencing, as well as a recently reported bioluminescent method for RNA quantitation (15). In bioluminescent applications the use of this substitute was found to significantly reduce the background luminescence from the reaction of dATP with firefly luciferase and therefore made this detection approach feasible (16). The polymerization rate of dATPaS by DNA polymerase I was reported to be 89% of dATP in the Mg²⁺ buffer (8). Studies have demonstrated that many triphosphate modifications do not markedly affect the incorporation efficiency of dNTPs toward some DNA polymerases (7,16,17).

In this report, the properties of different DNA polymerases for using the miRNA as a primer and incorporating dATPaS during nucleotide extension are presented. We found that various DNA polymerases are able to discriminate perfectly matched and mismatched miRNAs on the DNA template. Furthermore, the use of dATPaS as a substitute for dATP increases specificity of recognition between RNA species from the same miRNA family. The utilization of miRNA as a primer directly without the reverse transcription to cDNA in the polymerization reaction provides a straightforward approach to the miRNA detection, which is faster, simpler, and more accurate with less biases and errors.

MATERIALS AND METHODS

Reagents

The probe oligo and synthetic miRNAs were synthesized from Integrated DNA Technologies, Inc. (Coralville, IA). The sequences of the oligo probe (template) and miRNAs (primers) are listed in Table 1. To prevent non-specific hybridization and DNA extension due to the formation of self-dimers and hetero-dimers, the probe oligo was phosphorylated at the 3' end. The oligo probe was PAGE-purified. The miRNAs were RNase-free HPLC purified (Integrated DNA Technologies). Natural 2'-deoxynucleoside-5'-triphosphates (dATP, dGTP, dCTP and dTTP) were obtained from Promega Corporation (Madison, WI). The modified nucleoside triphosphate 2'-deoxyadenosine-5'-O-(1-thiotriphosphate) (dATPaS) was purchased from TriLink Biotechnologies (San Diego, CA). *E. coli* total RNA was purchased from Applied Biosystems (Austin, TX). The following commercially available DNA polymerases were purchased: DNA polymerase I large (Klenow) fragment (New England Biolabs, Ipswich, MA), 3'→5' exo⁻Klenow fragment (NEB), *Bst* DNA polymerase large fragment (NEB), TerminatorTM DNA polymerase (NEB), and *Taq* DNA polymerase (Promega). O'GeneRulerTM ultra low range DNA ladder was purchased from Fermentas (Glen Burnie, MD).

miRNA hybridization and DNA extension

DNA hybridization and extension experiments were performed in a 16 μl reaction volume containing 300 ng of oligo probe (0.9 μM), 300 ng (about 2.6 μM) of miRNA target spiked

into 0.5 μ g of *E. coli* total RNA, nucleoside triphosphates at 62.5 μ M each, 2x Denhardt's solution, and reaction buffer supplied with the respective polymerase (at 1x concentration). Either dATP or dATP α S was added to the reaction solution to investigate the incorporation efficiency of each enzyme. The mixture of the probe and miRNA target was incubated at 90°C for 5 min, cooled in ice immediately for at least 5 min, and then mixed with the reaction buffer as described above. The DNA polymerase reactions were carried out for one hour at the manufacturer's recommended optimum temperature for the respective enzyme. The reaction conditions for each enzyme were as follows: DNA polymerase I large (Klenow) fragment 10 units at 37°C, *exo*⁻ Klenow fragment 10 units at 37°C, *Bst* DNA polymerase 8 units at 65°C, TerminatorTM DNA polymerase 6 units at 65°C, and *Taq* DNA polymerase 5 units at 72°C. The 16 μ l of DNA extension reaction solution was mixed with 3.2 μ l 6x orange loading dye (Fermentas) and analyzed by 5% agarose gel stained with ethidium bromide.

Specificity of miRNA extension

miRNAs in the human let-7 family including let-7a, let-7b, let-7c, let-7d, and let-7e were used as targets to hybridize with the probe that was designed to perfectly match let-7d (see Table 1 for sequences). The DNA polymerization reactions were performed for an hour using *exo*⁻ Klenow fragment DNA polymerase 10 units at 37°C, *Bst* DNA polymerase 10 units at 65°C, and TerminatorTM DNA polymerase 6 units at 65°C. Both dATP and dATP α S were tested for the incorporation efficiency by each polymerase. The reaction products were separated by 5% agarose gel and stained with ethidium bromide.

RESULTS

miRNA-primed DNA polymerization and Incorporation of dATP α S

The structure of dATP α S is shown in Figure 1. The sulfur-bearing phosphorus at the α position is chiral, which results in the existence of two diastereomers – *Sp* (Fig. 1A) and *Rp* (Fig. 1B). It was reported that all DNA and RNA polymerases investigated so far exclusively use the *Sp* isomer (8). The polymerization rates of DNA polymerase I for dATP α S *Sp* isomer and *Rp* isomer were 89% and less than 0.2% of dATP, respectively (8). The dATP α S used in this study was a mixture of *Sp* and *Rp* isomers. Based on the dATP α S loading in the polymerase reaction, the amount of *Sp* isomer in the reaction mixture was sufficient enough for a rapid and efficient polymerization reaction.

Five DNA polymerases were studied for their ability to use miRNA as a primer and incorporate dATP α S. DNA polymerization reactions were carried out with oligo probe 061308 as a template and miRNA let-7d as a primer. The reaction temperatures were 37°C for mesophilic DNA polymerase I large (Klenow) fragment and *exo*⁻ Klenow fragment, 65°C for thermophilic *Bst* DNA polymerase and TerminatorTM DNA polymerase, and 72°C for *Taq* DNA polymerase. The reaction products were analyzed on a 5% agarose gel and stained with ethidium bromide (Figure 2). The band in lane 15 is the duplex of oligo probe 061308 and miRNA let-7d resulting from the hybridization of complementary bases. Lanes 1 and 6 show the strand extension products of DNA polymerase I large fragment using dATP α S and dATP, respectively. Compared to lane 1, the band in lane 6 has a higher molecular weight (about 70 bp) as measured by comparison with the co-migrating dsDNA markers, indicating that DNA polymerase I utilizes miRNA let-7d as a primer and generates DNA extension products using dNTPs as nucleotide substrates. However, lane 1 contains only the hybridization product with no detectable DNA extension products. The experimental results demonstrate that DNA polymerase I large fragment can use miRNA as a primer, but its incorporation efficiency of dATP α S is very low.

The DNA extension products for dATPaS and dATP from *Bst* DNA polymerase are shown in Figure 2 lanes 2 and 7, respectively. The lower band in lane 2 represents the duplex of the hybridized oligo probe and miRNA target, while the upper band represents the extended 69 bp double-strand product. The density ratio of upper band to lower band is about 1.4 calculated with ImageJ software version 1.41o (Wayne Rasband, National Institutes of Health, USA). Lane 7 contains an intense band approximately 70 bp, which corresponds to the product of the DNA polymerase reaction. The experimental results show that *Bst* DNA polymerase can utilize miRNA as a primer for DNA extension. However, only 58% of hybridized oligo and miRNA were extended when dATPaS was used as a nucleotide substrate, which is much lower than its natural substrate dATP.

Lanes 3 and 8 of Figure 2 contain the DNA polymerization products from Terminator™ DNA polymerase. It was reported that Terminator™ DNA polymerase was a 9°N_m™ DNA polymerase variant with an enhanced ability to incorporate modified nucleotides such as dideoxynucleotides, ribonucleotides, and acyclonucleotides (10, 18). We observed a stronger band density in lane 3 using dATPaS as a substrate than in lane 8, where dATP was used as a substrate. The results indicate that Terminator™ DNA polymerase has the capability to use the miRNA as a primer and incorporate dATPaS with comparable high efficiency to its natural substrate dATP.

Lanes 4 and 9 in Figure 2 show the products from *Taq* DNA polymerase reaction using dATPaS and dATP as a substrate, respectively. Compared to lanes 6–8, lane 9 contains only one band with approximately the same molecular size as the band in line 15, which is the duplex of the hybridized miRNA and oligo probe 061308. However, no double-stranded DNA extension product was detectable. Therefore, *Taq* DNA polymerase did not utilize miRNA as a primer, which is consistent with the observations reported by Greenough and Jack (19).

Lanes 5 and 10 in Figure 2 show the DNA polymerization products from exo⁻ Klenow fragment DNA polymerase. Both lanes contain clearly detectable double-stranded DNA extension products. The density ratio of the bands on lane 5 (dATPaS as a substrate) to lane 10 (dATP as a substrate) is about 0.7 as was measured using gel image processing software. The experimental results demonstrate that exo⁻ Klenow fragment can use miRNA as a primer and has a relatively high efficiency of incorporating dATPaS as a dATP substitute.

Table 2 summarizes the characteristics of the five DNA polymerases studied for using miRNA as a primer and dATPaS as a dATP substitute. The experimental results demonstrate that among the five DNA polymerases, only *Taq* DNA polymerase cannot be used with miRNA primers, while the other DNA polymerases can process miRNA primers and show different efficiencies for dATPaS incorporation.

Discrimination between miRNAs with high homology

The ability of each DNA polymerase to extend different primers with a high degree of homology was investigated using the miRNA let-7 family as primers. Among the five DNA polymerases, three DNA polymerases including 3'→5' exo⁻ Klenow fragment, *Bst* DNA polymerase, and Terminator™ DNA polymerase were further studied for their specific extension of miRNA primers with high sequence similarity. In previous experiments we established that *Taq* DNA polymerase cannot use miRNA as a primer and DNA polymerase I large (Klenow) fragment shows no detectable DNA extension product when dATP is substituted with dATPaS. Therefore, these two enzymes were excluded.

The abilities of the three DNA polymerases to discriminate between miRNAs that differ by as little as three nucleotides were tested using five synthetic miRNAs of let-7a, let-7b, let-7c,

let-7d, and let-7e. Figure 3A, B, and C show the DNA polymerization products of exo^- Klenow fragment, *Bst* DNA polymerase, and Terminator™ DNA polymerase, respectively. The oligo probe was probe 061308, which was perfectly matched to let-7d. For the five gel lanes for each DNA polymerase in Figure 3A, B, and C, the miRNA primers used were let-7a, let-7b, let-7c, let-7d, and let-7e from left to right, respectively. The dNTPs used in lanes 1–15 were dATPαS, dCTP, dGTP, and dTTP. The dATPαS was used in this study to investigate the ability of the polymerases to use modified dNTP analog as a substrate. Efficiency of DNA extension (%) is calculated as the ratio of the band density of the extended DNA product between the mismatched and perfectly matched miRNA primer, assuming 100% efficiency for the perfectly matched let-7d. The band density was measured using the gel image analysis software (ImageJ, v. 1.41o). Lanes 16–18 show products of DNA polymerization reactions using natural dNTPs and exo^- Klenow fragment, *Bst* DNA polymerase and Terminator™ DNA polymerase, respectively.

The results of this specificity study are summarized in Figure 3E. For exo^- Klenow fragment, the polymerization efficiency with primers of let-7a, let-7c, and let-7e were 24.2%, 10.7% and 100% respectively. The band intensity corresponding to the extension product of let-7e primer was indistinguishable from that of the perfectly matched let-7d miRNA primer. In addition, the bands on lane 4 and lane 16 show little or no difference, which demonstrates that the replacement of dATP by dATPαS substrate does not significantly reduce the nucleotide incorporation rate of exo^- Klenow fragment. Lanes 11–14 of Figure 2 correspond to the products of the polymerization reaction of the exo^- Klenow fragment polymerase with miRNAs let-7a, let-7b, let-7c, and let-7e primers and non-modified nucleotides substrates. In Figure 2 no significant difference in discrimination between similar miRNA sequences is observed when dATP is substituted by dATPαS.

The *Bst* DNA polymerase shows much higher specificity than exo^- Klenow fragment DNA polymerase. As can be seen from Figure 3B and 3E, *Bst* DNA polymerase does not generate non-specific primer extensions for the mismatched miRNA primers let-7a, let-7b, let-7c, and let-7e. However, compared to lane 17 where 100% of the hybridized duplex of the 69-nt oligo probe and 21-nt miRNA primer let-7d was extended with dATP/dCTP/dGTP/dTTP by *Bst* polymerase, only 56% of the hybridized oligo and miRNA primer let-7d was extended to generate products shown in lane 9 when dATPαS analog was used. The experimental results demonstrate that *Bst* DNA polymerase has higher specificity towards miRNA primer recognition, but its utilization rate of dATPαS is lower than exo^- Klenow fragment DNA polymerase.

Figure 3C shows the gel image for Terminator™ DNA polymerase reaction with dATPαS as dATP's substitute and miRNA primers of let-7a, let-7b, let-7c, let-7d, and let-7e from lane 11 to lane 15, respectively. Percentages of DNA extension products for the mismatched let-7a and let-7e primers were measured to be 4.3% and 100% of the perfectly matched let-7d primer. For Terminator™ DNA polymerase, the replacement of dATPαS analog (lane 14) did not cause any significant changes of the band density compared to the natural substrate dATP (lane 18).

DISCUSSION

In this study we investigated the characteristics of several DNA polymerases for their ability to use miRNA as a primer and incorporate dATPαS as a dATP analog for the miRNA extension on the DNA template. Among the five polymerases studied, the *Taq* DNA polymerase has the lowest, near non-existing efficiency for using miRNA as a primer on the DNA template. In contrast, the DNA polymerase I large (Klenow) fragment has a high efficiency for using miRNA as a primer. However, this polymerase shows very low

efficiency for the incorporation of dATP α S. Exo⁻ Klenow fragment DNA polymerase, *Bst* DNA polymerase, and TerminatorTM DNA polymerase present relatively high efficiencies of miRNA-primed DNA-directed polymerization and dATP α S incorporation.

The ability of these three DNA polymerases to discriminate between miRNA family members with the high nucleotide sequence homology was also demonstrated. Due to a limited thermal stability, the DNA polymerization reaction using exo⁻ Klenow fragment DNA polymerase was carried out at 37°C, under which the formation of self-dimers and hetero-dimers was possible. The binding of mismatched miRNA primer to the oligo template and the subsequent RNA-primed DNA polymerization reaction at 37°C formed the non-specific products. Figure 4 shows the formation of oligo template and miRNA primer complex. The number of complementary base pairs formed between the first eight bases at the 3' end of the miRNA primer and the oligo template was 3, 4, and 5 for miRNA let-7b, let-7c, and let-7a, respectively (Fig. 4B, C, A). Following the increase of the sequence homology at the primer's 3'-end the amount of reaction product is increasing from 0, 10.7% to 24.2 for let-7b, let-7c, and let-7a (Fig. 3E). When there are five or more complementary base pairs at the 3' end of the miRNA primer, as it takes place for let-7e (Fig. 4E), the exo⁻ Klenow fragment does not distinguish the partially and perfectly matched let-7e and let-7d primers (Fig. 3E).

Among the polymerases studied, thermophilic *Bst* DNA polymerase large fragment has the highest specificity for miRNA target recognition, which can discriminate between miRNAs with at least three mismatched bases. It was reported that *Bst* DNA polymerase, similar to *Taq* DNA polymerase, has a more than five-fold lower efficiency in extending a mismatched primer than matched primer (20). Due to the enhanced thermal stability, the *Bst* polymerase reaction can be carried out at the optimum temperature of 65°C vs. the optimum temperature of 37°C for the exo⁻ Klenow fragment polymerase. The increased reaction temperature could be a viable solution for the non-specific priming and reducing non-specific hybridization between miRNA probe and native total RNA present in the samples. In our experiments we found the *Bst* polymerase combined with the substitution of dATP by dATP α S provides the best specificity for discriminating between the miRNA let-7 family members. However, in some applications such as our previously reported bioluminescent RNA detection using the RNA-primed DNA polymerase reaction (15), the lower efficiency of dATP α S incorporation has to be taken into consideration. It was reported that the *Bst* polymerase incorporated nucleotide analogs, such as ddNTPs, dITP and 7-deaza-dGTP without undue discrimination (21). However, divergent biases in the recognition of modified nucleotide exist in a variety of DNA polymerases (10). Compared to the other two enzymes, the TerminatorTM DNA polymerase has higher specificity than exo⁻ Klenow fragment due to its thermostable property, but it has less discrimination capability than *Bst* DNA polymerase and cannot discriminate between let-7d and let-7e miRNAs.

To the best of our knowledge this report is the first systematic study of using miRNA directly as a primer without reverse transcription to cDNA. The approach to the miRNA extension on the DNA template can be used with various detection and RNA processing techniques, including Real-Time PCR, Rolling Circle Amplification and bioluminescent RNA detection with no need for miRNA reverse transcription and subsequent purification, which often are sources of biases and errors in the miRNA analysis. RNA hybridization has been widely used in Northern blotting for a long time. In Northern blotting, the freshly isolated RNA samples are fractionated by formaldehyde-denaturing agarose gel electrophoresis, transferred to a positively charged nylon or nitrocellulose membranes and immobilized by UV light. A labeled DNA or RNA probe is then used to hybridize and recognize the RNA of interest on the membrane. An important fact is that a standard Northern blotting experiment usually takes about 2 days and RNA molecules are stable

during the entire process. This indicates that RNA is a good source of material to be used directly in the hybridization experiment for the analysis and detection. High-quality and intact miRNAs are essential for the miRNA-primed DNA polymerase reaction and therefore preventing RNA degradation due to RNase contamination is critical to achieve accurate data in the assay for real samples.

Acknowledgments

This work is supported in part by the National Institutes of Health/National Cancer Institute Small Business Innovation Research grant 5R43CA126647 and National Institutes of Health/National Institute of General Medical Sciences grant RGM080037.

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Fig. 1. Structure of 2'-deoxyadenosine-5'-O-(1-thiotriphosphate) diastereomers. (A) dATPaS *Sp* isomer. (B) dATPaS *Rp* isomer.

Fig. 2.

Gel electrophoresis of miRNA-primed DNA extension products. The DNA extension experiments were carried out in the conditions described in the “Materials and methods”. The DNA extension products were isolated by 5% agarose gel and stained with ethidium bromide. For lanes 1–10, the probe was the oligo probe and target was miRNA let-7d with sequences listed in Table 1. M: DNA ladder. Lane 1: DNA polymerase I, dATPaS. Lane 2: *Bst* DNA polymerase, dATPaS. Lane 3: TerminatorTM DNA polymerase, dATPaS. Lane 4: *Taq* DNA polymerase, dATPaS. Lane 5: *exo*⁻ Klenow fragment, dATPaS. Lane 6: DNA polymerase I, dATP. Lane 7: *Bst* DNA polymerase, dATP. Lane 8: TerminatorTM DNA polymerase, dATP. Lane 9: *Taq* DNA polymerase, dATP. Lane 10: *exo*⁻ Klenow fragment, dATP. *Exo*⁻ Klenow fragment was used in lanes 11–14 and the miRNA targets were let-7a, let-7b, let-7c, and let-7e, respectively. Lane 15 was the mixture of 300 ng probe 061308 and 300 ng let-7d.

E

	Synthetic miRNA primers				
	Let-7a	Let-7b	Let-7c	Let-7d	Let-7e
3'→5' <i>exo</i> ⁻ (Klenow) fragment	24.2	0	10.7	100	100
<i>Bst</i> DNA polymerase	0	0	0	100	0
Therminator TM DNA polymerase	4.3	0	0	100	100

Note. Values are percentages of DNA extension product.

Fig. 3.

Specificity of DNA polymerase reaction using miRNA let-7 family as primers. M: DNA ladder. The DNA polymerization reactions were carried out in the conditions described in the "Materials and methods" using three different DNA polymerases: (A) 3'→5' *exo*⁻ Klenow fragment on lanes 1–5, (B) *Bst* DNA polymerase on lanes 6–10, and (C) TherminatorTM DNA polymerase on lanes 11–15. dCTP, dGTP, dTTP, and dATPαS were used as nucleotides. The miRNA primers used for A, B, and C are let-7a, let-7b, let-7c, let-7d, and let-7e from left to right. (D) DNA polymerase reactions using dCTP/dGTP/dTTP/dATP as nucleotides. DNA polymerases used to perform the DNA extension reactions were: lane 16 - 3'→5' *exo*⁻ Klenow fragment, lane 17 - *Bst* DNA polymerase, and lane 18 - TherminatorTM DNA polymerase. Lane 19 was the hybridized duplex of 300 ng probe 061308 and 300 ng let-7d. (E) Discrimination ability of miRNA let-7 family as primers for different DNA polymerases. Percentage of DNA extension product (%) is calculated as the ratio of the band density of the extended DNA product between perfectly matched and mismatched oligo probe and miRNA primer.

Figure 4.
 The formation of probe/miRNA let-7 complex. Lines represent complementary base pairs.

Table 1

Sequences of oligo probes and miRNAs

Gene symbol	Gene sequences
Probe 061308 *	5'-aagcaccagacaagacagcacaaagcacctatagtgagtcgtattata <u>tactatgcaac</u> tactacct -3'
let-7a	5'-ugagguaguagguuguauaguu 3'
let-7b	5'-ugagguaguagguugugugguu -3'
let-7c	5'-ugagguaguagguuguauugguu -3'
let-7d	5'-agagguaguagguugc <u>au</u> agu -3'
let-7e	5'-ugagguaggagguuguauagu -3'

* The bases designed to hybridize to the miRNAs are underlined.

Table 2

Characteristic summary of various DNA polymerases

DNA polymerases	miRNA as primer	dATP	dATPaS
DNA polymerase I	+ ¹	+++	ND ²
<i>Bst</i> DNA polymerase	+	+++	+
Therminator TM	+	+++	+++
<i>Taq</i>	–	–	–
exo ⁻ Klenow fragment	+	+++	+++

¹“+” means the capability to use miRNA as primer, incorporate dATP or dATPaS. “–” means no capability. The efficiency increases with the increase of “+”.

²ND – Not detectable.