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Isothermal sensitive detection of microRNA using an autonomous DNA machine recycling output as input

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ABSTRACT

An autonomous DNA machine recycling the output as the input for isothermal, sensitive, and specific detection of miRNAs has been developed. This machine shows considerably high signal amplification efficiency (~1000-fold) and thus a low detection limit (~20 amol). The machine also shows high specificity, discriminating 50 amol of synthetic miRNA from 100-fold larger amounts of its family member and from 100 ng of unrelated total RNAs. Moreover, it is available for practically detecting natural miRNAs in total RNAs.

MicroRNAs (miRNAs) are noncoding RNA molecules that play an important role in eukaryotic gene regulation.[1] They are very short nucleotides (17–25 nt), but long enough to bind to their perfectly or near-perfectly matched sequences in mRNA to induce degradation or translational repression of the mRNA. It has been reported that the expression levels or distribution of various kinds of miRNAs are directly linked to several significant cell activities; miRNAs are therefore expected to be used as diagnostic markers for cells.[2] However, it is challenging to detect them because they are small and scarce. To date, some inventive approaches for sensitively and specifically detecting these rare miRNAs have been reported, including microarray assays using locked nucleic acids,[3] real-time PCR assays,[4] and capillary electrophoresis assays in combination with rolling circle amplification (RCA).[5] Nonetheless, these assays require costly special equipment and/or cumbersome procedures. I report here a simpler method based on a DNA machine[6] for detecting miRNAs with high sensitivity and specificity.

In detecting miRNAs, it is valid to directly use the target miRNA as a primer in a polymerase-based reaction.[4,5,7] This is because the mismatches in some miRNA families are concentrated around the 3′-terminus[8] and additionally because polymerase requires a precise base-pairing of the primer's 3′-terminus to extend the primer. Moreover, higher temperatures increase the efficiency for discriminating the target from mismatched strands.[5,7] On the other hand, most DNA machines for detecting DNA work at lower than 40 °C, wherein the target DNA is used not as a primer but as a trigger for activating the machine and thus is reused with the help of other components of the machine for catalytic signal amplification and sensitive detection of itself.[6,9] Among these DNA machines, a polymerase-based DNA machine for sensitive DNA detection, which is composed of two DNA components (hairpin probe and template) in combination with two enzymes, has the potential to be used for sensitive miRNA detection.[9a] In this machine, the hairpin probe is opened by the target (= input DNA), and the opened probe is then used as a primer to produce detectable output DNA. However, to use the target as a primer instead of the hairpin probe, an issue must be resolved. Because the hairpin probe is required not only for recognition of the target but also for catalytic reuse of the target, another catalytic effect must be introduced for sensitive detection without the probe. I have therefore designed a novel DNA machine in which the target (first input) is used as a primer and the output DNA is automatically recycled as the second input for catalytic amplification of the output without any probe DNA (Fig. 1). This machine also works at a higher isothermal temperature (65 °C) for specific detection of the target.

The mechanism of this machine is as follows: (1) a target binds as the first input directly to an anti-target sequence on the DNA machine template (DM-template); (2) the target bound to the DM-template is extended by *Bst* DNA polymerase, which extends not only DNA but RNA, and the extended target is cleaved at the sequence 5′-G/CATTC-3′ by Nb.BsmI; this step is cycled a number of times due to the high strand displacement activity of the polymerase; (3) as a result of this cycle, a large amount of cleaved DNA (i.e., output DNA) is produced, although at this stage it is

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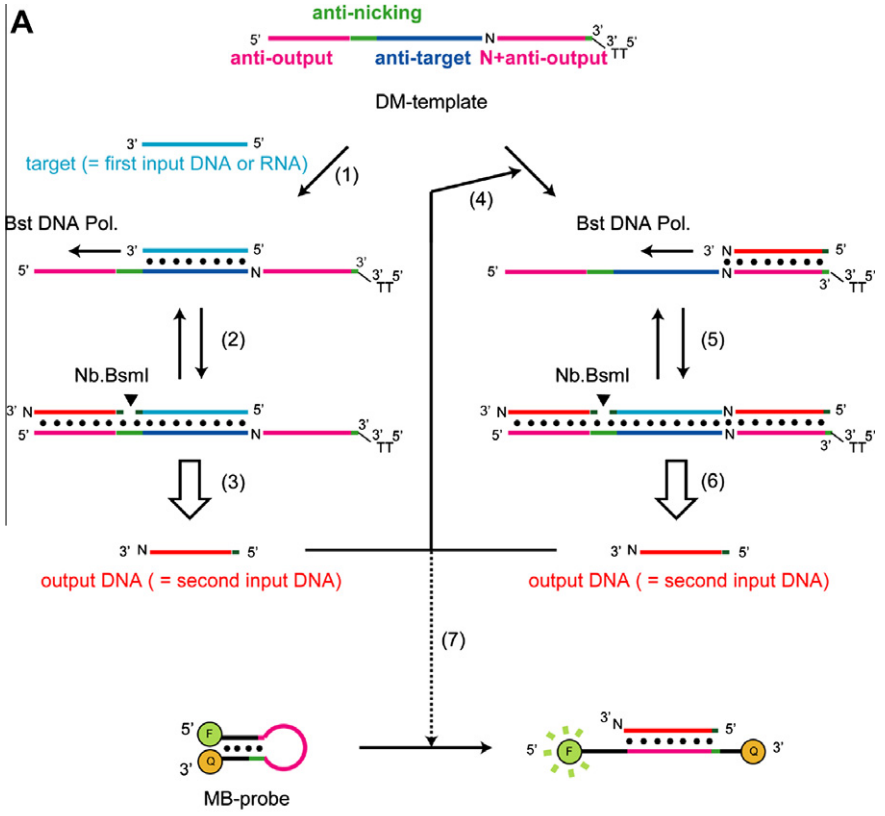

Figure 1. (A) Schematic mechanism of the autonomous DNA machine recycling the output as the input for isothermal sensitive detection of nucleic acids. To increase the discrimination efficiencies, *Bst* DNA polymerase and Nb.BsmI (nicking enzyme), both of which work at 65 °C, are used. (B) List of nucleic acids sequences. A 3′-terminus of DM-template is capped by two reverse Ts to inhibit its false extension. N represents a randomized nucleotide. Nb.BsmI nicks the sequence 5′-G/CATTC-3′ of the extended input DNA (complementary to DM-template).

not sufficient for sensitive detection (vide infra); (4) the output DNA released from the template binds as the second input to an anti-output sequence next to a randomized nucleotide N (N+anti-output) on another template rather than a 5′-terminal one because the output DNA has an extra nucleotide N on its 3′-terminus due to the polymerase lacking exonuclease activity; (5)(6) similarly to steps (2) and (3), the output DNA bound to the template automatically produces a large amount of itself, which is also used as the second input DNA in the step (4); (7) the amplified output DNA is simply detected by a standard molecular beacon technology. The reaction mixture contains only one component DNA (DM-template), *Bst* DNA polymerase, Nb.BsmI, dNTPs, and the target. All one has to do is incubate the reaction mixture at 65 °C for a few hours, and then add an MB-probe to the mixture to measure its fluorescence.

I first checked the amplification efficiency of this output-recycled machine by using DNA (DNA-122b in Fig. 1B) corresponding to one of the miRNAs, miR-122b (vide infra), as a target to be compared with that of other DNA-based machines. Figure 2A shows fluorescence signals in the presence of various amounts of DNA-122b at various reaction times under optimized conditions. Comparison of the fluorescence to a calibration curve (Supplementary Fig. S1) reveals that the efficiency of this machine for signal amplification is considerably high. Especially in the range between 5 and 50 amol of the target, a 120-min reaction produced more than 10,000-fold larger amounts of output DNA. This efficiency is slightly higher than that of standard RCA.[10] Therefore, the detection limit is as low as 500 zmol, which is equivalent to that of the original DNA machine containing the hairpin probe[9a] and other DNA-based machines.[9b,d,e,g] On the other hand, when a template without the N+anti-output sequence or without N was used instead of the DM-template, there was either no fluorescence signal or one much lower (approximately 20-fold), respectively, in the presence of 500 amol of DNA-122b with a 120-min reaction. These results clearly indicate that the anti-output sequence including the randomized nucleotide N on the DM-template is absolutely required for catalytic performance and sensitive detection.

miR-122b, one of the miRNAs highly expressed in the liver and believed to be involved in liver disease,[11] was targeted next. Figure 2B shows fluorescence signals in detecting various amounts of synthetic miR-122b. The fluorescence signals were all lower than those corresponding to DNA detection, probably due to the lower activity of the polymerase for RNA extension than for DNA. Nonetheless, the detection limit for miR-122b (20 amol) is slightly lower

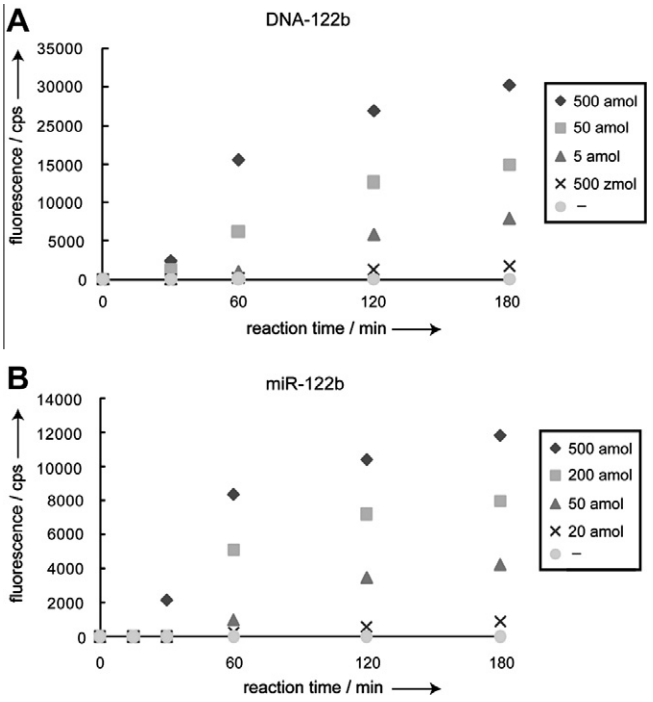

Figure 2. Fluorescence signals of the DNA machine in the presence of various amounts of DNA-122b (A) or miR-122b (B) at various reaction times. All samples were reacted in a total volume of 10 μL. Each relative fluorescence is calculated as the difference between before (0 min) and after the reaction.

than that of the RCA-based method for detecting miRNAs.[5] To further analyze the details regarding products of this machine, a denaturing PAGE analysis of reaction solution was performed (Fig. 3). The output DNA was clearly detected in the 120-min reaction with 500 amol of miR-122b (lane 4), while no output was detected in the absence of the target (lane 3) and before the reaction (lanes 1 and 2). It should be noted that the output DNA produced from the machine is slightly longer than the control DNA (output-N); this proves that *Bst* DNA polymerase actually adds an extra nucleotide to the 3′-terminus of the output DNA, which is in good agreement with the result that a template without N exhibits a much lower catalytic effect (vide supra). The amount of output DNA is estimated from the band intensity to be ~500 fmol, which agrees with the amount calculated from the fluorescence signals (Figs. 2B and Supplementary Fig. S1) and is approximately 1000 times larger than the input target. The thick band, which is slightly longer than the DM-template (lane 4), is estimated from its length to be a non-nicked product complementary to the DM-template. Its higher intensity than that of the DM-template indicates that the nicking enzyme (Nb.BsmI) was losing its activity during the reaction, given the fact that the increase rate of fluorescence decreases with the reaction time, as shown in Figure 2B.[12] A much thinner band corresponding to this non-nicked product was also detected in the absence of the target (lane 3), which is attributed to false amplification by *Bst* DNA polymerase, as recently reported.[13] However, because in addition to the gradually weakening Nb.BsmI a small amount of the output is caught by the abundant free DM-template, it is not detected with a molecular beacon (Fig. 2B, circles).

Finally, the specificity of this machine for the target was evaluated. Figures 4A and B show fluorescence signals of the machine with a 120-min reaction in the presence of various kinds of synthetic miRNAs and some mixtures of them, respectively. While 50 amol of miR-122b (sample 1) was clearly detected, 10-fold and 100-fold larger amounts (500 amol and 5 fmol, respectively) of miR-122a (samples 3 and 4, respectively), which is one of miR-122 family members and differs from miR-122b only in its 3′-terminal single nucleotide, produced no or a much weaker signal, respectively. Moreover, 1000-fold larger amounts (50 fmol) of a double mismatched RNA (2mis, sample 6) gave no signal. This result suggests that at least 50 fmol of foreign miRNAs belonging to families other than the miR-122 family should not be detected. In fact, 50 fmol of miR-23b and 50 fmol of miR-16 were not detected (samples 7 and 8, respectively). In competitive assays (Fig. 4B), 50 amol of miR-122b was specifically detected in the co-presence

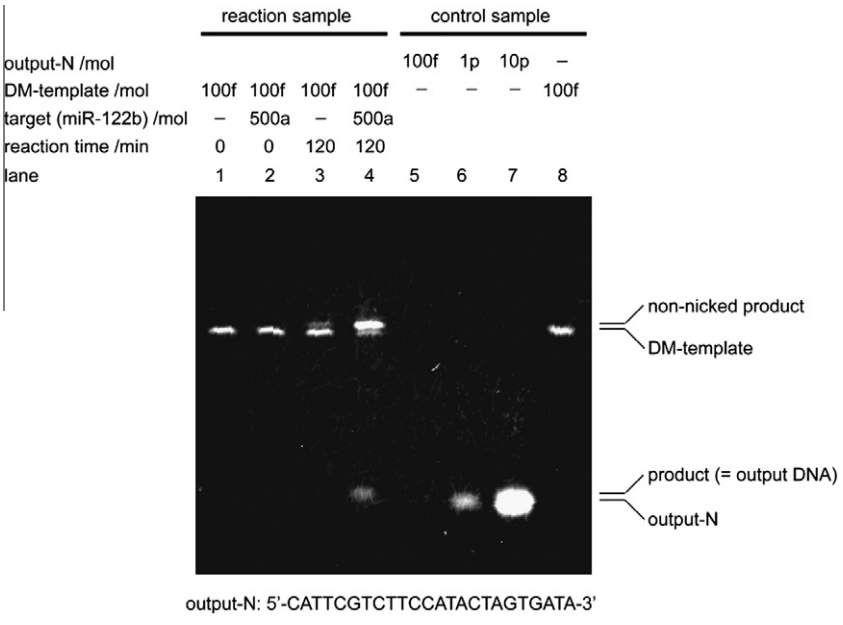

Figure 3. Denaturing PAGE analysis of reaction mixtures in the absence or presence of 500 amol of miR-122b before or after the 120-min reaction (lanes 1–4). Various concentrations of control DNA (output-N), which has almost the same sequence as a predicted output except lacking a 3′-terminal extra nucleotide N, and the same amount of DM-template as in the reaction sample were also loaded for comparison (lanes 5–8). Note that 100 fmol of output-N was not detected, although the 100 fmol of DM-template was detected because the longer EtBr-stained DNA is brighter. miRNA was too dilute to be detected.

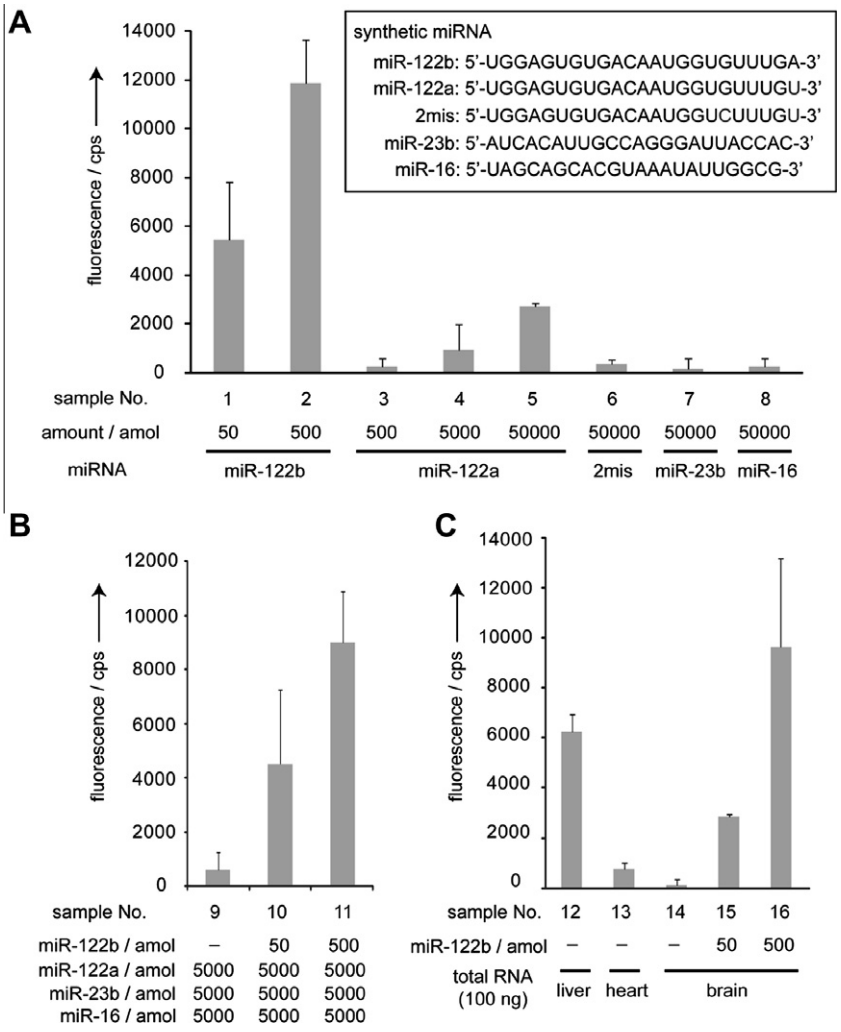

Figure 4. Fluorescence signals of the machine in the presence of various synthetic miRNAs (A), some mixtures of them (B), or various mouse total RNAs including natural (and synthetic) miRNAs (C). Each relative fluorescence is calculated as the difference from a target-free sample. All samples were reacted for 120 min in a total volume of 10 μL. The sequences of synthetic miRNAs are shown in the inset in (A).

of 5 fmol (100-fold larger amount) each of miR-122a, miR-23b, and miR-16 (sample 9 vs 10). Figure 4C shows the detection of miR-122b in 100 ng of total RNAs from mouse liver (sample 12), heart (sample 13), and brain (samples 14, 15, and 16). Natural miR-122b (and miR-122a,b) was detected only in the liver (sample 12), and not in the heart (sample 13) or the brain (sample 14), as previously reported,[8] and the amount was estimated to be 100–200 amol per 100 ng total RNA in the liver.[14] In addition, 50 amol of synthetic miR-122b was specifically detected in the co-presence of 100 ng of total RNA from brain, including many kinds of unrelated natural miRNAs and other RNAs (sample 15).

In summary, I have developed an autonomous DNA machine recycling the output as the input for isothermal, sensitive, and specific detection of miRNAs. This machine shows considerably high signal amplification efficiency: it can produce ~1000-fold larger amounts of output compared to miRNA input (~10,000-fold to DNA input) and thus achieve a detection limit as low as ~20 amol of miRNA (~500 zmol for DNA detection). This detection limit is comparable to that of some other complex methods, despite the low cost and simplicity of this machine with regard to both its preparation and use. The present machine also shows high specificity: it can discriminate 50 amol of synthetic miRNA from 100-fold larger amounts of 3′-terminal single-mismatched miRNA (one of the miRNA family members) and from 100 ng of unrelated total RNAs. Moreover, it is available for practically detecting natural miRNAs in total RNAs. Although a molecular beacon technology was used to detect mass-produced output DNA in this study, other methods for more sensitive DNA detection can be used instead, which may decrease the detection limit. In addition, because this machine can convert a certain nucleic acids sequence (input) to others (output) with amplification, it could potentially be used for other technologies such as complex logic networks. Further studies along these lines are in progress.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.08.055.

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12. This band is much thicker than a band corresponding to DM-template, indicating that non-nicked products were produced much more than DM-template. This is probably because the output DNA somewhat replaced the saturated non-nicked products on DM-template and extended by *Bst* DNA polymerase to be non-nicked products. Although the overproduced single-stranded non-nicked products have potential to open the MB-probe as well as the output DNA, the amount is estimated to be much smaller (~10%) compared to that of the output DNA even in the presence of 500 amol of target RNA. Incidentally, the possible reason why the band of DM-template in lane 4 is slightly thinner than those in other lanes (lanes 1–3) is the weakening Nb.BsmI falsely nicked DM-template similarly to normal restriction enzymes.
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