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Signal amplification of microRNAs with modified strand displacement-based cycling probe technology

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Micro ribose nucleic acids (miRNAs) play an important role in biological processes such as cell differentiation, proliferation and apoptosis. Therefore, miRNAs are potentially a powerful marker for monitoring cancer and diagnosis. Here, we present sensitive signal amplification for miRNAs based on modified cycling probe technology with strand displacement amplification. miRNA was captured by the template coupled with beads, and then the first cycle based on SDA was repeatedly extended to the nicking end, which was produced by the extension reaction of miRNA. The products generated by SDA are captured by a molecular beacon (MB), which is designed to initiate the second amplification cycle, with a similar principle to the cycling probe technology (CPT), which is based on repeated digestion of the DNA–RNA hybrid by the RNase H. After one sample enrichment and two steps of signal amplification, 0.1 pM of let-7a can be detected. The miRNA assay exhibits a great dynamic range of over 100 orders of magnitude and high specificity to clearly discriminate a single base difference in miRNA sequences. This isothermal amplification does not require any special temperature control instrument. The assay is also about signal amplification rather than template amplification, therefore minimising contamination issues. In addition, there is no need for the reverse transcription (RT) process. Thus the amplification is suitable for miRNA detection.

Introduction

miRNAs play an important role in biological processes, including cell differentiation, proliferation and apoptosis.[1] Abnormal expression of miRNAs leads to physical abnormalities and diseases. In many cancerous cells, such as in colon cancer and breast cancer cells, miRNA expression levels will change, and may have the effect of proto-oncogenes and tumour suppressor genes. In addition, the abnormal expression of miRNAs has been detected in many diseased tissues or cells. Therefore, further research on the quantitative detection of miRNAs in samples of tissue or cells will assist investigators to understand the relationship between miRNAs and disease development, and additionally provide a new method for early diagnosis of diseases. However, unique characteristics of miRNAs such as short length, minimal expression levels, and high sequence homology among family members, have made a great challenge for quantitative analysis.[2] At present, the main methods for miRNA detection are microarray chips, new-generation sequencing technology and various amplification strategies (mainly the RT-quantitative polymerase chain reaction (qPCR)).[3]

Microarray-based techniques are particularly attractive for miRNA profiling as they are capable of screening large numbers of miRNAs simultaneously. However, the sensitivity of a microchip is not high due to the small amount of the sample. In addition, the selectivity is also unsatisfactory as the hybrid environment of all miRNAs cannot be efficiently optimized at the same time, owing to the difference in the length and the composition of the miRNAs. Moreover, the cost is high.[4] Next-generation sequencing can detect the known and unknown miRNAs. However, the use of special equipment, associated high costs, and intense calculation and analysis on bioinformatics, limit its applicability.[5] Among various amplification strategies, real-time PCR is the most sensitive and practical.[6] However, the short length of miRNAs makes the primer design difficult. The most common solution is to design the stem-loop primers, using an SG fluorescent dye or the TaqMan probe method for detection. The former is low cost, but has poor specificity; the latter is specific, but each miRNA needs a

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corresponding TaqMan probe, therefore is expensive.[7] RT-qPCR relies on the synthesis of complementary DNA (cDNA), and if the reverse transcription efficiency is too low, is prone to false negative results. In addition, methods based on template amplification are compromised due cross contamination from aerosolised amplicons.

Thus an inexpensive and simple method for miRNA detection is still challenging.[8] In this paper, we present a novel signal amplification method for analyzing miRNAs. The method is composed of one enrichment step and two signal amplification cycles. miRNA was captured by the template coupled with beads, and then the first cycle based on SDA was repeatedly extended to the nicking end, which was produced by the extension reaction of miRNA. The products generated by SDA are captured by a molecular beacon (MB), which is designed to initiate the second amplification cycle, with a similar principle to the cycling probe technology (CPT), which is based on repeated digestion of the DNA–RNA hybrid by the RNase H.[9–11] The method is termed SDA-CPT. The key point of the SDA-CPT is the design of an oligonucleotide containing a sequence complementary to miRNA, a nicking site, and a sequence reverse complementary to the MB used to trigger CPT. As it is an isothermal amplification, no special temperature control instrument is required. This assay is underpinned by the signal amplification process, and therefore cross-contamination due to aerosol should be greatly suppressed. Most importantly, the assay is initiated by the direct extension of miRNA, hence no reverse transcription process is needed. This significantly simplifies the process of miRNA detection.

Experimental section

Materials

The HPLC-purified molecular beacon probe was synthesized by TaKaRa Biotechnology Co. (Dalian, China). DNA oligonucleotides were synthesized by Invitrogen Co. (Shanghai, China), and miRNAs were synthesized by GenePharma Co. (Shanghai, China) (Table 1).

Table 1 Sequences of the molecular beacon, template, and miRNAs

Name	Sequences (5′–3′)
MB probe	FAM CCG ACC CGC GAT GCC aaa aCA TAC CTA GGT CGG DABCYL[a]
MB complementary probe	TAG GTA TGT TTT GGC ATC GCG
Template	CGC GAT GCC AAA ACA TAC CTA AAC A**GA CTC** *CCT ACG ACT G*AA CTA TAC AAC CTA CTA CCT CA[b]
let-7a	UGA GGU AGU AGG UUG UAU AGU U
let-7b	UGA GGU AGU AGG UUG UGU GGU U
let-7c	UGA GGU AGU AGG UUG UAU GGU U

[a] The underlined regions in the MB probe identify the stem sequence, and the lowercase region identifies the RNA. [b] The underlined regions identify the nicking enzyme post-cut site, the bold region identifies the reverse complement of the nicking enzyme recognition site, and an additional italicized region at the end of the trigger-binding site.

Methods

Modified cycling probe technology. The MB probe (5 pmol) and 6 U of RNase H (TaKaRa Biotechnology Co. Ltd, Dalian, China) were added to 10 μl of SDA buffer containing 0.5 μl of Nt.BstNBI buffer, 1 μl of ThermoPol buffer and an MB complementary probe. The reaction mixture was heated to 55 °C for 90 min in a real-time PCR analyzer (MJ Research, Inc., USA).

Strand displacement amplification. The reaction mixtures for the SDA were prepared separately in two parts (part A and part B) on ice. Part A consisted of 0.5 μl Nt.BstNBI buffer, the amplification template, 0.25 μl dNTPs (10 mM each), and the 0.25 μl RNase inhibitor (40 U μl^{-1}); part B consisted of 1 μl ThermoPol buffer, 3 U nicking endonuclease Nt.BstNBI, 0.5 U Bst DNA polymerase, and DEPC-treated water. Parts A and B were mixed and the target was immediately added before being placed in the PCR system. The strand displacement amplification was performed in a final volume of 10 μl at 55 °C for 30 min in a PCR analyzer (Eastwin Life Sciences, Inc., China). In order to deactivate the enzyme, the reaction was incubated at 95 °C for 20 min.

Coupling the template with beads. The biotin-labelled template and streptavidin-coated beads (2 μl) (Dynabeads M-280 Streptavidin, Invitrogen Dynal AS, Norway) were washed thrice with water, before adding the 0.5 μl biotin labelled template (0.1 pmol μl^{-1}), 5 μl binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 M NaCl) and 4.5 μl water. The binding process was performed in a rotational mixer at 25 °C for 30 min.

Capturing miRNAs with the templates coated on magnetic beads. The beads coupled with the template and 10 μl annealing buffer (10×, 40 mM Tris-HCl, 20 mM $MgCl_2$, 50 mM NaCl, pH 7.5) were added to 100 μl target solution. The mixture was incubated at 30 °C for 90 min in a rotational mixer. The supernatant was discarded, and the beads were washed twice using distilled water. After the enrichment, the SDA reaction was performed, and denatured at 70 °C for 1 min. The supernatant was removed for the next CPT reaction.

Results and discussion

Principle of SDA-CPT assay

The principle of the assay included one sample enrichment step and two amplification cycles (Fig. 1). Firstly, we designed a ssDNA strand (termed "template") with three different segments containing sequences: (i) complementary to miRNA, (ii) to be recognized by a nicking nuclease, and (iii) reverse complementary to the MB used in CPT. The third sequence part is used to bridge the two amplification cycles. We modified the 3′ end of the template, and then combined it with magnetic beads. The template modified with beads captured the sample through the hybridization reaction. Beads were collected using a magnet to enrich the sample. In the SDA, miRNA is extended with the template by the Bst DNA polymerase. The products from the extension reaction would be subsequently cleaved by the nicking enzyme, and trigger an extension reaction by the polymerase to displace a strand complementary to the MB.

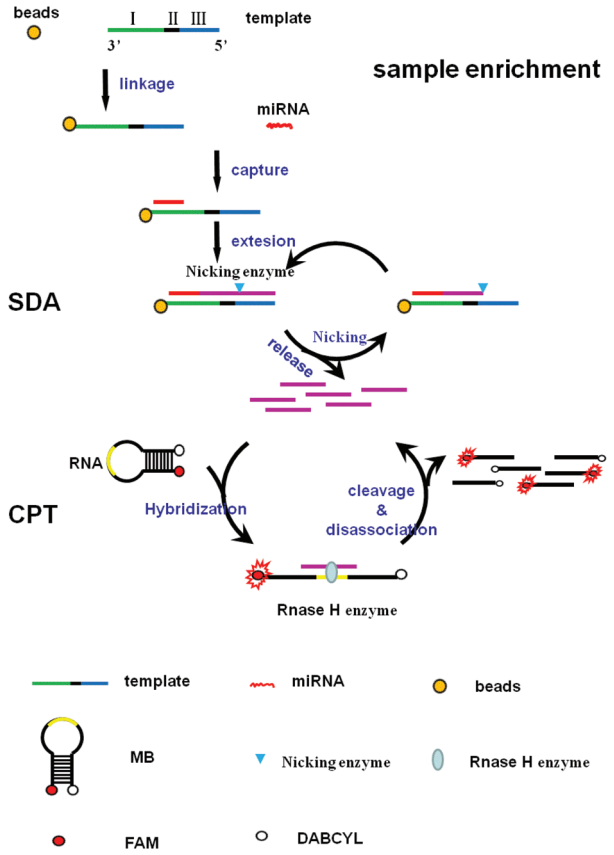

Fig. 1 The principle of signal amplification of miRNAs. This assay involves three steps: (1) the template modified with beads captures the sample; (2) strand displacement amplification generates abundant universal triggers in the presence of a nicking enzyme and polymerase; and (3) the amplified DNA strand hybridized with the MB initiates a new CPT cycle.

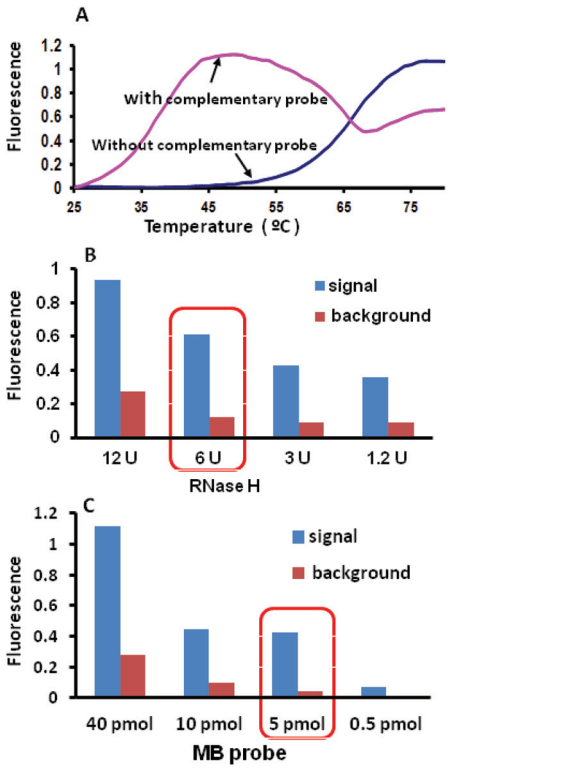

Fig. 2 Optimization of modified cycling probe technology. (A) The melting curve of the MB probe (from 25 °C to 80 °C). (B) CPT reaction with different amounts of RNase H (1.2, 3, 6, and 12 U). (C) CPT reaction with different amounts of the MB probe (40, 10, 5, and 0.5 pmol).

The cycle of the extension and nicking reactions generates a large amount of strands (purple lines in Fig. 1) complementary to MB, forming the first signal amplification reaction of miRNAs. To further amplify the strands, the MB was designed to contain a short RNA sequence (yellow curve) in the loop region. Once the amplified DNA strand hybridized with the MB, the RNA strand in the DNA–RNA duplex would be digested by RNase H yielding a signal. Since the DNA strand would be captured again by the intact MB, the second signal amplification cycle is achieved due to the iterative digestion of MB by RNase H. The sequence of the target (the strand of the purple lines in Fig. 1) for RNase H-mediated MB cleavage is independent of miRNAs, and universal to any miRNA of interest. The assay is simple to set-up as it is only dependent on the design of the targeted miRNA template.

Optimization of modified cycling probe technology

In conventional CPT, the probe is of linear strand.[9] In this assay, the probe is designed as a type of molecular beacon thus reducing the proximity between the fluorophore and quencher and thus the background signal becomes smaller when there is no complementary sequence.[12,13] The T_m of the stem is designed at 55 °C because the first SDA always occurs at this temperature. The melting curve of the MB was detected and the complementary probe was added in addition for comparison. The incubation was performed from 25 °C to 80 °C, increasing by 1 °C every 10 seconds (Fig. 2A). The background signal of MB is the smallest at 45 °C and the difference in the signal at 55 °C between the molecular beacon only and the molecular beacon with the complementary probe is obvious, so the design of the molecular beacon was successful.

In the CPT reaction, increasing the amount of RNase H and the molecular beacon would improve the detection signal, but the background would also increase, so it was necessary to investigate the amount of RNase H and MB. At constant MB (5 pmol) and MB complementary probe (1 fmol), the various amounts of the enzyme (12, 6, 3, and 1.2 U) were tested. As shown in Fig. 2B, when the 12 U of RNase H was used, the signal was the strongest, however the background was also high and so we used 6 U RNase H in the following reactions.

To optimize the amount of MB needed in the assay, constant amounts of RNase H (6 U) and the MB complementary probe (1 fmol) were tested against various amounts of MB (40, 10, 5, and 0.5 pmol). The results showed (Fig. 2C), 10 or 5 pmol of MB; the signal was relatively high and the background

was very low, therefore 5 pmol of the molecular beacon was chosen as the optimal amount in the final step.

Optimization of the strand displacement amplification

Firstly, we investigated the appropriate usage of the template in the SDA reaction, because the template and the MB would compete to combine with the amplified products. In the CPT reaction were added 1 pmol, 0.1 pmol, 0.05 pmol and 0.02 pmol of the template, respectively. The MB complementary probe was 0.01 pmol. As shown in Fig. 3A, the amount of the template inhibited the CPT reaction. When the amount of the template was 1 pmol, the signal was relatively weak. However, inhibition to the CPT reaction is minimal with the 0.02 pmol template.

In the most conventional SDA reaction, Vent DNA polymerase is used.[14] Therefore, we performed the first step (linear amplification reaction) according to the literature conditions, in which Vent DNA polymerase was 0.5 U. In parallel, we performed the CPT reaction with a molecular beacon complementary probe, as a positive control experiment. As shown in Fig. 3B, amplification with Vent DNA polymerase did not generate any significant signal. However, the positive control showed normal results, demonstrating that the MB cycle amplification experiment system was not compromised. It was reasoned that the "no signal" could be due to the inhibition of the first step linear amplification by the CPT reaction. We postulated that the Vent enzyme was difficult to inactivate and hence affected the following experiment. MB, combined with the amplified product in the first cycle, could be cut by RNase H but the still active Vent DNA polymerase might make the molecular beacon continue to extend. Therefore, the amplified products in the first step firmly complemented, and could not combine with another MB for the iterative cycles. Therefore, the CPT reaction was suppressed and the reaction signal was greatly reduced.

Therefore, we proceeded to use Bst DNA polymerase instead of Vent DNA polymerase as the former is easily deactivated. In order to investigate the influence of Bst DNA polymerase on the CPT reaction, we added Bst DNA polymerase to the CPT reaction, and compared by adding Bst DNA polymerase after high-temperature inactivation. As shown in Fig. 3C, the addition of Bst DNA polymerase showed a significant decrease in the CPT system, the inactive Bst DNA polymerase generated a signal consistent with the positive control. The result indicated that Bst DNA polymerase would affect the CPT reaction. This principle may be the same as that for Vent DNA polymerase. However, after heat inactivation, the influence of DNA polymerase can basically be eliminated.

In the SDA reaction, if the amount of Bst DNA polymerase was increased, the detection signal would improve. However, the background would also increase, so it was necessary to optimize the amount of Bst polymerase. The two-step signal amplification reaction was performed using three different amounts of Bst polymerase (0.23, 0.4, and 0.8 U). As shown in Fig. 3D, 0.4 or 0.8 U of Bst polymerase generated a relatively strong signal. However, the background was also significantly high. Only 0.23 U of Bst polymerase generated a sufficient signal with an acceptable level of the background. Therefore 0.23 U was utilised for subsequent experimentations.

In a previous experiment, the reaction time of the first-cycle amplification was 30 min. Two other reaction times (10 and 20 min) were tested to identify the optimal amplification time. As shown in Fig. 3E, the reaction time of 30 min generated a significant difference between the 10 pM RNA sample and the blank. However, upon reduction of the reaction time to 20 min and 10 min, the positive signal could not be distinguished from the blanks. Therefore, 30 min was regarded as the suitable reaction time.

According to the literature, the optimum temperature of the SDA reaction is 55 °C, therefore we tested the effect of temperature of the SDA reaction.[15] Three temperatures for the reaction were investigated (50 °C, 53 °C and 57 °C). As shown in Fig. 3F, reducing the temperature did not improve amplification efficiency. At 50 °C, there was almost no amplification. Increasing the reaction temperature from 55 °C to 57 °C was also tested. The result shown in Fig. 3F indicated that the amplification efficiency was lower at 57 °C indicating that the reaction efficiency was the highest when the temperature was 55 °C.

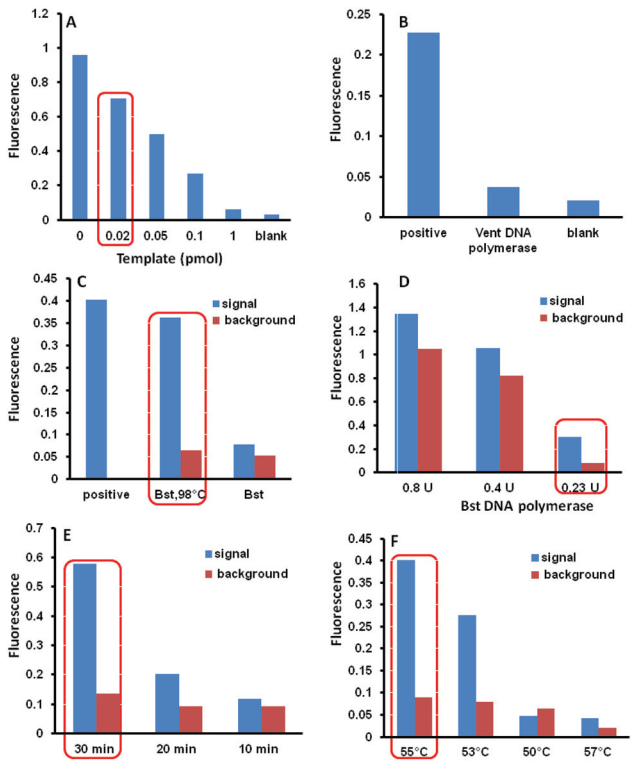

Fig. 3 Optimization of the SDA reaction. (A) SDA reaction with different amounts of the template (1, 0.1, 0.05, and 0.02 pmol). (B) Effect of Vent DNA polymerase on the SDA reaction. (C) Effect of Bst DNA polymerase on the SDA reaction. (D) SDA reaction with different amounts of Bst DNA polymerase (0.23, 0.4, and 0.8 U). (E) The effect of reaction time (30, 20, and 10 min). (F) Changing the reaction temperature of the SDA reaction (from 55 °C to 50, 53 and 57 °C).

Sample enrichment

In order to further improve the sensitivity of the method, we designed a sample enrichment step before the two-cycle amplification. The difference between the solid and liquid phase amplification reactions was the 3′ end modification of the template and a denaturation at 70 °C after the first-stage reaction respectively. In order to investigate whether the addition of beads would affect the amplification reaction, we compared the solid phase amplification with the liquid phase. As shown in Fig. 4, the solid phase amplification efficiency did not decrease, but slightly increased. The reason could probably due to the removal of the template by the beads. For the reduced amount of the template, it was weak to compete with the MB to combine with the amplified products in the second reaction.

Previous experiments have demonstrated that the solid phase amplification with a modified template has the same amplification efficiency as the liquid amplification. To further investigate the efficiency of the sample enrichment experiment, we amplified let-7a miRNA with solid phase amplification, including the enrichment step and compared directly with liquid amplification. We put the 1 fmol target into the hybridization system, and combined it with the template. Then we carried out the solid phase amplification with the captured products. In comparison, we also amplified the 1 fmol target with the liquid amplification at the same time. As shown in Fig. 5, the amplification efficiency of the two processes was almost coincident, indicating the higher capture efficiency. When the sample enrichment step was used, the sensitivity increased by nearly 100 times, from 100 pM to 10 fM.

Detection of let-7a miRNA

Using let-7a as a template, we investigated the sensitivity of the signal amplification reaction using the optimized experimental conditions. As shown in Fig. 6A, 10 amol let-7a miRNA could be detected with strand displacement amplification-based modified cycling probe technology.

The rate of the sensitivity curve from 5 to 15 min was calculated using the miRNA amounts of 10, 30, 100, 300 amol and 1 fmol in 100 μl (that is, the miRNA concentrations of 0.1, 0.3, 1, 3 and 10 pM) to be 0.0104, 0.0612, 0.1004, 0.1921 and 0.2532 respectively (Fig. 6A). A standard curve was constructed by plotting the rate of the sensitivity curve *versus* the log of the concentrations of let-7a miRNA. As shown in Fig. 6B, the standard curve displays a linear response (R = 0.9906). Therefore, the linear relationship was ample in the range of the tested amounts, and can be used for quantitative work.

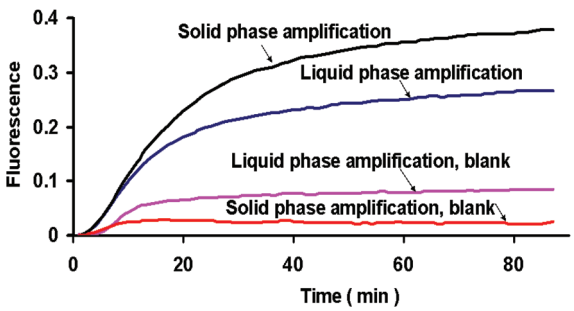

Fig. 4 Comparison of the solid phase amplification and liquid phase amplification.

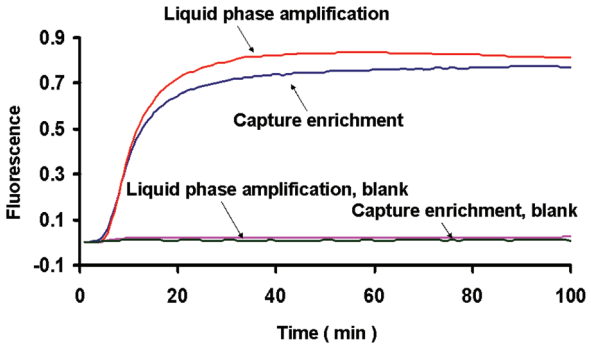

Fig. 5 Comparison of the amplification after the enrichment and the amplification directly.

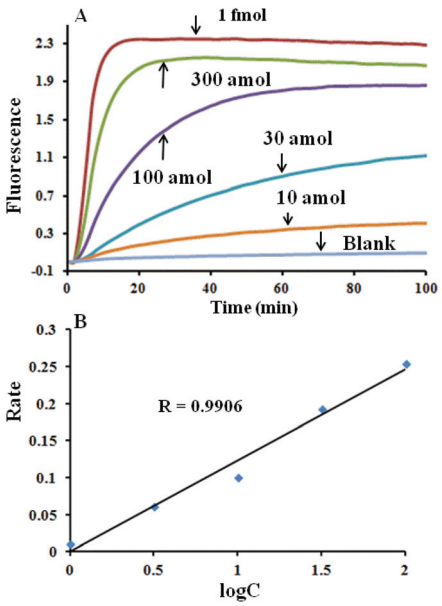

Fig. 6 Detection sensitivity of SDA coupling of the CPT reaction. (A) Real-time fluorescence curves in response to different amounts of let-7a (10, 30, 100, 300 amol and 1 fmol in 100 μl). (B) The value of the rate of the sensitivity curve is a log-linear correlation with the let-7a concentration in the range from 0.1 to 10 pM.

Specificity

To evaluate the specificity of the proposed miRNAs assay, we selected let-7a–c as a model system due to their high sequence homology. We amplified the selected miRNAs using identical conditions as the let-7a sample. As shown in Fig. 7, let-7a

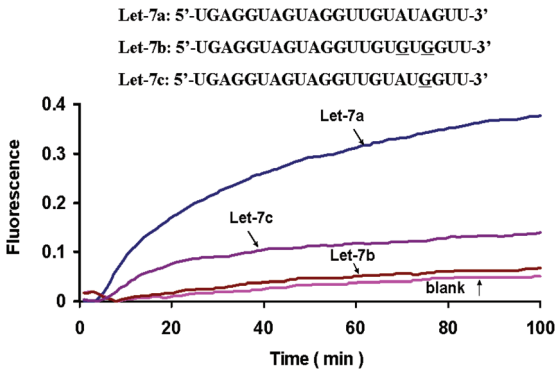

Fig. 7 Detection specificity of SDA coupling of the CPT reaction. Typical real-time fluorescence curves in response to let-7a, let-7b, let-7c, and the blank. Inset: sequences of let-7a, let-7b, and let-7c. Bases underlined represent differences in the sequence from let-7a.

could be clearly distinguished from 7b and 7c. The assay was able to efficiently discriminate let-7b (two base difference) from 7a and 7c (one mismatched base).[16]

Conclusions

This paper offers a new method of sensitive signal amplification for miRNAs on the basis of modified cycling probe technology with strand displacement amplification. After one sample enrichment and two steps of signal amplification, 0.1 pM of let-7a can be detected. The miRNA assay exhibits a great dynamic range of over 100 orders of magnitude and high specificity to clearly discriminate a single base difference in miRNA sequences. In conventional RT-PCR, detection with a SG dye has poor specificity. The TaqMan probe method has high cost due to the need to design a corresponding probe for each miRNA. However, in this study, the molecular beacon probe is universal, so the cost of experiments is low. Meanwhile, the molecular beacon probe in the CPT reaction reduces the background and improves the sensitivity compared with the linear probe in conventional CPT. The assay designed is also about signal amplification rather than template amplification, therefore minimising contamination issues. In addition, the assay is an isothermal amplification, needs no reverse transcription process, and hence it does not require any specialised temperature control instrument. Thus this amplification is very suitable for the detection of clinical diseases relevant to miRNA expression.

Acknowledgements

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Notes and references

1 H. Jing, Q. Song, Z. Chen, B. Zou, C. Chen, M. Zhu, G. Zhou, T. Kajiyama and H. Kambara, *ChemBioChem*, 2011, **12**, 845.
2 S. Daniels, F. Sillé, A. Goldbaum, B. Yee, E. Key, L. Zhang, M. Smith and R. Thomas, *Cancer Epidemiol., Biomarkers Prev.*, 2014, **23**, 2658.
3 J. Koshiol, E. Wang, Y. Zhao, F. Marincola and M. Li, *Cancer Epidemiol., Biomarkers Prev.*, 2010, **19**, 907.
4 W. Zhou, Y. Chen and R. Corn, *Anal. Chem.*, 2011, **83**, 3897.
5 O. Morozova and M. Marra, *Genomics*, 2008, **92**, 255.
6 E. Dempsey, F. Dervin and P. B. Maguire, *Blood*, 2014, **124**, 2760.
7 E. Miotto, E. Saccenti, L. Lupini, E. Callegari, M. Negrini and M. Ferracin, *Cancer Epidemiol. Biomarkers Prev.*, 2014, **23**, 2638.
8 P. Nelson, W. Wang, B. Wilfred and G. Tang, *Biochim. Biophys. Acta*, 2008, **1779**, 758.
9 F. Bekkaoui, I. Poisson, W. Crosby, L. Cloney and P. Duck, *BioTechniques*, 1996, **20**, 240.
10 X. Chen, J. H. Ren, H. Guo, L. H. Lin and Q. X. Yao, *Nanfang Yike Daxue Xuebao*, 2008, **28**, 1210.
11 L. T. Dickinson, D. C. Mah, R. T. Poirier, F. Bekkaoui, W. E. Lee and D. E. Bader, *Mol. Cell. Probes*, 2004, **18**, 341.
12 Y. Wang, J. Li, J. Jin, H. Wang, H. Tang and R. Yang, *Anal. Chem.*, 2009, **81**, 9703.
13 H. Kashida, T. Takatsu, T. Fujii, K. Sekiguchi, X. Liang and K. Niwa, *Angew. Chem., Int. Ed.*, 2009, **48**, 7044.
14 H. Jia, Z. Li, C. Liu and Y. Cheng, *Angew. Chem., Int. Ed.*, 2010, **49**, 5498.
15 T. Eric, E. Barbara, D. Shale, F. Tanya, B. Megan, I. Bruce, V. Karl and N. Angelika, *Biochemistry*, 2008, **47**, 9987.
16 S. Roush and F. J. Slack, *Trends Cell Biol.*, 2008, **18**, 505.