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COMMUNICATION

One-step ultrasensitive detection of microRNAs with loop-mediated isothermal amplification (LAMP)†

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An ultrasensitive microRNA assay was developed with one-step loop-mediated isothermal amplification (LAMP) initiated by the target microRNA.

MicroRNAs (miRNAs), a class of endogenous, nonprotein-coding small RNAs, play important roles in gene expression regulation by the formation of an RNA-induced silencing complex (RISC) with target mRNAs, which have critical functions in a wide range of physiologic and pathologic processes.[1] Recent works have also revealed that miRNA expression can informatively reflect the developmental lineage and differentiation state of the tumours.[2] miRNAs are attractive candidates of biomarkers for early cancer diagnosis.[3] Therefore, detection of miRNAs has great significance not only for the studies of miRNAs' biological functions but also for clinical diagnosis.

Currently, Northern blotting and microarray methods are widely used for miRNA analysis.[4] However, the sensitivity and selectivity of these methods cannot satisfy the needs of miRNA detection, due to the short length, similar nucleotide sequences, and low expression levels of miRNAs.[5] Various novel methods for miRNA detection have been proposed in recent years, such as nanoparticle-labeling methods,[6] modified Invader assay,[7] ribozyme-based methods,[8] conjugated-polymer-based methods,[9] bioluminescence detection[10] and electrochemical detection.[11] These reported methods have greatly improved the sensitivity of miRNA detection. Nevertheless, most of these methods need probe modification, expensive labels, and/or sophisticated procedures. Recently, a number of enzyme-based exponential amplification strategies have also been reported, including quantitative RT-PCR,[12] branched rolling circle amplification,[13] and ligase chain reaction (LCR).[14] More recently, we also reported an ultrasensitive method for miRNA detection with exponential isothermal amplification.[15] These methods not only achieve high sensitivity but also improve the selectivity for discrimination of similar miRNA sequences based on enzymatic specificity. However, all these methods require multiple enzyme-catalytic reactions, resulting in expensive cost and complex steps.

Loop-mediated isothermal amplification (LAMP), based on auto-cycling strand displacement DNA synthesis,[16] is an outstanding DNA amplification procedure, in which the reaction can be processed with one type of DNA polymerase under isothermal conditions.[17] Owing to its simplicity and operativity, LAMP has widely been used for detection of specific DNA sequence,[18] single nucleotide polymorphisms,[19] viruses and other pathogenic microorganisms.[20] In this communication, by using miRNAs to initiate the LAMP reaction, we report a simple, rapid, specific and cost-effective miRNA assay.

The mechanism and LAMP reaction steps for miRNA detection are illustrated in Fig. 1. The LAMP system, which consists of a template DNA, forward inner primer (FIP), backward inner primer (BIP) and outer primer B3, is designed according to the literature[16] with some modifications. The template DNA successively contains the sequences of B3, B2, B1, F1c, F2c and M. The sequence of M is perfectly complementary to the target miRNA. FIP contains F1c, a TTTT spacer and the sequence F2 complementary to F2c. In the initial step, FIP hybridizes to F2c in the template DNA and extends in the presence of *Bst* DNA polymerase and dNTPs. After that, miRNA, which is a few bases shorter and lower in concentration than FIP, hybridizes to M in the template DNA and initiates the strand displacement DNA synthesis (SDS) based on the catalytic activity of RNA extension along a DNA

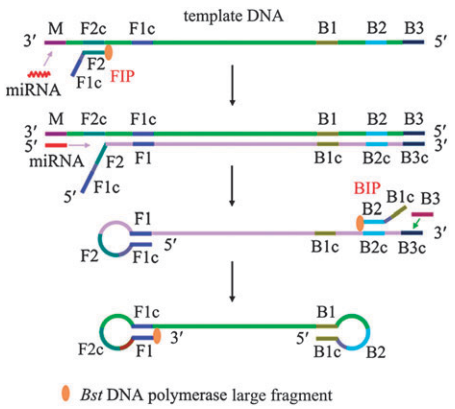

Fig. 1 Schematic representation of the LAMP reaction initiated by the target miRNA (let-7a). All the sequences of the DNA template, FIP primer, BIP primer, B3 primer and let-7a miRNA are listed in ESI.†

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template[21] and the strand displacement activity[22] of *Bst* DNA polymerase, releasing a FIP-linked single strand DNA (ssDNA), which can form a stem-loop structure at the 5′-terminus through the hybridization between F1 and F1c. BIP contains the sequence B1c, a TTTT spacer and B2 complementary to B2c. At the 3′-terminus of the ssDNA, BIP hybridizes to B2c to perform the primer extension and then the outer primer B3 hybridizes to B3c in the 3′-terminus of the ssDNA to perform the SDS, releasing a BIP-linked ssDNA which can form double stem-loop structures at 3′- and 5′-terminus, respectively. As demonstrated in the previous reports,[16,17] the double stem-loop DNA quickly converts a stem-loop DNA by self-primed DNA synthesis at its 3′-terminus. Then, FIP hybridizes to the F2c sequence in the loop and primes SDS, generating two stem-loop DNAs containing the B2c sequence in the loops. BIP then hybridizes to the B2c sequences in the loops and primes SDS again to form more stem-loop structures in which the loops contain the sequences of F2c and B2c, respectively. Thus, more FIP and BIP, respectively, hybridize to F2c and B2c in the loops to form more and more stem-loop structures through SDS. The auto-cycling SDS can be repeated continuously and leads to exponential amplification of DNA, which can amplify the DNA to a similar magnitude to that of PCR (see ref. 16 for details). In this communication, oligreen, picogreen and SYBR Green (SG) are tested for real-time measurement of the LAMP reaction and SYBR Green is selected as the fluorescent dye for real-time miRNA detection (see ESI†, Fig. S5). POI (point of inflection), which is defined as the time corresponding to the maximum slope in the real-time fluorescence curve, is adopted for quantitative detection of miRNA target.

The optimization of experimental conditions for the LAMP reaction, including the reaction temperature and the concentration of various components, was performed. The temperature of 55 °C, 0.6 μM FIP and BIP primer, 50 pM B3 primer and 4U *Bst* DNA polymerase were found to be optimum (see ESI†, Fig. S1–S4). Under the optimized experimental conditions, the relationship between the POI value and the amount of miRNA target was investigated by using let-7a miRNA as a model. As depicted in Fig. 2, the POI values are linearly dependent on the logarithm (lg) of target miRNA amounts in the range of 1.0 amol to 1.0 pmol. The correlation equation is POI = $-33.25 - 6.89 \lg A_{\mathrm{miRNA}}$ (A: amounts of target miRNA, mol) and the corresponding correlation coefficient R is 0.9968. Therefore, as low as 1.0 amol miRNA can be quantitatively detected with the LAMP-based method, and the dynamic range spans over 6 orders of magnitude. From Fig. 2a, one can also see that the blank signal is the main factor to limit the sensitivity of miRNA detection. In the absence of *Bst* DNA polymerase, the fluorescence signal of the control reaction (NTC) is maintained at a very low level with increasing reaction time. Therefore, the blank signal should be attributed to the non-specific amplification arising from the DNA polymerase.

To evaluate the specificity of the LAMP-based miRNA assay, the let-7 miRNA family members (let-7a–e) are selected as a model system because their sequences are highly similar and their expression levels are closely associated with cell development and human cancers.[23] When the sequence M in

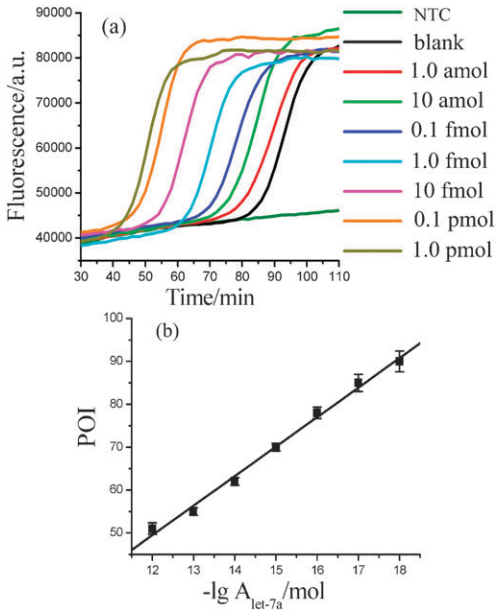

Fig. 2 (a) The real-time fluorescence curves of LAMP reactions triggered by let-7a miRNA with different amounts. The blank was treated in the same way as the miRNA detection but without let-7a miRNA. NTC (no-target control) was a control reaction in which the LAMP reaction was performed in the absence of target miRNA and *Bst* DNA polymerase. (b) The relationship between POI value and lg amount of let-7a miRNA. The results were average values with three repetitive measurements. The LAMP reactions were performed in the volume of 10 μL. The final concentration: [DNA template] = 50 pM, [FIP] = [BIP] = 0.6 μM, [B3] = 50 pM, [*Bst* DNA polymerase large fragment] = 0.4 U μL^{-1}, [SYBR Green] = 0.4 ng μL^{-1}, and [each dNTP] = 0.2 mM.

the template DNA used in the LAMP reaction is perfectly complementary to let-7a miRNA, as shown in Fig. 3, the real-time fluorescence signal produced by let-7a can be completely separated from those produced by other let-7 miRNAs, indicating that the proposed miRNA assay can clearly discriminate one-base difference among the miRNAs. At the POI of the let-7a signal along the time axis, the signals of other let-7 miRNAs except let-7e have not reached their exponential period yet. Let-7a, let-7c and let-7e only differ by one base. Compared to let-7a, the different base in let-7c is near to its 3′-terminus. Therefore, let-7c can be efficiently discriminated from let-7a by the extension reaction. However, the different base in let-7e is near to the middle position. So let-7e is relatively difficult to be discriminated from let-7a. Even in this case, according to the correction equation mentioned above, the interferences for detection of let-7a arising from the signals produced by let-7e, let-7d, let-7c and let-7b were estimated to be 6.9%, 0.9%, 0.06% and 0.06%, respectively (see the estimation in ESI†). Thus, the proposed miRNA assay can be characterized with high selectivity.

The proposed miRNA assay has been applied to determine the amount of let-7a in human lung total RNA samples. With the simultaneously constructed calibration curve, the amount of let-7a in the 10 ng total RNA samples was estimated to be 50 amol by five repetitive measurements. The standard deviation (SD) and coefficient of variation (RSD) were 4.3 amol

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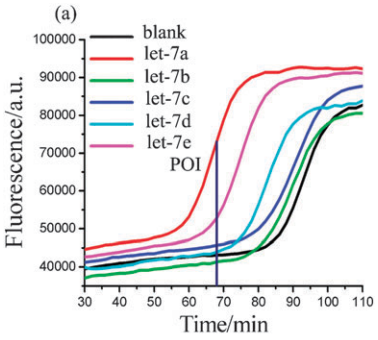

Fig. 3 (a) The real-time fluorescence curves of LAMP reactions triggered by 1.0 fmol let-7a–e, respectively. (b) The sequences of let-7a–e. The different bases compared to let-7a in other miRNAs are marked in red. The experimental conditions are the same as those in Fig. 2.

and 8.6%, respectively. To further confirm the result, 200 amol synthetic let-7a was added into 10 ng total RNA samples, in which the average amount of let-7a was determined as 252.9 amol by five repetitive measurements (SD = 32.4 amol, RSD = 12.8%). The average recovery was thus estimated as 105.9%. Therefore, the proposed miRNA assay should be used for quantitative detection of miRNAs in total RNA samples down to amol level.

In summary, we have demonstrated that the LAMP reaction can be applied to ultrasensitive detection of miRNAs. By using target miRNA to initiate the LAMP reaction, as low as 1.0 amol miRNA can be accurately determined. The determination can be accomplished with one-step operation under isothermal conditions within 90 min. Moreover, this proposed miRNA assay does not require any modified or labeled DNA probes and only one type of DNA polymerase is needed, which should significantly reduce the cost and simplify the experimental procedure. Due to the high specificity of the LAMP reaction,[16] the proposed miRNA assay exhibits high selectivity to clearly discriminate one-base difference among miRNA sequences. A conceivable disadvantage of the proposed miRNA assay is the requirement of a template DNA and FIP, BIP, B3 primers for the LAMP reaction. However, a LAMP reaction only needs 0.5 fmol template DNA, 6.0 pmol FIP and BIP primers, and 0.5 fmol B3 primer. Therefore, the proposed miRNA assay is still cost-effective. On the other hand, just because the FIP, BIP, B3 primers and the target miRNA need to recognize six distinct sequences in the template DNA for the LAMP reaction, the LAMP-based assay can be performed with high selectivity. In this communication, the LAMP-based assay was only applied to miRNA detection in the total RNA sample. If the assay is to be applied to real biological samples, such as extraction of cells and tissues, the miRNA detection will encounter the possible interference from proteins and DNA *etc*., which needs to be investigated in the future.

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