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Rolling circle extension-actuated loop-mediated isothermal amplification (RCA-LAMP) for ultrasensitive detection of microRNAs

Weimin Tian[a,b], Pengjie Li[a,b], Wenli He[a,b], Chenghui Liu[a,b,*], Zhengping Li[a,b,*]

[a] Key Laboratory of Applied Surface and Colloid Chemistry, Ministry of Education, School of Chemistry and Chemical Engineering, Shaanxi Normal University, Xi'an 710062, Shaanxi Province, PR China

[b] Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Province, School of Chemistry and Chemical Engineering, Shaanxi Normal University, Xi'an 710062, Shaanxi Province, PR China

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ABSTRACT

Rolling circle amplification (RCA) is an elegant and well-recognized isothermal nucleic acid amplification mechanism that has been widely used for the detection of various kinds of genetic biomarkers. However, traditional RCA is a linear signal amplifying mechanism so that the amplification efficiency is generally not satisfactory. Herein, we rationally combine RCA with efficient loop-mediated isothermal amplification (LAMP) to establish a rapid and ultrasensitive RCA-LAMP method for the detection of microRNAs (miRNAs). In the RCA-LAMP, the target let-7a miRNA can directly template the ligation of a padlock probe to trigger RCA reaction, producing long and tandem amplification products. Only such RCA-produced long DNA repeats can act as the template to generate a lot of double stem-loop DNAs with functional sequences, which are the essential starting materials to initiate subsequent LAMP. Finally, the products of LAMP reaction, the amount of which is dependent on the initial miRNA dosage, can be fluorescently monitored in a real-time manner. Through the combination of ligation-mediated RCA with LAMP, the amplification efficiency and the detection sensitivity has been significantly improved. As a result, even 10 aM miRNA target can be clearly and accurately detectable. Despite the excellent analytical performance for miRNA analysis, compared with conventional RCA-based miRNA assays, the combination of RCA with LAMP does not introduce any additional reaction steps or sample transfer operations. Both the RCA and LAMP are fulfilled in a single step. Therefore, this facile and ultrasensitive RCA-LAMP assay provides a new promising tool for miRNA analysis and can be extended to the detection of various kinds of genetic biomarkers.

1. Introduction

MicroRNAs (miRNAs) are a group of non-coding small RNAs that can be widely find in animals, plants and viruses (Git et al., 2010; Lee et al., 1993), which play vital roles in the gene expression processes (Ambros, 2004). More and more studies have shown that the aberrant expression levels of miRNAs are closely associated with the occurrence of many human diseases including cancers (Dong et al., 2013; Sita-Lumsden et al., 2013; Tavazoie et al., 2008). As a result, miRNAs are considered as important markers for both cancer diagnosis and treatment (Cao et al., 2011; Peng and Gao, 2011; Ventura and Jacks, 2009; Wang et al., 2013). So, it is imperative to establish highly sensitive and specific miRNA assays to better understand their biological functions or for biomedical applications. However, sensitive and accurate miRNA quantification is still challenging due to their unique characteristics such as small size, sequence similarity and low abundance in biological samples.

In recent years, some elegant nucleic acid amplification-aided assays, particularly isothermal amplification-based protocols, are extensively applied to detecting miRNAs because they can greatly improve the detection sensitivity under a constant temperature without requirement of precise thermal cycles (Cheng et al., 2009; Deng et al., 2017; Duan et al., 2013; Jonstrup et al., 2006; Li et al., 2016; Liu et al., 2017, 2016; Ma et al., 2017; Yu et al., 2018; Yue et al., 2017). Among the various isothermal signal amplification protocols, rolling circle amplification (RCA) is no doubt the most attractive and prominent for both *in vitro* and *in vivo* miRNA analysis owing to its advantages of simplicity and versatility. Jonstrup et al. first applied the padlock probe-based RCA method to miRNA analysis (Jonstrup et al., 2006). Nevertheless, the labour-intensive and time-consuming electrophoretic

* Corresponding authors at: Key Laboratory of Applied Surface and Colloid Chemistry, Ministry of Education, School of Chemistry and Chemical Engineering, Shaanxi Normal University, Xi'an 710062, Shaanxi Province, PR China.

E-mail addresses: liuch@snnu.edu.cn (C. Liu), lzpbd@snnu.edu.cn (Z. Li).

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separation and radioactive tracing are needed to detect the RCA products. Our group introduced a second primer to fabricate a branched-RCA reaction for the detection of miRNA (Cheng et al., 2009), where the products can be directly and easily detected by fluorescence dye SYBR Green I (SG). However, the amplification efficiency of the branched-RCA is still not high enough and it needs as long as 6 h to accomplish the whole RCA reaction. Up to now, although several kinds of modified RCA protocols have been proposed for the detection of miRNAs, nevertheless, due to the intrinsic linear signal amplifying mechanism of RCA, the sensitivity of RCA-based assay are generally not satisfactory (typically in the range of fM~pM) to detect low levels of miRNA (Hong et al., 2016; Liu et al., 2013; Wang et al., 2015; Zhang et al., 2014; Zhou et al., 2010), which hampers their wider applications.

Loop-mediated isothermal nucleic acid amplification (LAMP) is another attractive isothermal strategy with high amplification efficiency, which requires only one kind of DNA polymerase (Notomi et al., 2000; Tomita et al., 2008). Our group has pioneered the target-initiated LAMP for miRNA analysis (Li et al., 2011). It is worth noting that double stem-loop DNA structures with elegantly designed sequences, are the essential starting materials for LAMP. In conventional LAMP reaction, an extremely long single-strand DNA (ssDNA) template including at least six pre-defined sequences, and four primers are required to form such starting DNA structures, which render the probe sequence design extremely stringent and sophisticated. Besides, one target can only produce one molecule of double stem-loop DNA, resulting in the low formation efficiency of the LAMP starting structures.

Herein, we wish to propose a facile and ultrasensitive method for the detection of let-7a miRNA by elegantly integrating the distinct advantages of RCA and LAMP (denoted as RCA-LAMP strategy). In the RCA-LAMP assay, the miRNA directly templates the ligation of a rationally designed padlock probe to initiate the RCA reaction, producing long ssDNA with multiple tandem repeats. A rationally designed stem-loop primer can bind with such tandem repeats one by one to initiate cascading extension and displacement reactions along the RCA products to generate double stem-loop DNA structures. As such, aided by efficient RCA, each target can generate numerous double stem-loop structures with different stem lengths, which can independently initiate subsequent LAMP. So, the RCA-LAMP greatly improves the detection sensitivity than RCA methods or LAMP assays. By using the proposed RCA-LAMP, even 10 aM of miRNA target is unequivocally detectable. Notably, compared with conventional RCA-based miRNA assay, the RCA-LAMP method does not introduce any extra experimental operations. Both the RCA reaction and LAMP reaction are proceeded simultaneously in a single step by using only one kind of polymerase without any sample transfer steps. Therefore, the proposed RCA-LAMP provides a robust tool towards the facile and ultrasensitive miRNA analysis in biological and biomedical studies.

2. Experimental

2.1. Materials and apparatus

T4 RNA ligase 2 and its reaction buffer, Bst DNA polymerase large fragment and the corresponding ThermoPol buffer, were all purchased from New England Biolabs (USA). Betaine and salmon sperm DNA were supplied by Sigma (USA). Ribonuclease inhibitor, dNTPs, and the RNase-free water were all obtained from Takara (Dalian, China). All nucleic acids used in this work including miRNAs, padlock probe, forward inner primer (FIP), stem-loop primer (SLP), and backward inner primer (BIP) were also synthesized and purified by Takara (Dalian, China). The sequences of all nucleic acids were listed in Table S1. The real-time fluorescence measurements of the RCA-LAMP reactions were proceeded on a StepOne (Applied Biosystems) real-time PCR instrument.

2.2. Standard procedures of the RCA-LAMP miRNA assay

In the RCA-LAMP, the pre-ligation of the padlock probe is first conducted. Typically, in 10 μL T4 RNA ligase 2 buffer, 2 nM padlock probe, 1 U T4 RNA ligase 2, 4 U Ribonuclease inhibitor and appropriate amounts of the target miRNA or total RNA sample were mixed and incubated at 39 °C for 30 min to accomplish the ligation reaction.

Then, 2 μL of the ligation products were pipetted into an RCA-LAMP reaction mixture containing 100 nM SLP, 0.8 μM FIP/BIP, 1 M betaine, 2 mM dNTPs, 4 U Bst DNA polymerase large fragment, and 0.4 ng/μL SYBR Green I in 1x ThermoPol reaction buffer with a final 10 μL volume. Then the 10 μL solution was put into StepOne real-time PCR instrument to conduct the RCA-LAMP under a constant temperature of 65 °C. The fluorescence intensity of the RCA-LAMP reaction system was real-time monitored at intervals of 30 s.

2.3. Gel Electrophoresis

To check the RCA-LAMP products, the non-denaturing polyacrylamide gel electrophoresis (16% PAGE) was performed in 1x TBE buffer, which was run at 110 V constant voltage for 80 min. The gel was stained by 2x SYBR Gold and then visualized on a Gel Doc EZ Imager (Bio-Rad).

2.4. Preparation of total RNA cell extracts

HCT-116, a human colon cancer cell line, was obtained from the cell bank of Chinese Academy of Sciences. The HCT-116 cells were cultured in DMEM Medium (GBICO) which contains 1% $NaHCO_3$, 100 U/mL penicillin, 10% (v/v) fetal calf serum, and 100 μg/mL streptomycin at 37 °C with 5% CO_2. The HCT-116 cells were collected in the exponential phase of growth, and the total RNA was extracted with Trizol Reagent (Invitrogen). The extracted total RNA was quantified with a Nanodrop 2000 Spectrophotometer (Thermo Scientific).

3. Results and discussion

3.1. Design principle of the RCA-LAMP strategy for miRNA analysis

The new RCA-LAMP strategy for miRNA detection is schematically illustrated in Fig. 1, where let-7a is chosen as the model target. The padlock probe contains several critical segments including target hybridization regions at its 5′ and 3′ terminals and three functional regions (SHSc, F2c and F1c). In the presence of let-7a miRNA, both the 5′-end and 3′-end sequences of the padlock probe will respectively hybridize with the target miRNA and thus become adjacent enough to be ligated by T4 RNA ligase 2, forming a circular ssDNA. In order to improve the ligation efficiency, the 3′-terminal of the padlock probe is modified with two ribonucleotides (Zhang et al., 2013, 2011).

Subsequently, the RCA-LAMP reaction is conducted in a single step by mixing the circular ssDNA, the forward inner primer (FIP), the backward inner primer (BIP), the stem-loop primer (SLP) and only one kind of DNA polymerase. First, the FIP with F1c (c means complementary) and F2 sequences, can specifically bind with the F2c of the circular ssDNA to trigger RCA reaction. The RCA product containing long tandem sequences can form a stem-loop structure (F1/F1c and F2) at its 5′-end. At the same time, the SLP consisting of another stem-loop structure (B1/B1c and B2) and a 3′-end ssDNA, can hybridize with the SHS (SLP hybridization site) regions in the RCA product to initiate reverse extension reaction along the RCA product. As the tandem RCA product contains multiple SLP binding sites, the first elongated SLP can be displaced by the extension of the second SLP. The displaced extension products of the first SLP can form double stem-loop structures at its 3′ and 5′ terminals respectively due to the hybridization between F1/F1c and B1/B1c. Meanwhile, the extended product of the second SLP will be also displaced by the third SLP and so on, releasing many SLP

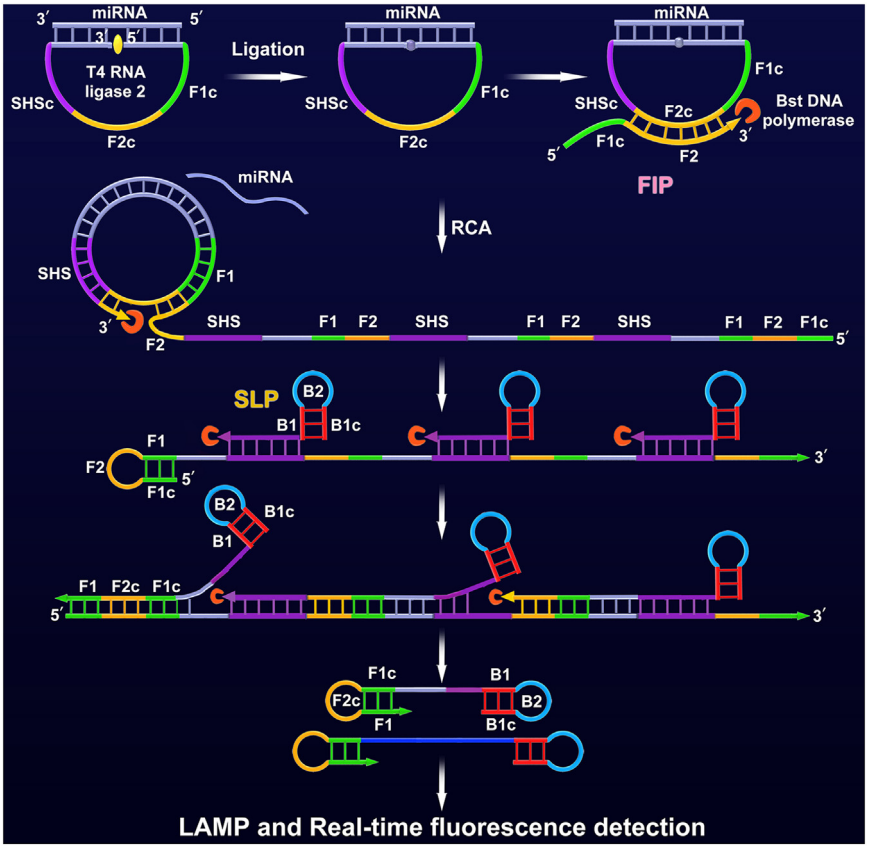

Fig. 1. Schematic illustration of the RCA-LAMP method for miRNA detection.

extension products. As such, many double stem-loop DNA structures with different stem lengths are produced. It should be noted that only such double stem-loop DNAs can act as the starting materials to initiate LAMP for auto-cycling DNA synthesis with the assistance of FIP/BIP, which can produce a lot of stem-loop DNAs with varying stem lengths (Du et al., 2016; Li et al., 2011; Sun et al., 2017; Wang et al., 2016). The detailed principles of LAMP have been well introduced in previous reports (Du et al., 2016; Li et al., 2011; Notomi et al., 2000; Sun et al., 2017; Tomita et al., 2008; Wang et al., 2016), which is also schematically illustrated in the Supplementary information (Fig. S1). The amount of LAMP products, which can quantitatively reflect the original miRNA level, can be real-time detected with the dsDNA-staining dye SYBR Green I. In contrast, if target miRNA is absent, the padlock probe will not be circulated and thus RCA reaction will not occur. As a result, no double stem-loop DNAs can be produced to initiate subsequent LAMP.

The RCA-LAMP approach exhibits several unique merits. On the one hand, different from traditional LAMP-based miRNA assay (Li et al., 2011), the miRNA target-generated tandem RCA products are elegantly employed as the template to produce the double stem-loop DNA structures, the essential initiating materials of LAMP. So, the challenging chemical synthesis of quite long ssDNA template (typically ~200 nucleotides) in conventional LAMP is no longer required. More importantly, each miRNA can produce not one, but a lot of double stem-loop DNA structures, greatly amplifying the efficiency of subsequent LAMP. On the other hand, compared with traditional RCA-based assays, by using exponential LAMP as the signal transducing mechanism, the detection sensitivity of RCA is significantly improved while no additional experimental procedure is introduced. Except for the indispensable padlock probe pre-ligation reaction, both the RCA and LAMP can be simultaneously accomplished in one step without extra sample transfer operations. Therefore, the combination of RCA with LAMP has provided a facile but highly efficient strategy for miRNA detection.

3.2. Analytical performance of the RCA-LAMP assay for the detection of let-7a

With the systematically optimized reaction parameters (Fig. S2–S5), we have evaluated the analytical performance of the RCA-LAMP assay by detecting let-7a miRNA with different concentrations. Fig. 2a displays the real-time fluorescence curves aroused by varying concentrations of let-7a in the range of 10 aM to 10 pM From Fig. 2a, one can find that the fluorescence signal produced by as low as 10 aM (0.1 zmol) let-7a target can be obviously discriminated from that of the blank control. The point of inflection (POI) value, which is the time corresponding to the maximum slope of the fluorescence curve, is recorded and used to quantitatively determine the let-7a level. As depicted in Fig. 2b, the POI values exhibit an excellent linear relationship with the logarithm (lg) of the let-7a concentrations ranging from 10 aM to 10 pM The correlation equation is POI = – 45.13 –6.91 lg $C_{\text{let-7a}}$/M with a correlation coefficient (R^2) of 0.9943.

As mentioned above, our group has previously developed a branched RCA assay for the fluorescent detection of let-7a miRNA, where the detection limit of let-7a is ~10 fM (Cheng et al., 2009). It is obvious that the sensitivity of the RCA-LAMP strategy has been increased more than 10^3-fold compared with the branched-RCA assay. This significant improvement is undoubtedly ascribed to the use of efficient LAMP as the signal transducing manner, which successfully converts the fluorescence detection of RCA products to an exponential mode by simply introducing a SLP primer. Furthermore, Li et al. have first applied the traditional LAMP design to the detection of let-7a miRNA (Li et al., 2011). Because of the low formation efficiency of the target-generated double stem-loop DNA structure, such LAMP-based miRNA assay can only detect the lowest 1 amol (100 fM) let-7a miRNA. In this study, the

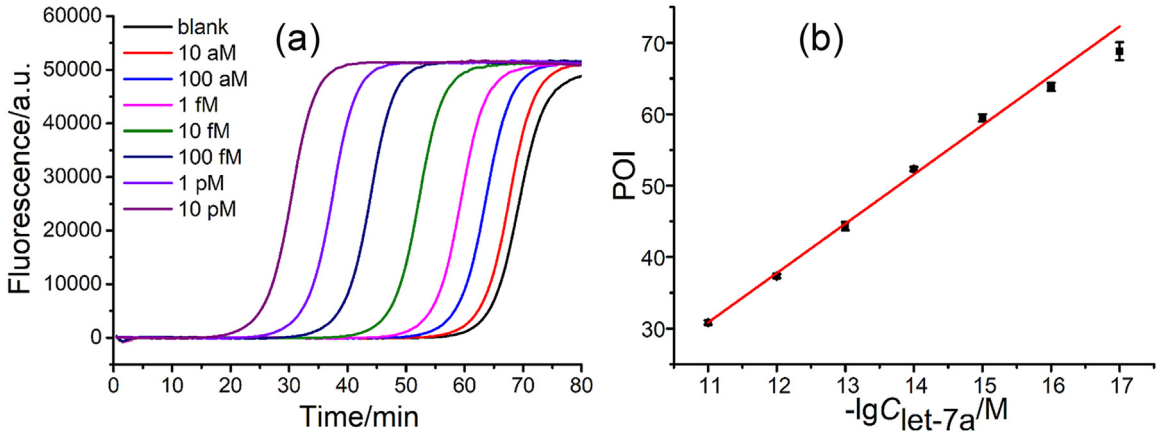

Fig. 2. (a) Real-time fluorescence curves of the RCA-LAMP system in the presence of different concentrations of let-7a miRNA. From right to left, the concentrations of let-7a is 0 (blank), 10 aM, 100 aM, 1 fM, 10 fM, 100 fM, 1 pM, and 10 pM, respectively. (b) The linear relationship between the corresponding POI values and logarithm (lg) of the let-7a concentrations.

RCA-LAMP design greatly increases the formation efficiency of the double stem-loop DNAs and thus significantly accelerates the amplification efficiency of LAMP. As a result, the detection limit of let-7a by our RCA-LAMP method is also about 10^4-fold lower than that by using traditional LAMP design. Moreover, Table S2 has summarized the previously reported miRNA assays based on either modified RCA or LAMP strategies, one can see that the sensitivity of the proposed RCA-LAMP is superior or at least parallel to these listed methods.

The occurrence of let-7a-actuated efficient RCA-LAMP is further confirmed by the non-denaturing polyacrylamide gel electrophoresis (PAGE). As shown in Fig. 3, in the presence of 10 pM and 10 fM let-7a, the RCA-LAMP reaction take place efficiently accompanied with multiple ladder-like amplification products (lane 2–3), which may be ascribed to the stem-loop structure DNAs with difference stem lengths (the characteristic products of LAMP). If let-7a is absent, no such ladder-like bands can be observed (lane 5), indicating that no RCA-LAMP can be triggered without the target miRNA. Meanwhile, as can be seen from Fig. 2a, with a reaction time of 60 min of the RCA-LAMP, 10 aM let-7a cannot arouse obvious fluorescence response. So, in the PAGE results, 10 aM let-7a cannot produce observable amplification bands (lane 4). Furthermore, if only 10 fM let-7a is introduced while the T4 RNA ligase 2 is absent, no ladder-like bands can be observed (Fig. S6), which further confirms that the proposed RCA-LAMP assay is indeed dependent on the target-templated specific ligation of the padlock probe.

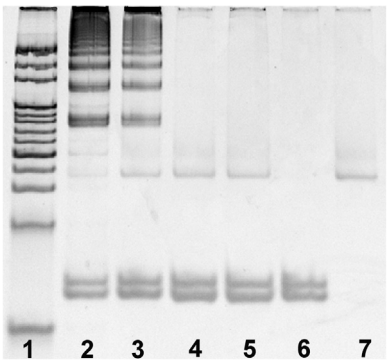

Fig. 3. PAGE analysis of the products in the RCA-LAMP reaction. Lane 1: DNA markers (20 bp DNA ladder); Lane 2–5: amplification products produced by 10 pM, 10 fM, 10 aM and 0 fM (blank) let-7a miRNA, respectively. Other experimental conditions in Lane 2–5: padlock probe, 2 nM; SLP, 100 nM; FIP and BIP, 0.8 μM; RCA-LAMP reaction time, 60 min. Lane 6: FIP + BIP (0.8 μM); Lane 7: SLP (100 nM).

3.3. Specificity of the RCA-LAMP for the detection of let-7a

In order to test the specificity of the RCA-LAMP approach for let-7a analysis, some non-complementary miRNAs such as miR31, miR143, miR155, miR205, miR92a and ath-miR156a are randomly selected and detected by using let-7a-specific padlock probe. As shown in Fig. 4a, all of these miRNA sequences exhibit negligible interference for the detection of let-7a. Furthermore, let-7 miRNA family members, including 7a to 7 g and 7i, are highly similar in their sequences, which differ by only 1–4 nucleotides. A practical miRNA assay should be specific enough to be capable of discriminating such quite similar sequences. To further interrogate the specificity of the RCA-LAMP strategy, let-7 family members with the same concentration of 10 fM are respectively detected by the proposed method by using let-7a-specific padlock probe. According to the principle of RCA-LAMP, the specificity is highly dependent on the ligation reaction. It is worth noting that let-7c, let-7e and let-7f have only one base difference from let-7a. As displayed in Fig. 4b, let-7f produces little nonspecific signal (2.14%) because the mismatched base in let-7f to let-7a is at the 3′-end of the padlock probe (the exact ligation site). For let-7c and let-7e, since their mismatched bases are not at the ligation site, they produce larger interference signals, which are both ~18. 84%. Comparing with let-7a, let-7b and 7d both have two mismatched bases, and the mismatched bases are far from the ligation site. Therefore, they produce 13.52% and 11.43% interference signals, respectively. In addition, the interferences of let-7g and let-7i are both negligible (0.35% and 0.29%, respectively) for let-7a detection. Moreover, we have also investigated the specificity of the RCA-LAMP assay for the detection of let-7 family members in a complex background of 2 ng salmon sperm DNA (Fig. S8), and the obtained results are similar to those obtained in pristine buffer solution. These results demonstrate that our proposed RCA-LAMP assay has satisfactory specificity which allows the discrimination of one-base difference between homologous miRNA family members even in complex biological media.

From the aforementioned results, one can see that although the interferences of the non-complementary miRNA sequences are all negligible, the highly similar homologous sequences with only 1–2 bases difference to let-7a may lead to some nonspecific ligation of the padlock probe, which finally arouse some false positive interference signals for the detection of let-7a. Actually, it is still a big challenge for most of the hybridization-based miRNA assays to completely eliminate the false positive responses aroused by quite similar sequences. We now envision that by using a set of peptide nucleic acid (PNA) probes to effectively clamp other homologous miRNA sequences except for the target sequence (Das et al., 2015, 2016), the false-positive interference

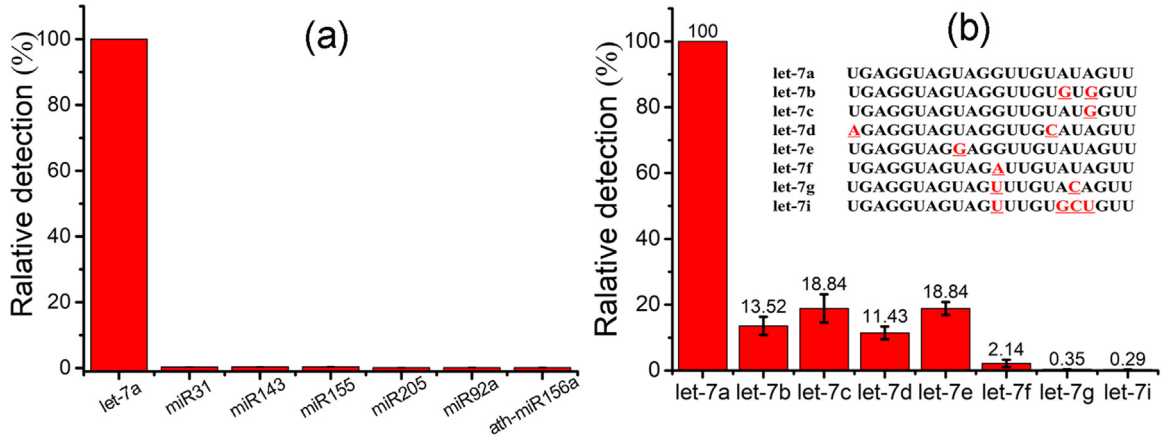

Fig. 4. (a) Specificity evaluation of the RCA-LAMP strategy treated with different non-complementary miRNA targets by using the let-7a-specific padlock probe. (b) Specificity evaluation of the RCA-LAMP assay for the detection of let-7 family members by using let-7a-specific padlock probe.

Table 1
Determination of let-7a in total RNA extracted from HCT-116 cells by the RCA-LAMP.

Sample	Amount of let-7a (zmol)	Average value (zmol)	Recovery (%)
500 pg total RNA (3 parallel determination)	91.0 (sample 1) 99.5 (sample 2) 108.2 (sample 3)	99.5	–
500 pg total RNA + 400 zmol let-7a (3 parallel determination)	479.0 (spiked sample 1) 483.5 (spiked sample 2) 482.0 (spiked sample 3)	481.5	95.5

of the homologous miRNAs may be greatly suppressed.

3.4. Determination of let-7a in total RNA extracted from cancer cells

To further test the feasibility of the RCA-LAMP assay for miRNA quantification in real complex samples, it is applied to evaluating let-7a level in the total RNA extract of HCT-116 cells, and the results are shown in Table 1. The amount of the let-7a miRNA in 500 pg total RNA extract is calculated to be 99.5 zmol. To further verify the accuracy of this method, we prepared spiked samples containing 500 pg total RNA and 400 zmol synthetic let-7a miRNA. The determined let-7a miRNA in the spiked sample is 481.5 zmol with a recovery of 95.5%. Furthermore, we also compared our result with that obtained by using a well-recognized stem-loop RT-PCR commercial Kit (Applied Biosystems, TaqMan® MicroRNA Assays Kit). By using the commercial Kit, the let-7a level in 500 pg total RNA is determined to be 102.6 zmol. This result is well consistent with the value obtained from the RCA-LAMP method. All of the above results clearly suggest that RCA-LAMP is a practical and reliable method which can accurately detect let-7a miRNA in complex biological sample.

4. Conclusion

In summary, we have established a versatile and ultrasensitive miRNA assay which integrates the distinct advantages of both RCA and LAMP. Compared with traditional linear RCA or LAMP-based miRNA assays, the amplification efficiency of the RCA-LAMP is greatly improved and thus it exhibits much higher detection sensitivity with a detection limit lowered down to 10 aM. Moreover, compared with traditional RCA, both the RCA and LAMP reactions can be fulfilled in a single step without the involvement of any extra operation steps. What's more, we believe that besides miRNA, this versatile RCA-LAMP design can be expanded to the detection of various kinds of genetic biomarkers, which is of great potential in the fields of biological research and biomedical studies.

CRediT authorship contribution statement

Weimin Tian: Methodology, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Pengjie Li:** Investigation, Data curation. **Wenli He:** Investigation, Data curation. **Chenghui Liu:** Conceptualization, Supervision, Funding acquisition, Resources, Writing - review & editing. **Zhengping Li:** Conceptualization, Methodology, Supervision, Resources, Funding acquisition.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.bios.2018.12.041.

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