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Light Scattering Technology-Combined Ligation-Dependent Loop-Mediated Isothermal Amplification (LL-LAMP) for Sensitive Detection of RNA

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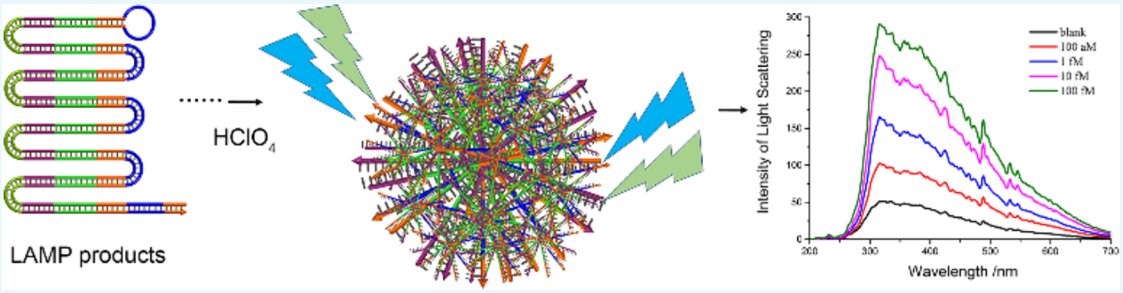

ABSTRACT: Loop-mediated isothermal amplification (LAMP) has been widely used in nucleic acid assay because of its high specificity, sensitivity, and isothermal property. However, the complexity of amplification product detection is still a major challenge for its wide applications. Herein, we developed a light scattering technology-assisted, low-cost, and simple detection manner of LAMP products without expensive reagents and complicated instruments. Only needing to add a kind of strong acid to the amplification products, the amplification products can aggregate into large particles in a strongly acidic medium, and large particles can produce strong light scattering, which shows a good proportional relationship with the number of amplification products in a wide range. The proposed method shows excellent sensitivity and high specificity that can quantify RNA as low as 100 aM with a single-base resolution.

INTRODUCTION

RNAs including microRNAs (miRNAs) and messenger RNAs (mRNAs) have always been very important research topics due to their vital roles in the fundamental process of life.[1,2] As RNA is considered a hallmark for identifying pathogens,[3] and its expression profile is relevant to the pathogenesis of the disease,[4] RNA analysis has become an important content of current biological research. Thus, a simple and sensitive RNA detection method is particularly important because of its great value in disease pathophysiology[5] and medical diagnostics.[6]

Up to now, many ways for RNA analysis have been established from simple to complex and multistep procedures. Among these ways, reverse transcription-polymerase chain reaction (RT-PCR) is the most popular amplification technology with a wide dynamic range.[7] However, several main limitations of RT-PCR are its thermal cycle process, requirement of reverse transcription, and false-positive signal.[8] Furthermore, since miRNA is quite short, it is difficult to be amplified by the direct use of PCR, which makes the PCR construction very complex.[9] As promising alternatives to PCR, isothermal amplification methods of miRNA,[10] such as digital miRNA detection,[11] loop-mediated isothermal amplification (LAMP),[12−14] rolling circle amplification (RCA),[15,16] exponential amplification reaction (EXPAR),[17,18] nucleic acid sequence-based amplification (NASBA),[19] duplex-specific nuclease signal amplification (DSNSA),[20] catalyzed hairpin assembly (CHA),[21] and hybridization chain reaction (HCR),[22] are effective amplification methods under isothermal conditions. Gines et al. reported an isothermal digital detection method by designing DNA circuits that inhibits nonspecific amplification common in nucleic acid amplification methods and provides absolute quantification of miRNAs.[11] CHA and HCR are enzyme-free methods that rely on competitive hybridization, which can be used to probe endogenous miRNAs in living cells.[22] LAMP, RCA, NASBA, EXPAR, and DSNSA are well-known enzyme-based amplification strategies that can be used for quantitative analysis of miRNAs through their exponential or linear amplification mechanism. Among the numerous enzyme-based isothermal methods, LAMP possesses impressive sensitivity. Generally, LAMP can amplify

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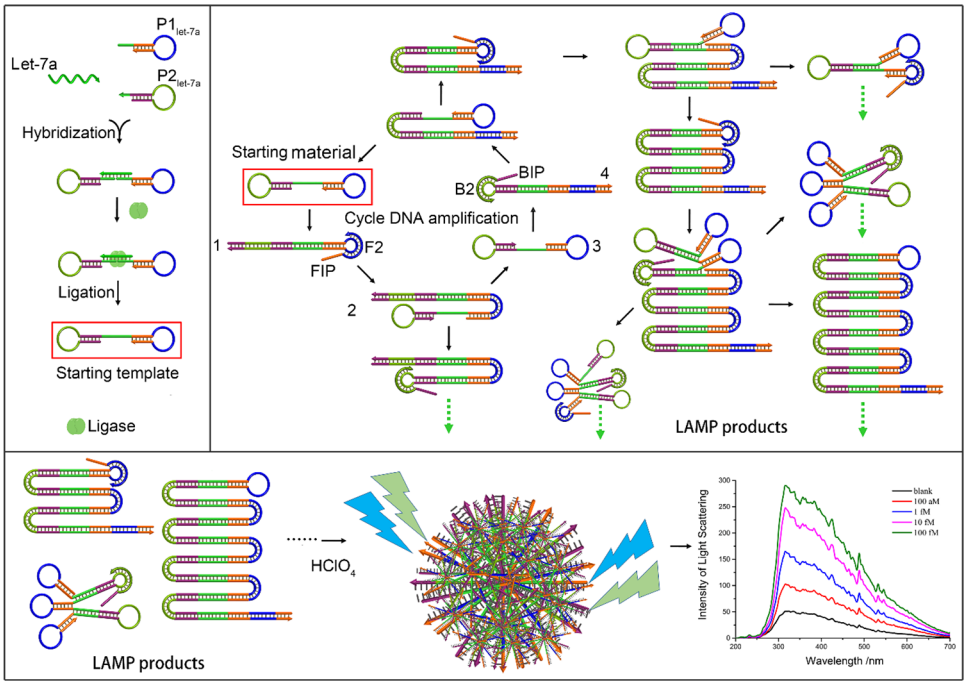

Figure 1. Principle of light scattering technology-combined ligation-dependent loop-mediated isothermal amplification for miRNA assay.

a few copies of nucleic acids to detectable capability at a constant temperature. Therefore, LAMP technology has been transformed into a popular method for the quantitative assay of biomarkers[23,24] and pathogen-caused infectious diseases, such as COVID-19.[25−27] Classic LAMP uses four primers to target six areas on the target sequence (more than 130 bp), which results in the requirement of long target sequences and carefully designed primers.[28] Du et al. used ligation-dependent LAMP to amplify miRNA target sequences, in which one pair of stem-loop probes can be specifically linked together to generate double stem-loop structure DNA to start up LAMP.[12] The strategy of initiating LAMP based on double stem-loop DNA greatly simplifies the design of the primer for LAMP.[29] Although LAMP is quite robust, the detection manner of amplification products is still a challenge.

LAMP reaction is a highly efficient nucleic acid amplification reaction, which can produce a mass of stem-loop structure DNAs with a variety of stem lengths.[28] So far, many ways have been used for the analysis of amplification products including gel electrophoresis,[30,31] naked eye monitoring,[32,33] real-time fluorescence,[34] electrochemical methods,[35] and so on. Magnesium pyrophosphate can be produced as a by-product during nucleic acid amplification by DNA polymerase. Based on this characteristic, Notomi and co-workers put calcein in the reaction solution of LAMP[33] and the amplification reaction can be detected by the naked eye through the color change of calcein-manganese (orange) turning into calcein-magnesium (green fluorescence signal) as the reaction proceeds. But this assay found that calcein would reduce the sensitivity of LAMP compared with reaction results without adding additional reagents. This is mainly due to the finding that calcein combined with magnesium can reduce the activity of the enzyme[36] and the interaction between calcein and double-stranded DNA leads to reduced sensitivity.[37] For example, by adding SYBR Green I in an amplification reaction,[34] a kind of embedded fluorescent dye, the fluorescence signal can be collected and the process of LAMP reaction can be detected in real time. However, this method requires a precise real-time fluorescence detection system. Recently, a CRISPR/Cas12a-assisted LAMP product detection method was reported.[38] This method can improve the specificity of amplification product detection, but it needs to use a fluorescence probe and CRISPR/Cas12a protease to generate fluorescence signals, which greatly increases the detection cost. Therefore, simple and low-cost detection manners are desirable for popularizing the application of LAMP.

In this work, we establish research on light scattering technology-combined ligation-dependent LAMP (LL-LAMP) for RNA detection to develop a convenient and low-cost detection method for the analysis of nucleic acid amplification products without sophisticated instruments. We just need to add perchloric acid into the amplification products, and the DNA double-helix structure of products denatures into single-stranded nucleic acids since hydrogen bonding between the bases is destroyed in a strongly acidic medium. Then, single-stranded nucleic acids aggregate into large particles with a hydrated radius of 200−700 nm.[39] The large particles produce a very strong signal of light scattering, which shows a good proportional relationship with the number of amplification products in a wide range.

EXPERIMENTAL SECTION

Materials and Reagents. SYBR Green I (20 ng/μL) was supported by Fanbo Biochemicals Co., Ltd. (Beijing, China). T4 RNA ligase 2 and Bst DNA polymerase were obtained from New England Biolabs (NEB, China). Deoxy-ribonucleotide triphosphate dNTPs, Recombinant RNase Inhibitor (RRI), and RNase-free water were obtained from Takara Biotechnology Co., Ltd. (Dalian, China). MCF-7 cells were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). TRIzol reagent was purchased from Invitrogen (USA). Stock solutions for RNA analysis were respectively prepared in RNase-free water. All oligonucleotides

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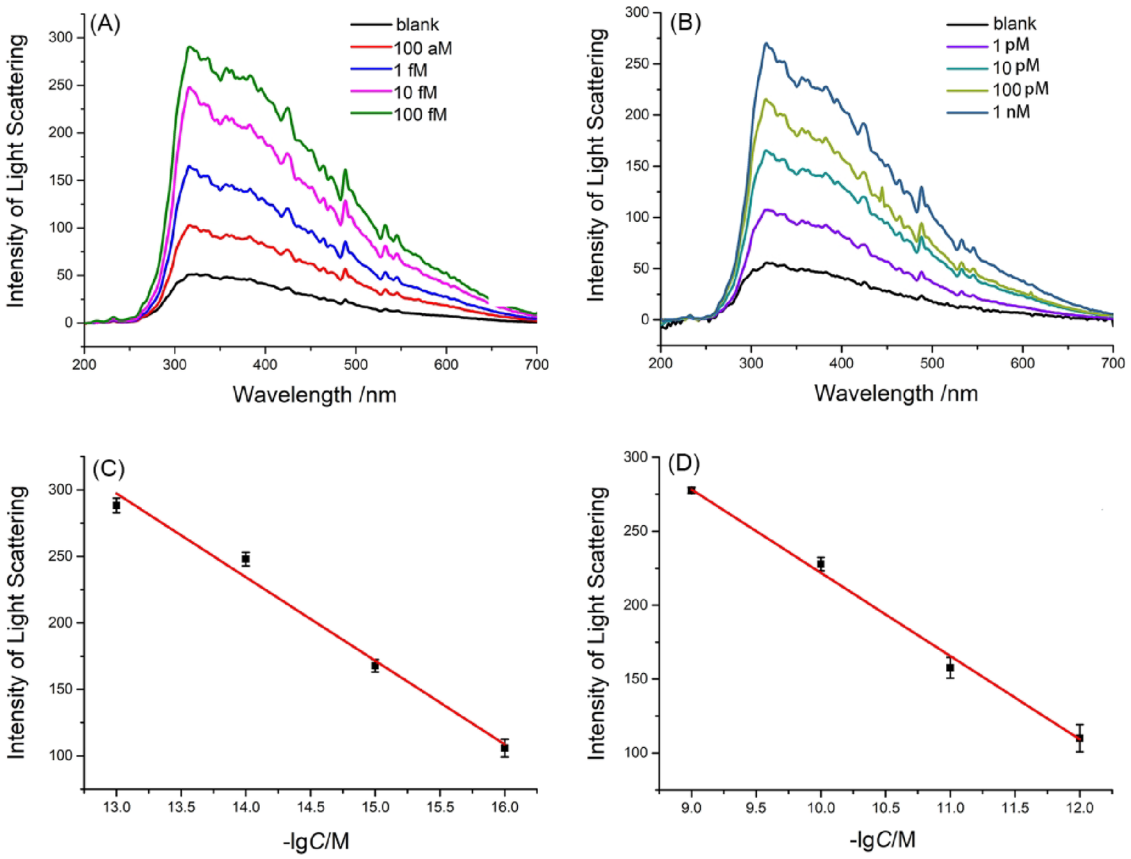

Figure 2. (A, B) Light scattering spectrum of let-7a with different concentrations by the LL-LAMP protocol. (A) From bottom to top, the concentrations are 0 (blank, without let-7a in the LL-LAMP system), 100 aM, 1 fM, 10 fM, and 100 fM, respectively. The LL-LAMP reaction time is 35 min. (B) From bottom to top, the concentrations are 0, 1 pM, 10 pM, 100 pM, and 1 nM, respectively. The LL-LAMP reaction time is 15 min. (C, D) Linear relationship between the light scattering intensity (at 320 nm) and the logarithm of concentrations. Error bars are the standard deviation of three replicates in panels (C) and (D).

used in this research were obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China), and they are listed in Table S2.

Extraction of Total RNA. MCF-7 cells were kept in 7 mL of DMEM medium (Gibco) containing 100 μg/mL streptomycin, 3 mmol/L L-glutamine, 10% (v/v) fetal calf serum, and 100 U/mL penicillin. The experimental process of extracting total RNA from MCF-7 cells was based on the protocol of the TRIzol reagent. The concentration of total RNA was detected using a NanoDrop One (Thermo Scientific, USA) and then stocked at −80 °C.

Experimental Process of LL-LAMP-Based RNA Assay. For the ligation reaction, 1 μL of total RNA sample or 1 μL of target RNA (microRNA or mRNA) was added to 5.0 μL of the reaction solution including 2.3 μL of RNase-free water, 1 μL of 200 nM $P1_{let\text{-}7a}$ (or $P1_{mRNA}$), 1 μL of 200 nM $P2_{let\text{-}7a}$ (or $P2_{mRNA}$), 0.1 μL of 40 U/μL RRI, and 0.6 μL of 10× T4 RNA ligase 2 buffer (10 mM DTT, 500 mM Tris−HCl, 4 mM ATP, and 20 mM $MgCl_2$). The reaction solution was put at 65 °C for 2 min and then at 39 °C for 5 min to accomplish the hybridization reaction of target RNA and probes. Then, 3.5 μL of RNase-free water, 0.4 μL of 10× ligase 2 buffer, and 0.1 μL of ligase were put in the reaction solution, and the solution was heated at 37 °C for 30 min for ligation reaction.

For the amplification reaction, a volume of 2 μL reaction product mentioned above was put into 8 μL of LAMP mixture including 4.3 μL of RNase-free water, 1.0 μL of 10× ThermalPol buffer (20 mM $MgSO_4$, 200 mM Tris−HCl, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, and 1% Triton X-100), 0.5 μL of 8 U/μL Bst polymerase, 0.2 μL of SYBR Green I, 0.5 μL of 10 μmol/L FIP, 1.0 μL of 2.5 mmol/L dNTPs, and 0.5 μL of 10 μmol/L BIP with a final system of 10 μL. The reaction mixture was immediately put into a 2720 PCR system (Applied Biosystems, USA) to carry out the LAMP process at 65 °C. LAMP amplification times were 35 min (the concentration of RNA ranges from 100 aM to 100 fM) and 15 min (the concentration of miRNA ranges from 1 pM to 1 nM).

Ten microliters of $HClO_4$ (1 M) and 80 μL of water were added to the above 10 μL of amplified products. One hundred microliters of the mixture was mixed and added to the cuvette. An LS55 fluorescence spectrophotometer was used to scan the scattering spectrum.

RESULTS AND DISCUSSION

The Working Principle of LL-LAMP for RNA Assay. The method described here applies to any known RNA. Figure 1 gives an illustrated example for the microRNA (let-7a) assay. According to the sequence of let-7a (arrow represents the 3′-terminus), we design two stem-loop probes including $P1_{let\text{-}7a}$ and $P2_{let\text{-}7a}$. $P1_{let\text{-}7a}$ is composed of the complementary region with a half sequence of let-7a and the stem-loop structure region from the 5′-terminus direction to the 3′-terminus direction. $P2_{let\text{-}7a}$ is composed of the stem-loop structure region and complementary region with a half sequence of let-7a from the 5′-terminus direction to the 3′-terminus direction. $P2_{let\text{-}7a}$ is

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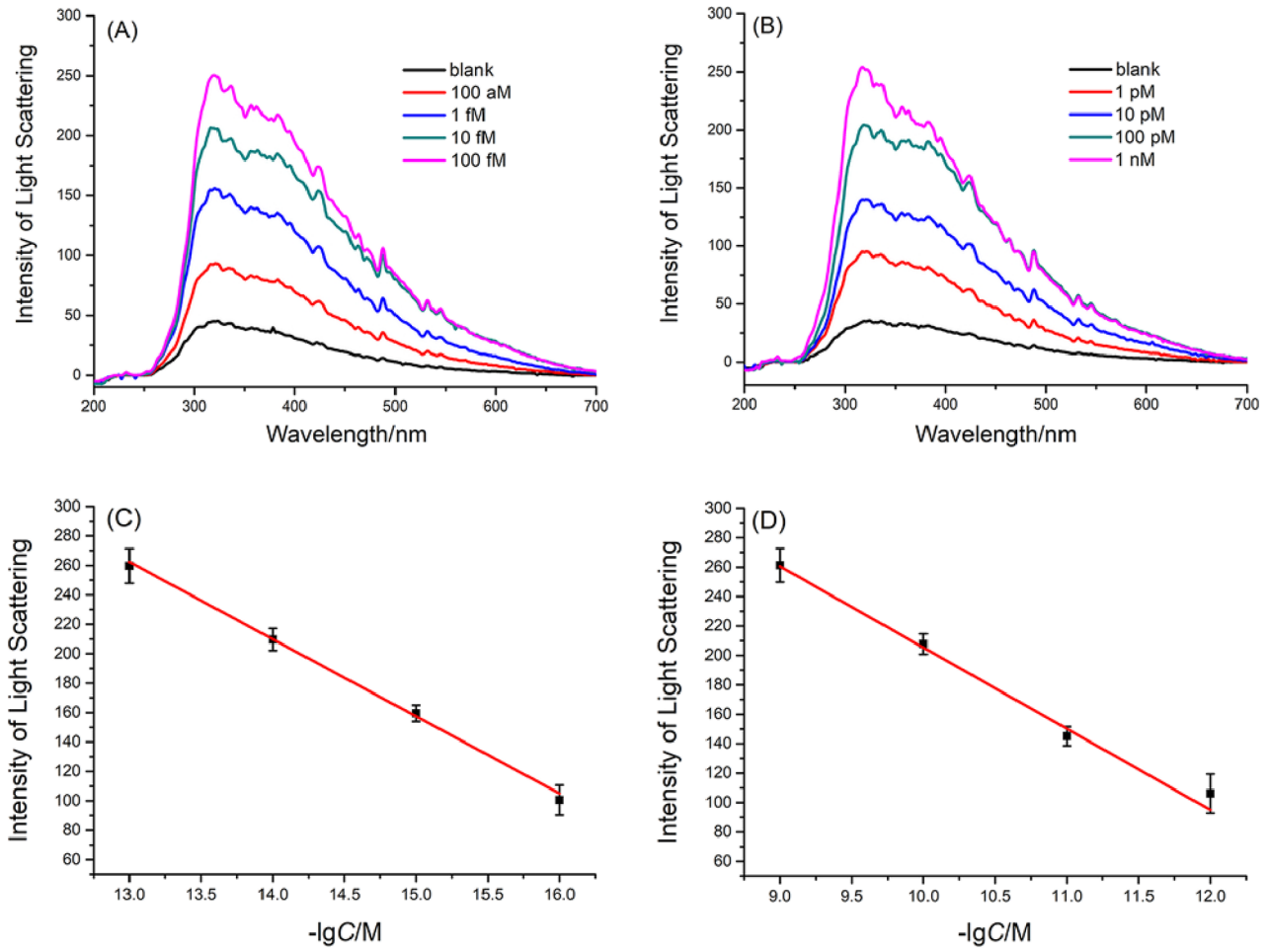

Figure 3. (A, B) Light scattering spectrum of mRNA with different concentrations by the LL-LAMP protocol. (A) From bottom to top, the concentrations of mRNA are 0 (blank, without mRNA in the LL-LAMP system), 100 aM, 1 fM, 10 fM, and 100 fM, respectively. The LAMP reaction time is 35 min. (B) From bottom to top, the concentrations of mRNA are 0, 1 pM, 10 pM, 100 pM, and 1 nM, respectively. The LL-LAMP reaction time is 15 min. (C, D) Linear relationship between the light scattering intensity (at 320 nm) and the −lg of concentrations of mRNA. The linear correlation equations are $I_{320\ nm} = 944.2 + 52.4\ lg\ C/M$ ($R^2 = 0.9958$) with the concentration range from 100 aM to 100 fM and $I_{320\ nm} = 756.7 + 55.1\ lg\ C/M$ ($R^2 = 0.9828$) with the concentration range from 1 pM to 1 nM. Error bars represent the standard deviation of three replicates in panels (C) and (D).

modified with two ribonucleotides at the 3′-end, which effectively improves the connection efficiency.[40] In the presence of let-7a, $P1_{let\text{-}7a}$ and $P2_{let\text{-}7a}$ respectively hybridize adjacently with let-7a and are ligated together by the catalysis activity of ligase to form a special DNA molecule with a double stem-loop structure. The special DNA with a double stem-loop structure is a starting template for the subsequent cycle DNA amplification process, which is exactly a similar process to loop-mediated isothermal amplification (LAMP).[20] Once the starting template is formed, it can initiate a cycle DNA amplification reaction in the presence of FIP (forward inner primer) and BIP (backward inner primer) under isothermal conditions.[29] Cycle DNA amplification reaction is catalyzed by Bst polymerase with chain replacement activity. First, double stem-loop DNA will initiate an extension reaction along with itself as a template to form single stem-loop DNA consisting of a stem and a loop (structure 1). F2 of FIP hybridizes with a loop of structure 1 to continue the extension reaction while displacing the lower strand to form structure 2. Structure 2 continues the extension reaction to form structure 3 and structure 4. Briefly, DNA strand displacement occurs in two processes: self-primed extension reaction and FIP (or BIP)-mediated extension reaction. DNAs released by the strand displacement process can self-hybridize to continue the self-primed extension reaction, and then FIP (or BIP) will go on hybridizing with a loop to start the extension reaction. Finally, such a cycle DNA amplification reaction can produce a large number of stem-loop DNAs with different inverted repeats and cauliflower-like structures with multiple loops. When perchloric acid is added to the LAMP amplification products, the DNA double-helix structure is first denatured into single strands and then polymerized into large particles with a radius of 200 to 700 nm,[39] which generates strong light scattering. Moreover, the intensity of scattered light depends on the original target RNA dosage, which can realize the highly specific and sensitive analysis of RNA.

The Performance of LL-LAMP for RNA Analysis. The influence of various conditions, including types of acids, the concentration of $HClO_4$, and the stability of the light scattering signal, on the performance of LL-LAMP has been optimized (Figures S1−S3). By testing a series of different amounts of let-7a miRNA as target RNA, the analysis performance of LL-LAMP was verified under the optimum conditions. As shown in Figure 2A, the intensity of the light scattering signal

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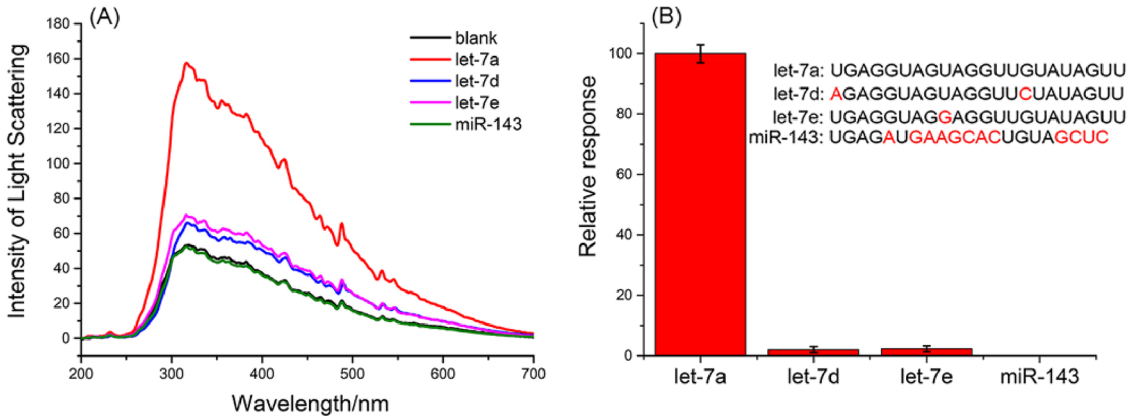

Figure 4. (A) Light scattering spectrum of the LL-LAMP-based assay of different miRNAs. (B) Relative light scattering responses (at 320 nm) of other miRNAs compared to let-7a. The relative response of let-7a is defined as 100% and calculates the relative responses of other miRNAs based on the linear equation in Figure 2. Error bars are the standard deviation of three replicates.

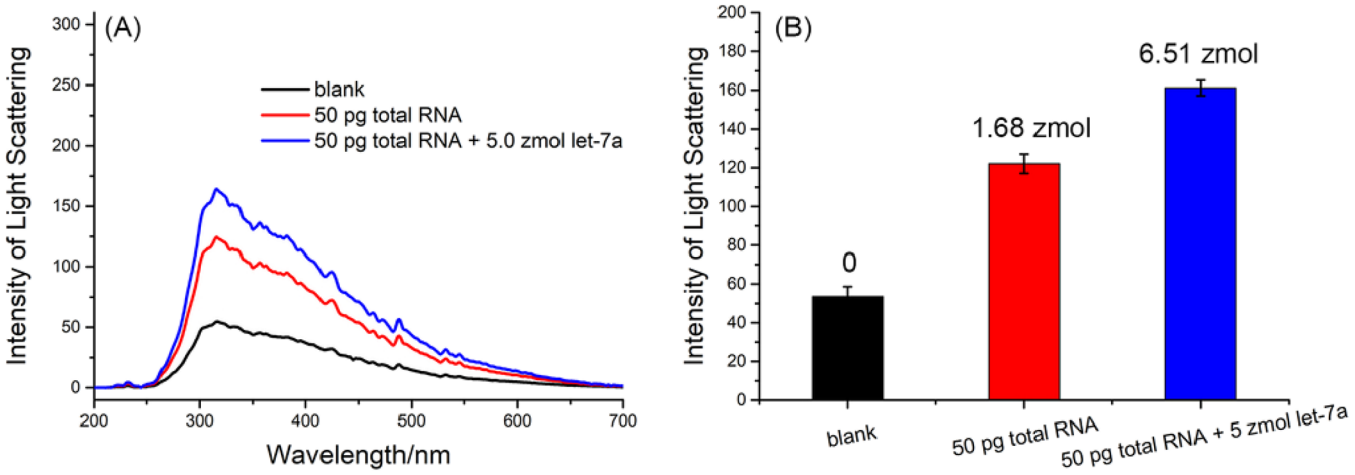

Figure 5. Quantification of let-7a in 50 pg of total RNA samples by using LL-LAMP. (A) Light scattering spectra for the assay of let-7a in total RNA samples. (B) Let-7a content in total RNA. Error bars indicate the standard deviation of five replicates.

increases gradually with the increase in miRNA concentration and as low as 1 zmol (100 aM) miRNA can be distinguished from the blank (without target RNA in LL-LAMP reaction). When the light scattering intensity at 320 nm was plotted against the negative logarithm concentration of miRNA (−lg C/M) in the range of 100 aM to 100 fM, the standard curve showed a good linear relationship (Figure 2C). The correlation equation is $I_{320\ nm}$ = 1114.2 + 62.8 lg C/M with a linear correlation coefficient R^2 = 0.9923, suggesting that the LL-LAMP method is sensitive enough to quantitatively detect miRNA with the detection limit of 100 aM. Typically, the miRNA concentration range is about 100 aM to 800 fM in 1 ng of total RNA samples, that is, the dynamic range of the proposed method is sufficient to detect the vast majority of miRNAs in unknown actual samples.[37] In addition, LL-LAMP can expand the testing range by shortening the amplification time (Figures 2B,D). Similarly, the intensity of light scattering at 320 nm has a linear relationship with the negative logarithm of the concentration of target miRNA (−lg C/M) in the range from 1 pM to 1 nM. The linear equation is $I_{320\ nm}$ = 785.2 + 56.3 lg C/M (R^2 = 0.9921), which indicates that higher concentrations of miRNA can also be analyzed by reducing the amplification time. It is worth noting that the timescale of LAMP amplification is different for the different ranges of miRNA (Figure S4), which has been discussed in detail in the Supporting Information.

To further demonstrate the generality of the method, p53 mRNA was chosen as another model target. According to the sequence of p53 mRNA, $P1_{mRNA}$ and $P2_{mRNA}$ were designed respectively by changing complementary regions of the target sequence in $P1_{let\text{-}7a}$ and $P2_{let\text{-}7a}$. As displayed in Figure 3, LL-LAMP can sensitively quantify mRNA with the same performance of let-7a. These results confirm the superior applicability of the proposed method to the quantitative analysis of other RNA molecules.

The Specificity of LL-LAMP for miRNA Detection. Accurate single-base identification of target RNAs for genetic biomarker analysis is an important aspect, especially those miRNA sequences that are extremely similar. To evaluate the specificity of the LL-LAMP protocol for RNA assay, the same amount (1 fM) of let-7a family (let-7a, let-7d, and let-7e) and a random miRNA (miR-143) were simultaneously detected by using let-7a-specific probes ($P1_{let\text{-}7a}$ and $P2_{let\text{-}7a}$). As shown in Figure 1, $P1_{let\text{-}7a}$ and $P2_{let\text{-}7a}$ can perfectly hybridize with let-7a. Let-7a can be used as a template to effectively ligate the let-7a-specific stem-loop probes, start the subsequent amplification reaction, and generate a clear light scattering signal. On the contrary, the mismatched nucleotide from the other miRNA can form the overhang with several nucleotides, which can

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avoid effective ligation reactions. As displayed in Figure 4A, the light scattering signal of let-7a can be distinguished from the other kinds of miRNA. It is worth noting that let-7a, let-7d, and let-7e are different from one to two nucleotides. One can see from Figure 4B that let-7e and let-7d produce 2.29 and 2.0% nonspecific signals, respectively. For miR-143, the light scattering signal is the same as the blank, indicating that there is no interference from miR-143. Overall, these results demonstrate that the LL-LAMP protocol has high specificity and the ability to identify highly similar RNAs with only single-base differences.

Quantification of miRNA in the Total RNA Sample. Furthermore, to investigate the practicability of the LL-LAMP system for miRNA assay in complex biological samples, let-7a in total RNA extracted from MCF-7 cells was detected. As shown in Figure 5, 50 pg of the total RNA sample produced a well-defined light scattering curve. With the calibration curve in Figure 2C, the amount of let-7a in 50 pg of total RNA is estimated to be 1.68 zmol. Upon adding 5 zmol of synthetic let-7a to 50 pg of the total RNA sample, after five repeated determinations, the average let-7a content was 6.51 zmol, and the average recovery rate was 96.6%. These results indicate that LL-LAMP is a reliable and practical method for the quantitation of miRNA in biological samples.

CONCLUSIONS

In summary, we developed a convenient and low-cost method (LL-LAMP) for RNA analysis based on light scattering technology and ligation-dependent loop-mediated isothermal amplification. In the proposed method, the efficient mechanism of LAMP ensures the sensitivity of RNA assay. The target RNA-templated ligation reaction of two stem-loop probes enables LL-LAMP with single-base resolution. More importantly, the amplification products can aggregate into large particles after adding perchloric acid and produce strong light scattering. Thus, the target RNA can be quantitatively detected by using light scattering technology without expensive reagents. By using miRNA and mRNA as proof-of-concept RNA targets, we have demonstrated that LL-LAMP is a practical and reliable method for RNA analysis. These features highlight that the LL-LAMP protocol has great potential in the field of nucleic acid assay.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c01759.

Optimization of experimental conditions (Figures S1−S3), results of real-time fluorescence monitoring of amplified products (Figure S4), comparisons of different optical methods for the detection of RNA (Table S1), and sequences of the oligonucleotides (Table S2) (PDF)

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Author Contributions

Honghong Wang, Hui Wang, and Z.L. conceived the work. Honghong Wang and S.W. planned and performed the experiments. Honghong Wang, Hui Wang, and Z.L. discussed and analyzed the data. Y.L. and Y.J. carried out confirmatory experiments. Honghong Wang, Hui Wang, and Z.L. wrote and revised the paper. All authors declare no competing financial interest and agree to contribute.

Notes

The authors declare no competing financial interest.

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