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Highly Effective and Low-Cost MicroRNA Detection with CRISPR-Cas9

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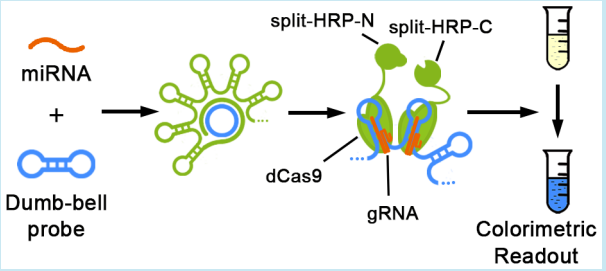
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Supporting Information

ABSTRACT: MicroRNAs have been reported as related to multiple diseases and have potential applications in diagnosis and therapeutics. However, detection of miRNAs remains improvable, given their complexity, high cost, and low sensitivity as of currently. In this study, we attempt to build a novel platform that detects miRNAs at low cost and high efficacy. This detection system contains isothermal amplification, detecting and reporting process based on rolling circle amplification, CRISPR-Cas9, and split-horseradish peroxidase techniques. It is able to detect trace amount of miRNAs from samples with mere single-base specificity. Moreover, we demonstrated that such scheme can effectively detect target miRNAs in clinical serum samples and significantly distinguish patients of non-small cell lung cancer from healthy volunteers by detecting the previously reported biomarker: circulating let-7a. As the first to use CRISPR-Cas9 in miRNA detection, this method is a promising approach capable of being applied in screening, diagnosing, and prognosticating of multiple diseases.



KEYWORDS: *miRNA detection, CRISPR-dCas9, rolling circle amplification, split reporter, biomarker*

MicroRNAs (miRNAs) are small noncoding RNAs 18−24 nucleotides long and are important for post-transcriptional regulation of gene expression.[1] Recently, miRNAs have been found essential in the genesis and development of numerous diseases, and thus promoted multiple studies focusing on their regulation mechanisms. Besides, their expression patterns were also proven related to numerous clinical variables of cancer, wherein miRNA profiling can be used for cancer detection and classification.[2,3] Circulating miRNAs, for instance, were identified as potent biomarkers for point-of-care diagnosis of multiple diseases because they can remain stable under extreme conditions such as RNase exposure, multiple freeze−thaw cycles, and extreme pH.[4,5] Hence, the ability to detect miRNAs with high efficacy and low cost may aid in noninvasive diagnosis, prognosis and other general laboratory tasks.

Currently, traditional methods, such as Northern blot analysis, microarray, and qRT-PCR, are our main approaches for detecting and quantifying miRNAs.[6] However, due to the short length, low expression level, and high homologous sequence similarity of miRNAs, the efficacy of these techniques, as well as some other new approaches, such as bioluminescence[7] and nanopore sensors,[8] remains improvable (especially in biofluid samples like serum, where the miRNA concentration ranges from fM level to pM level). The cumbersome detection devices, and relatively high costs of these methods also limited their further applications.

Hence, improvements are necessary for detecting miRNAs. Recently, isothermal amplification techniques have been used for *in vitro* amplification of nucleotides in multiple researches,[9−12] rolling circle amplification (RCA) technique has also been proven to be able to amplify miRNA *in vitro*[13] and has also been applied to amplify RNA for *in situ* imaging[9] or logic gates construction[14] recently. These methods elongate short-length RNAs into considerably regular structures, thereby providing potential clues for the primary amplification of miRNA input signal. Moreover, the CRISPR-Cas systems may provide new approaches given its highly specific nucleic acid binding ability.[15,16] Recent studies have developed nucleotide detection techniques based on these approaches.[17,18] However, their application on short-length miRNA detection remained constrained. Notablely, the production of Cas9 mutants with inactivated nuclease activity, termed as catalytically dead Cas9 (dCas9), has been produced to introduce functional proteins, especially enzymes and reporters, to specific genome loci for different applications such as "base editing", imaging, and signaling using a protein-fusing manner.[18−20]

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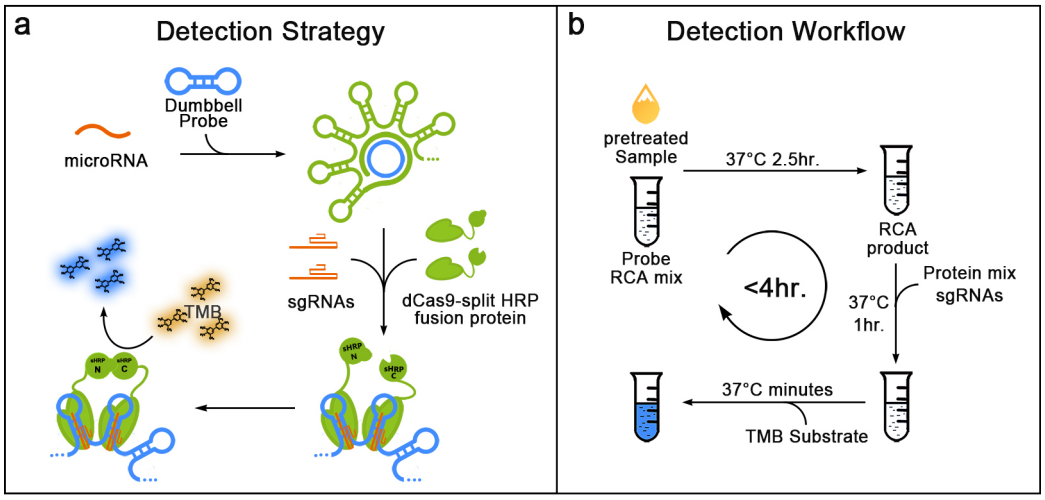


Figure 1. Schematic representation for the blueprint and detection workflow of the RCH method for detecting miRNAs. (a) Our strategy is to use RCA as a primary amplifier for inputting miRNA signals to generate large DNA fragments with repeated miRNA complementary sequences and regular stem-loop structures. For this part, dumbbell-like probes were bound specifically to the target miRNAs to initiate a strand-replacement reaction and change its structure to a suitable substrate for a phi-29-mediated RCA. Subsequently, introducing a CRISPR-dCas9 based technique targets the products from the first-step-amplification, thereby achieving secondary amplification and signal output. For such matter, dCas9 proteins were fused in advance with split-HRP reporting fragments. Guided by specific sgRNAs, split-HRP-dCas9 fusion proteins can be recruited around the scaffold-like structure of the RCA products, thereby performing the HRP activity. The HRP activity can be detected using 3,3′,5,5′-tetramethylbenzidine (TMB) color reaction. (b) The detection shall be completed with simple equipment such as tubes, peptides and incubators. The whole detection workflow shall last for less than 4 h.

Here, we demonstrate the novel RCA-CRISPR-split-HRP (RCH) method built with synthetic biocomponents for the low-cost and convenient detection of miRNAs with a fM-level sensitivity and single-base specificity (Figure 1a). For the detection test, miRNAs were isothermally amplified *via* RCA and used for *in vitro* dCas9 binding and split HRP activity assessment.[21] A color change of the TMB substrate from light yellow to blue indicated the existence of specific miRNAs. The whole flow should take no more than 4 h, with portable and relatively cheap reagents and equipment (Figure 1b).

Using let-7a as a target, we first examined whether the probes can initiate RCA reactions in complex liquid samples like serum. For such matter, we used SYBR I to detect the double-stranded RCA reaction products (Figure 2a). Four probes for let-7a were designed and synthesized as 5′-phosphorylated oligo-DNA. The synthesized DNA was ligated by T4 DNA ligase and unligated fragments were digested by exonucleases I and III (Supplementary Figure S1a). All four probes were proved to be able to initiate RCA reaction triggered by 10 nM of let-7a (Supplementary Figure S1b), with the most efficient ones chosen for subsequent tests. The gradient amount of the trigger miRNA was spiked into either water or human serum (7%) to closely mimic clinical liquid samples and determine if RCA is inducible and can maintain its sensitivity under such conditions. To further investigate the significance of the signal among the intensive concentration gradients under the optimal reaction time, an end-point fluorescence experiment was performed. A fluorescent−concentration curve was obtained by fitting the data with exponential curves (Figures 2b and 2c, R^2 = 0.9763 and 0.9685, respectively). These results indicated the ability to amplify miRNA signal with relatively high detection sensitivity of the RCA reaction.

Similarly, we assessed the RCA specificity in water or serum solution by comparing the results using 10 nM of let-7a as input with the ones using 10 nM of three different miRNAs in the let-7 family, namely let-7c (one base mismatch against let-7a), let-7f (one base mismatched), and let-7g (two base mismatched), as inputs (Figures 2d and 2e). Under both solution environments, the fluorescent intensities obtained from let-7a group are both significantly higher than those obtained from other miRNA groups (Figures 2d and 2e). The results demonstrated the RCA feature of single base specificity to be an amplifier of the input miRNA signal. SYBR I may provide a primary evaluation of the RCA products; however, its high background and unstable fluorescent output remained significant, which was inadequate for detecting circulating miRNA level. Such disadvantage seemed to result from poor specificity of the SYBR I staining and the lack of second-stage amplification mechanisms. Similar concerns were raised in other new detecting techniques, including quantum dots tagging[22] and ATP-Releasing Nucleotides.[23]

To further improve the miRNA detection sensitivity, we then introduced a CRISPR/dCas9-combined split-HRP based reporter system, intended to reach a highly specific output and an enzyme-based second-stage amplification of the RCA output signal (Figure 3a). HRP-reporter assay was conducted to test the sensitivity and specificity of the RCH method by inputting a sample of let-7a suspended in water or 7% human serum as a prototype. For such matter, we fused the two split-HRP fragments (sHRP-C and sHRP-N) onto the N-terminus of the dCas9 protein (termed as sHRP-C-dCas9 and sHRP-N-dCas9) with a ten-amino acid linker (Supplementary Figure S2a). The reconstituted activity of the split HRP system have been proven to be close to that of full-length HRP.[21] Both proteins were successfully expressed and purified to a high degree of up to 90% (Supplementary Figure S2b). The sgRNA Cas9-binding was designed based on the dumbbell-shaped probe. By placing the coding DNA sequence under control of a T7 promoter (Supplementary Figure S3a), sgRNA was effectively obtained through *in vitro* transcription and examined through electrophoresis (Supplementary Figure S3b).

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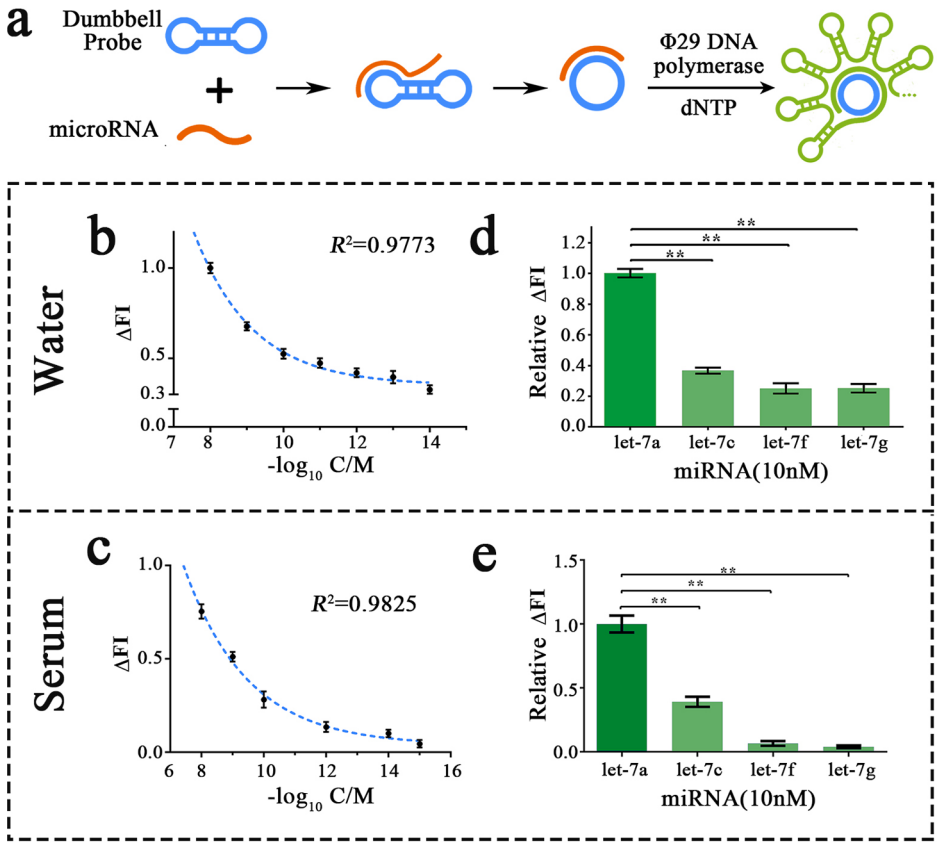


Figure 2. Evaluation of RCA-initiating ability of selected probes targeting synthesized miRNAs. (a) Schematic representation of the procedure used for probe evaluation. (b,c) The plot of fluorescent intensity variation against the negative logarithm of the let-7a concentration using water- (b) or 7% serum-diluted miRNA (c) were used as input. (d,e) Specificity determination of the RCA reaction was initiated by the collaboration of the designed probe and let-7a, let-7c, let-7f, or let-7g. The bases marked red in the sequences of the tested miRNAs displayed below are mismatched bases against let-7a. The miRNA was diluted to 10 nM in water (d) or 7% serum (e). The excitation and emission wavelengths were 495 and 515 nm, respectively. Fluorescence intensity variation was calculated by subtracting the fluorescent intensity of the blank group. For the calculation of relative fluorescence intensity variation, the let-7a group was set arbitrarily at 1.0, and the levels of the other groups were adjusted correspondingly. The 7% human serum used in the experiment was a mixture of serum samples from healthy volunteers. The error bars were obtained from at least three independent experiments. The squared correlation coefficient (R^2) was analyzed using a Graphpad Prism 6.0. $^{**}p < 0.01$.

Protein concentration affects the system performance; therefore, a mathematical model based on the Michaelis–Menten equation and probability analysis were developed to select the proper protein concentration for the wet-lab experiments. According to the prediction, approximately 1.5 μg sHRP-C-dCas9 and 1.5 μg sHRP-N-dCas9 proteins may be the optimal concentration for the split-HRP-based reporter assay (Supplementary Figure S4). Thus, in wet-lab experiments, each supplementation of 1.5 μg fusion proteins into a 50 μL reaction mix containing the proper buffer, sgRNA, and RCA products generated from different water-diluted input miRNA concentrations did obtain a good OD_{450}-miRNA concentration relation (Figure 3b) with a colorimetric difference (Figure 3c). Such results suggested that the response of the RCH system to 1 fM miRNA input remained concentration-sensitive supplemented with proper amount of proteins. The detection limit (blank+3 × SD) was estimated to be around 3.540×10^{-17} mol/L (~35.4 aM), with the dynamic range spanned over 3 orders of magnitude. In contrast, the reaction mix supplemented with 0.15 μg protein showed a significantly poor performance (Figures 3b and 3c). Similar results were consistent for the RCA products generated from serum-diluted miRNA input at 100 fM to 10 nM (Figure 3e), whereas the results below the 100 fM input showed no significance (data not shown), indicating the hundred-fM-level let7a imbedded in the serum from healthy volunteers, which corresponded with the previous report. Our RCH method showed higher significant specificity than the first step of distinguishing let-7a from other let-7 family miRNAs using RCA under different input conditions (water- or serum-diluted) (Figures 3d and 3f). These results demonstrated the fM-level sensitivity and the one-base-level specificity of the RCH method.

To demonstrate the potential clinical use of the RCH method, we then applied our scheme on serum samples from Non-Small Cell Lung Cancer (NSCLC) patients and healthy volunteers to detect circulating let-7a (Figure 4a). Circulating let-7a has been previously reported significantly downregulated in NSCLC patients, thus making it a promising biomarker for NSCLC.[24−26] Two other miRNAs, miR-195 (a previously determined biomarker of breast cancer[27]) and miR-16, were used as control groups to represent the irrelevant biomarker and internal control,[28] respectively. The dumbbell-like probes for miR-195 and miR-16 were designed, prepared, and validated using previously addressed methods (Supplementary Figure S5). Serum samples collected from 16 NSCLC patients and 16 healthy volunteers were used in the exam. The serum samples (100 μL) were diluted ten times with diethyl pyrocarbonate (DEPC)-treated water to reduce the possible inhibitory effects

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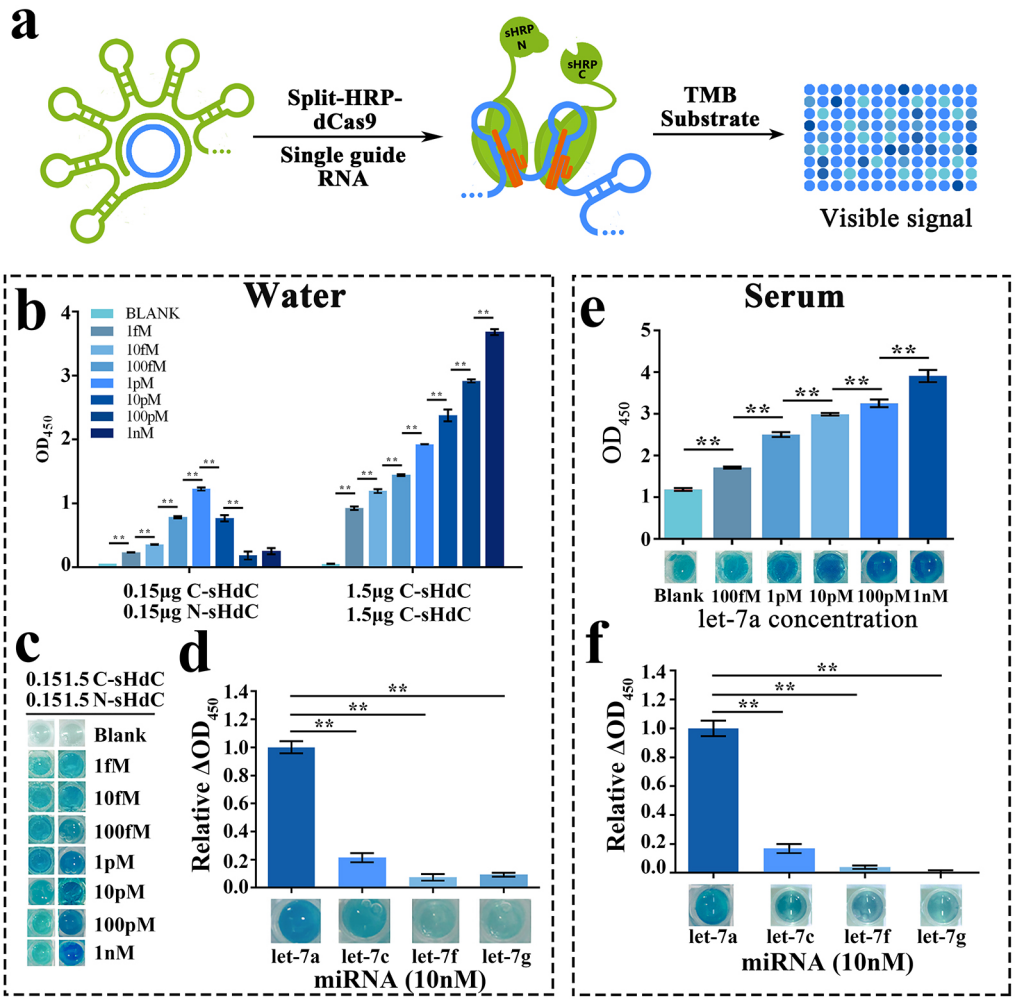


Figure 3. Assessment of the sensitivity and specificity of RCH method. (a) Schematic representation of *in vitro* dCas9 binding and HRP activity assessment. (b) OD_{450} value of gradient let-7a concentration (water-diluted) groups under different protein concentrations. (c) Color difference among different let-7a (serum-diluted) concentrations under 1.5 and 0.15 μg of each fusion proteins. Images were taken before terminating the reaction with H_2SO_4. (d) Specificity determination of the RCH using water-diluted let-7a, let-7c, let-7f, or let-7g. Images below show the color difference against different miRNA inputs. OD_{450} variation was calculated by subtracting the OD_{450} of the blank group. The OD_{450} of let-7a group was set arbitrarily at 1.0. (e) OD_{450} value of gradient concentration groups of let-7a (serum-diluted). Images below show the color difference against different concentrations of let-7a inputs. (f) Specificity determination of the RCH using serum-diluted let-7a, let-7c, let-7f, or let-7g. Images below showed the color difference against different miRNA inputs. Error bars were obtained from at least three independent experiments. $^{**}p < 0.01$.

of the serum on downstream reactions.[29] The diluted sample was boiled in 95 °C for 10 min before use. Results showed that for let-7a, the OD450 output level of the patient group was significantly lower than in the healthy group (p = 0.0002), whereas the miR-195 and miR-16 groups showed no significance (p = 0.6030 and p = 0.3976, respectively) (Figure 4b). To verify our results, a standard Taq-man quantitative RT-PCR assay based on stem-loop primers was performed on the same samples. A significant downregulation on the let-7a level in NSCLC group compared with the healthy group (p = 0.0004) could be observed, which was consistent with the previous report,[24] whereas the miR-195 concentration showed no significance between two groups (p = 0.5176) (Figure 4c). Notably, improved data significance and homogeneity was observed in our RCH method against the traditional qRT-PCR technique.

In summary, we described a novel method for miRNA detection. As with our original protocol, the miRNA level can be rapidly detected with fM-grade sensitivity and one-base specificity. Comparing to other RCA based methods,[9,13] our scheme may provide wider dynamic range while maintaining high sensitivity due to the cascade amplification of dCas9-split-HRP system. Also, our scheme may potentially reduce the affection of dCas9 off-target effect since the RCA reaction is highly specific. With the rapid development of smartphone-based ELISA detection devices[30] and paper-based techniques,[29] our scheme might be used as solution-based or paper-based colorimetric readouts in low-tech conditions with limited supplies in future (Supplementary Figure S6). Moreover, the success of the let-7a demo in distinguishing NSCLC patients from healthy volunteers implied the potential application of the scheme in screening and diagnosing multiple diseases, such as cancers and schizophrenia. Besides, we also demonstrated that our scheme was also applicable for miRNA detection in cell lysate (Supplementary Figure S7), where the miRNA concentration was significantly higher than the serum sample. For different miRNA, a RCH probe/sgRNA can be redesigned and synthesized in days, the cost for each test is estimated to be as low as $1.727 (Supplementary Table S3), which may further decrease by industrializing the protein producing procedure.

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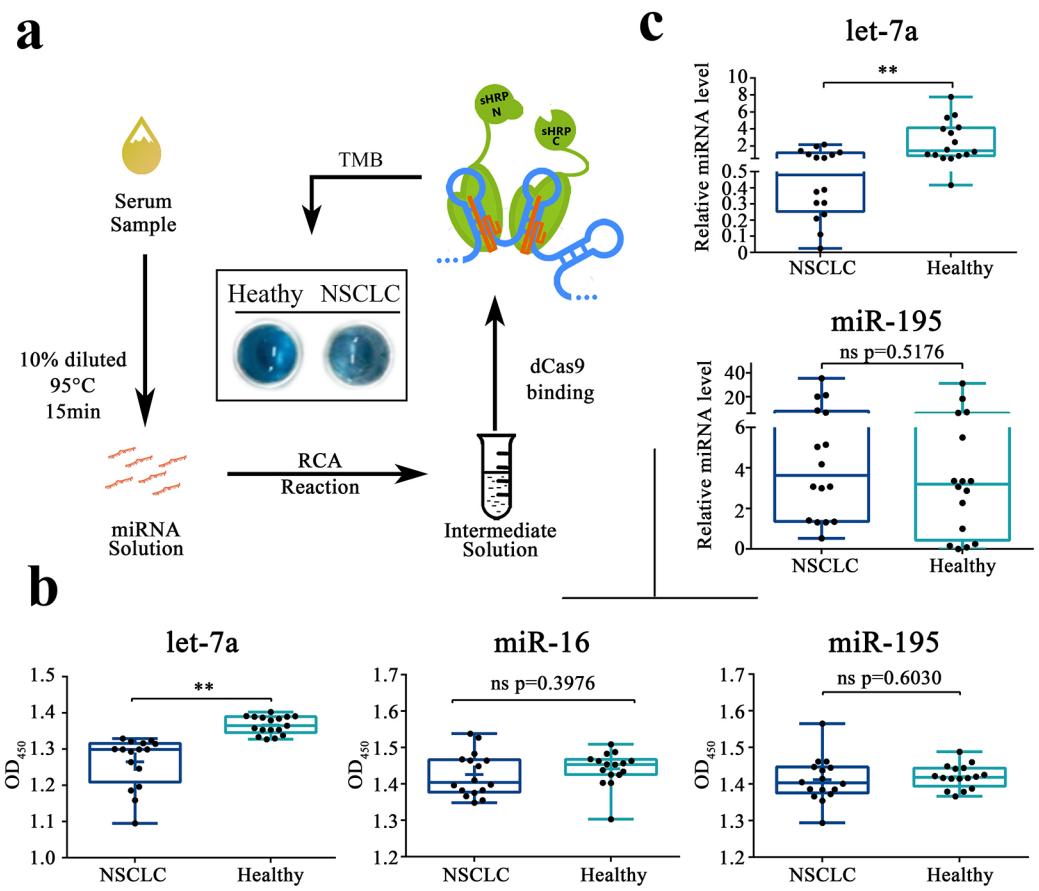


Figure 4. miRNA detection in clinical serum samples using RCH method. (a) Schematic representation of RCH detection of the miRNA. (b) Plots of OD_{450} value obtained from RCH of samples from 16 healthy volunteers and 16 NSCLC patients on let-7a, miR-195, and miR-16. (c) Quantitative RT-PCR assay detection of let-7a and miR-195 in NSCLC and healthy samples. Relative gene expression level was normalized to miR-16. The median of the healthy group was arbitrarily set to 1.0. Error bars were obtained from at least three independent experiments. $^{**}p < 0.01$.

However, this scheme remains room for improvement. For example, proteins concentration, optimal RCA reaction time, and dCas9 binding time can still be further determined for different miRNAs and their corresponding probes. Furthermore, with the rapid development of isothermal amplification systems and split-reporting systems, the sensitivity and dynamic range of our current assay may still be improved by implementing other isothermal amplification systems[31] or other reporting systems, such as split-GFP,[32] split-luciferase,[19] and split-β-galactosidase.[33]

In general, by confronting unsolved issues in the previous miRNA detecting methods, this RCH method provided an approach with lower cost and remarkable efficacy for detecting miRNAs. Future applications in highly efficient diagnosis of different diseases under different conditions are highly promising. Meanwhile, to the best of our knowledge, our study is the first to implement the CRISPR-Cas9 technique in miRNA detection and can broaden the applications of such genome-editing tools.

METHODS

Preparation of Dumbbell Probe. The dumbbell probe was prepared based on the synthesized 5′-phosphorylated oligo-DNA synthesized from Synbio Biotechnologies Co., Ltd. (Supplementary Table S1). The oligo-DNA was ligated using T4 DNA ligase (Life Technologies) to form the desired structure, and unsealed structures were digested using Exonuclease I (Life Technologies) and Exonuclease III (Life Technologies). The synthesized oligo-DNA was rehydrated into a 10 mM concentration. The ligation was conducted in a 20 μL mixture containing 1 μL oligo-DNA, 2 μL 10× ligase buffer, and 17 μL DEPC-treated H_2O. The ligation reaction was conducted in 22 °C for 4 h before the ligase was denatured at 65 °C for 10 min. After that, exonucleases I and III were added into the mixture, and the reaction mixture was incubated in 37 °C for 2 h and before the exonucleases were denatured at 85 °C for 20 min. The prepared dumbbell probes were examined by electrophoresis in 1.5% agarose (TaKaRa Biotech) gel.

RCA Reaction. The miRNAs were synthesized by TaKaRa Biotech Company (Dalian, China). The RCA reaction was conducted in a 25 μL mixture containing 1 μL of the prepared probe, 2.5 μL 10× reaction buffer, 0.5 μL BSA (10 mg/mL), 6 μL dNTP (10 mM each), 2.5 μL serum sample or target miRNA solution, 12 μL DEPC-treated H_2O, and 0.5 μL phi29 DNA polymerase (10 U/μL, Life Technologies). The reaction mixture was incubated at 37 °C for 2 h, and quenched at 65 °C for 10 min. The reaction product was used for electrophoresis/fluorescence analysis, or further *in vitro* dCas9 binding and HRP activity assay. For electrophoresis, 5 μL of the product was mixed with 1 μL 6× loading buffer (TaKaRa Biotech) and loaded in 0.7% EB-stained agarose gel under 120 V for 40 min. Images were obtained with a gel imaging system (Proteinsimple).

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sgRNA Production. The DNA sequence-coding sgRNAs were designed and synthesized under control of T7 promoter before being subcloned to pUC-57 plasmid vectors (Synbio Biotechnologies Co., Ltd.). A SpeI site was added after the sgRNA-coding sequence for plasmid linearization. The *in vitro* transcription was conducted with a T7 *in vitro* transcription kit (Viosolid), following manufacturer instructions, with a linearized plasmid DNA as input. Transcription products were stored at −20 °C after being validated through 3% agarose gel electrophoresis.

***In Vitro* dCas9 Binding and HRP Activity Assessment.** The reaction condition of the dCas9 binding and HRP activity assay is established based on previous reports[15] and trial experiments (Supplementary Figure S8). Each reaction mix in this assay was constructed into a 50 μL volume system containing 1.5 μg (or 0.15 μg) each of purified sHRP-N-dCas9/sHRP-C-dCas9 protein, 2.80 μg (or 0.28 μg for 0.15 μg protein group) sgRNA, 5 μL 10× Cas9 buffer (Novoprotein), and 1 μL RNase inhibitor (Novoprotein), and DEPC-treated H_2O was added to fill the remaining volume. Heme (Life Technologies) was supplemented into the reaction mix to trigger the interaction of sHRP-C and sHRP-N based on the previously reported volume.[21] The reaction mix was incubated in 37 °C for 1 h for the dCas9 to bind and the sHRP fragments to interact. Then, the reaction mixture was transferred to a 37 °C prewarmed 96-well plate (Corning) and 100 μL TMB substrate solution (Life Technologies) was added to the mixture for HRP detection. The plate was incubated in 37 °C for 3 min, and reactions were terminated using 0.16 M H_2SO_4. Results were obtained through OD_{450} measurement of each sample (Multiscan FC Microplate Reader, Thermo Fisher).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00446.

Supplementary methods, figures and tables (PDF)

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Author Contributions

‖X.-Y.Q., L.-Y.Z., and C.-S.Z. contributed equally to this work. X.-Y.Q., L.-Y.Z., and L.Z. conceived the original idea. X.-Y.Q., L.-Y.Z. and L.Z. designed the experiments. X.-Y.Q., L.-Y.Z., C.-S.Z., J.-X.M., S.-S.X., L.M., and D.-A.T. conducted the experiments. T.H. prepared the clinical samples. X.-M.W. and D.-Y.Z. provided important advising and experimental support. X.-Y.Q., L.-Y.Z., and L.Z. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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