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CRISPR-Cas14a-integrated strand displacement amplification for rapid and isothermal detection of cholangiocarcinoma associated circulating microRNAs

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Abstract

Circulating microRNAs (miRNA) can serve as key biomarkers for early diagnose of cholangiocarcinoma. Herein, an assay that uses circulating miRNA to trigger strand displacement amplification (SDA) and a CRISPR-Cas14a system to report the SDA process has been developed. In the proposed method, SDA directly amplifies miRNAs without reverse transcription. The reporter, CRISPR-Cas14a, can reduce the risks of non-specific amplification and offers a sequential amplification that improves the sensitivity for miRNA detection. The assay, termed Cas14SDA, can discriminate miRNAs with similar sequences and can detect as low as 680 fM miR-21 (miRNAs overexpressed in cholangiocarcinoma) within 1 h. In particular, Cas14a was efficiently activated by a single-stranded SDA amplicon which improved the sensitivity by 2.86 times compared to that using Cas12a. This research has demonstrated that the Cas14SDA assay can discriminate cholangiocarcinoma patients from healthy donors by testing miR-21 in their blood samples. The Cas14SDA assay developed broadens the toolbox for miRNA biomarker analysis.

Keywords

CRISPR-Cas14a; microRNA; Cholangiocarcinoma; Strand displacement amplification; Circulating biomarker

1. Introduction

Cholangiocarcinoma is one of the most common hepatic diseases [1]. It is a type of aggressive tumor that is difficult to diagnose, and when found in patients, they are usually at an advanced stage of the pathology [2]. Circulating microRNAs (miRNAs) are short RNA sequences that modulate the expression of proteins by interacting with mRNAs [3]. Thus, miRNAs are associated with main processes in cells, such as growth, division and canceration [4], and they can be frequently found circulating in blood. The level of miRNAs, such as miR-21, has been associated with the occurrence of cholangiocarcinoma [5-8]. Thus, circulating miRNAs are now recognized as promising biomarkers for diagnosing cholangiocarcinoma.

The analysis of miRNAs is challenging because they are short (about ~20 nt). This makes it harder to detect than other nucleic acid biomarkers such as genes, mRNAs, and long non-coding RNA. Currently, the most frequently used methods for miRNA detection are northern blotting, microarray analysis and real time polymerase chain reaction (qPCR) [9,10]. Northern blotting is the gold standard method for miRNA detection. This microassay can be highly multiplexed, however, it usually has insufficient sensitivity for detecting low levels of miRNAs in blood. In contrast, qPCR can be highly sensitive. However, the short sequence of features in miRNAs makes the design of primers for qPCR complex. Besides, the high cost of the instruments for qPCR analysis hinders the use of the assay.

Isothermal amplification strategies have been studied for detecting miRNA and they

have become and advanced tool for profiling miRNA [11,12]. Rolling circle amplification (RCA) [13], loop-mediated isothermal amplification (LAMP) [14], strand-displacement amplification (SDA) and exponential amplification reaction (EXPER) [15] have been designed to detect miRNAs by using miRNAs as primers or templates. These platforms are coupled with fluorescent, electrochemical, and electrochemiluminescence sensing platforms, which lead to highly sensitive detection of miRNAs. However, amplification processes may contribute to non-specific amplification of miRNAs due to the lack of procedures for accurate identification of the desired amplicons [16-18].

CRISPR-Cas systems, evolved from bacterial immunity, have been developed for nucleic acid tests [19,20]. CRISPR-Cas system, such as Cas12a and Cas13a can be specifically activated via binding with target genes or RNAs [21-24]. The activation of Cas protein can cleave single stranded RNA or DNA sequences: termed *trans*-cleavage process [25-27]. By labeling short DNA/RNA sequences with fluorophores and quencher groups, CRISPR-Cas systems can serve as reporters for nucleic acid amplification, such as PCR, or isothermal amplification including recombinase polymerase amplification (RPA) and LAMP [25,28-30]. Besides, the integration of nanomaterials in the system facilitates the creation of rapid, sensitive and equipment-free biosensors for detecting biomolecules of interest [31-34]. The Hou group advanced the use of CRISPR/Cas system in isothermal amplification such as EXPAR [27], SDA [24] and RCA [35], and based on the same principle, they constructed assays for ssRNA,

ssDNA[36], glycosylase [37,38], alkaline phosphatase [27] and ATP[24]. Cas12a and Cas13a usually require the target sequences with defined nucleotides, for example, Cas12a need a protospacer adjacent motif (PAM), TTTV [19]. Besides, a number of isothermal amplification methods such as RCA, SDA and EXPER, produce single-stranded DNA products [39,40]. The *trans*-cleavage activity of Cas12a can be reduced using single-stranded DNAs as the activator compared to double-stranded DNAs [41]. Recently, it was reported that Cas14a could be used to identify sequences without nucleotide restriction [42-45]. Cas14a is particularly favorable to be activated by single-stranded DNA. In addition, Cas14a is small and compact [19], which makes it a competitive reporter for the nucleic acid amplification that produces single-stranded DNA amplicons.

In this work, for the first time, we introduce CRISPR-Cas14a as reporter of isothermal amplification, SDA [46-48], to develop and propose a rapid and isothermal assay for detecting the cholangiocarcinoma cancer biomarker miR-21The design of SDA is very simple, and only involved a single DNA sequence to serve as the template. The amplification triggered by target miRNAs can be strictly checked by CRISPR-Cas14a. The detection of miRNAs can be finished within 1 h. We used the Cas14SDA assay to test blood samples from cholangiocarcinoma patients and heathy ones. The rapid and simple Cas14SDA assay may facilitate the transition of miRNA for clinical diagnosis.

2. Materials and Methods

2.1. Reagents

The DNA sequences used in the proposed method are listed in Table S1. They were synthesized by Sangon Biotech (Shanghai, China). The 5'- and 3'- terminals of the reporter were modified with 6-carboxyfluorescein (FAM) fluorophores and black hole quencher 1 (BHQ1), respectively, and they were purified by high performance liquid chromatography. Other sequences were purified by polyacrylamide gel electrophoresis. Two Easy PCR SuperMix were purchased from Transgen (Beijing, China). T7 RNA polymerase (20 U/μL) with five T7 RNA polymerase buffers, RevertAid™ Master Mix and PlatinumTM SYBRTM Green qPCR SuperMix-UDG w/ROX were obtained from Thermo Fisher Scientific. (Waltham, USA). Nt.BstNBI, Bst DNA polymerase with 10 × Isothermal Amp Buffer, DNase I with 10× reaction buffers, ribonucleotide solution mix (rNTPs) and deoxynucleotide solution mix (dNTPs) were bought from New England Biolabs (Beijing, China). Cas14a and Cas12a proteins were obtained from Tolo Biotech. (Shanghai, China). Molecular biology grade H_2O was provided by Corning Incorporated (New York, USA).

2.2. Sample preparations and miRNA extraction

Samples were collected from 4 patients with histologically proven diagnosis of cholangiocarcinoma (labelled as C) and 4 healthy participants (labelled as A) from Fujian Medical University Union Hospital (Ethical Approval no. 2021KJCX026). Samples were stored at -80 °C until analysis. Briefly, blood samples (100 μL) were

added Trizol (1 mL). The homogenate specimens were transferred to 1.5 mL- tubesand placed at room temperature for 5 min to completely isolate the nucleoprotein complex. After the separating phases following the addition of chloroform (0.2 mL) and centrifugation (12,000× g, 15 min), the aqueous phase was transferred to a separate EP tube, where RNA was precipitated by adding isopropyl (0.5 mL). The RNA precipitate was washed with 75% ethanol (1 mL) in water. The washed RNA was dissolved in H_2O (30 μL). The extracted RNA can be used immediately or be preserved at -80 °C.

2.3. Preparation of sgRNA

PCR was used to amplify Cas14a plasmid (16 ng/μL) using primers of Cas14a-sgRNA-F (0.4 μM) and Cas14a-sgRNA-R (0.4 μM) to obtain the DNA template of Cas14a-sgRNA. Following, 5 × Transcription buffer (16 μL), T7 RNA polymerase (2 μL, 20 U/μL), rNTPs (3 μL of 25 mM each for ATP, GTP, CTP, and TTP) and H_2O (51 μL) were added to PCR(8 μL) products and they were let to react at 37 °C for 12 h. Subsequently, the residual DNA template was removed by replenishing DNase I (4 μL) for 3 h at 37 °C. The DNase I was inactivated by heating at 85 °C for 15 min.

2.4. miRNA detection

The SDA reaction was carried out by mixing of the SDA template (4 μL, 1 μM), 4 μL from different concentrations of miR-21, 2 μL Isothermal Amp Buffer, 1 μL dNTPs (10 mM each for dATP, dGTP, dCTP, and dTTP), 0.5 μL Nt.BstNBI (10 U/μL), 0.5 μL Bst DNA polymerase (10 U/μL) and 14 μL H_2O. The mixture was left to react at 55 °C for 30 min. Then, NEB buffer 3.1 (4 μL), Cas14a (4 μL, 1 μM), synthesized sg-

RNA (4 μL, 2 μM) and reporter (4 μL , 5 μM) were added to the above mentioned mixture. The mixture was then incubated at 37 °C for 40 min. A microplate reader Synergy H1 was used to measure fluorescent emission at 510 nm when the samples were excited at 480 nm. All samples were analyzed in triplicate.

2.5. Gel electrophoresis

Each step of the SDA reaction was analyzed using 3 % (w/v) agarose gel. Gel-loading solution had a final reaction volume of 6 μL (including 5 μL of oligonucleotides and 1 μL of gel loading buffer). The gel had 3 % agarose, 1× TAE buffer and 0.4× Gelred. The process was accomplished in 1× TBE buffer at 150 V for 30 min. Gel images were observed using Gel Doc XR+ system (Bio-Rad, USA).

2.6. Reverse transcription and Real-time PCR detection of miRNA

Total extracted RNAs from blood samples were analyzed by Cas14SDA and RT-qPCR. Thes solution containing RNA was diluted to 100 ng/μL with H_2O. The sequence of the stem-loop RT primer, real-time PCR primers and the procedure of qPCR were constructed in accordance with the reported miR-21 RT-qPCR assay. Firstly, the RT process of the miRNA21 was carried out with a volume containing 1 μL of total extracted RNA (100 ng/μL), 0.5 μL RT probe, 0.5 μL enzyme mix (RevertAid Master Mix), 5 μL RT buffer mix (RevertAid Master Mix) and 3 μL of H_2O. The mixture was incubated at 42 ℃ for 30 min, 95 ℃ for 5 min and finally preserved at 4 ℃. Next, the qPCR detection reaction was carried out by adding 2 μL RT products to the qPCR reaction mixture with 4 μL RT-qPCR-F (2.5 μM), 4 μL RT-

qPCR-R (2.5 μM), 10 μL Platinum™ SYBR™ Green qPCR SuperMix-UDG w/ROX. The qPCR reaction was carried out using QuantStudio®3 Real-Time PCR Instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA). The qPCR reaction was set 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min.

3. Results and Discussion

3.1. Design and working principle of the Cas14SDA assay

The Cas14SDA assay uses CRISPR-Cas14a to report the SDA reaction triggered by target miRNAs These steps have been illustrated in Fig. 1. The SDA only involves a single DNA strand serving as the template. The template contains three modules: the miRNA recognition site, the Cas14a activation site and a nicking site. The target miRNA can be identified with the template, resulting in an extension of the template with the involvement of Bst DNA polymerase. Following the polymerization, a DNA duplex which contained sequence-specific nicking sites of the nicking endonuclease, Nt.BstNBI was produced. The Nt.BstNBI cleaved the nicking sites of the DNA duplex, Cas14a activation sequences (termed activators) were then released from the template via the strand-displacement process induced by the polymerase. Then, the obtained activators can activate the trans-cleavage activity of Cas14a via specific hybridization with sgRNA, triggering the cleavage of the ssDNA reporter. The reporter ssDNA was terminally labeled with 6-FAM (F) fluorophore and BHQ1 (B) quencher. Once the 6-FAM-BHQ1 donor-quencher pair was destructed via Cas14a cleavage, the fluorescence

183 of 6-FAM fluorophore would restore. In this way, the presence of target miRNA was
184 transformed into fluorescence change which was measured using fluorometers. Hence,
185 using of Cas14a can identify SDA amplicon, thus increasing the specificity of SDA and
186 reducing the non-specific signal output.

187 We chose as biomarker of cholangiocarcinoma miR-21 as the target miRNAs for the
188 Cas14SDA assay. To confirm the working principle of the Cas14SDA assay for miRNA
189 detection, fluorescence measurement and gel electrophoresis analysis of its each step
190 were performed. The activation process of Cas14a was first tested.. In the absence of
191 activator, the fluorescence of the reporter remained low (Fig. 2B) because fluorescence
192 of 6-FAM was quenched by BHQ1 group. The addition of activator dramatically
193 increased fluorescence, indicating that the trans-cleavage activity of Cas14a was
194 activated to cleave the single-stranded reporter. For the SDA reaction, neither the
195 absence of Nt.BstNBI enzymes nor Bst DNA polymerase led to remarkable
196 fluorescence enhancement compared the absence of target of miR-21 (Fig. 2A). The
197 presence of miR-21 with Nt.BstNBI enzymes and Bst DNA polymerase contributed to
198 marked enhancement of fluorescence signal (from 2015 to 12881 emission intensities).

199 The SDA process was confirmed with electrophoresis analysis (Fig. 2C). The
200 reaction was initiated by miR-21 (Lane 1). It was hybridized with the template (Lane
201 2) to start the reaction. Incomplete double-stranded DNA/RNA duplex moved slowlier
202 than miR-21 and the template was formed (Lane 3). Subsequently, the SDA reaction
203 was carried out by the addition of Nt.BstNBI enzymes and Bst DNA polymerase (Lane

4 and Lane 5). The hybridization of miR-21 and the template could trigger the Bst DNA polymerase-mediated extension and there would appear a band lagging. Furthermore, the use of the nicking enzyme Nt.BstNBI made possible recognizing and cleaving DNA duplex at nicking sites to generate activators of Cas14a, which emerged as a band advancing. The bands of double-stranded template and activators were not formed in the absence of miR-21 (Lane 5). This result indicated that the miR-21 initiated the SDA reaction.

3.2. Optimization of assaying conditions

The cleavage time of the CRISPR-Cas14a system could substantially affect the fluorescence response of the Cas14SDA assay, thus it was optimized early in the development (Fig. 3A). The fluorescence intensity in the presence of miR-21 was significantly increasing with increasing the duration of the reaction until 40 min. The background fluorescence in absence of miR-21 only induced minor changes in when the duration of the cleavage was up to 80 min. Finally, 40 min was selected as the optimized CRISPR-Cas14a cleavage reaction time. Furthermore, fluorescence intensity also became affected by the concentration of reporter (Fig. 3B). Fluorescence intensities of both the negative group (absence of the miR-21) and the positive group (presence of miR-21) rose with an increased amount of reporter, and the maximum signal-to-background (S/B) ratio reached 7.13 using a ratio of 1:5 (Cas14 to reporter). Subsequently, the fluorescence intensity of the positive group grew slightly, while that of the negative group dramatically increased, resulted in a reduction of S/B ratio.

225 Therefore, Cas14a-to-reporter ratio was chosen as 1:5 serving as the optimized
226 experimental condition.

227 *3.3. Detection performance*

228 Different concentrations of miR-21 were added to individual reaction mixtures to
229 investigate the sensitivity of the proposed Cas14SDA assay under optimal experimental
230 conditions. Resulting fluorescence emission spectra are shown in Fig. 4A. Fluorescence
231 intensity gradually increased with increasing concentrations of miR-21. A good linear
232 relationship between the fluorescence intensity and the logarithmic (lg) value of miR-
233 21 concentration was obtained in the range of 0.68–50 pM (Fig. 4B). The detection
234 limit is estimated to be 680 fM based on 3σ/S calculation, where σ is the standard
235 deviation for the blank solutionand S is the slope of the calibration curve. The
236 regression equation was $Y = 625.89X + 2205.7$ ($R^2 = 0.9826$), where X and Y were the
237 logarithmic (lg) value of concentrations of miR-21 and the fluorescence intensity of the
238 Cas14SDA assay, respectively. These results showed that the Cas14SDA assay can be
239 a sensitive sensing platform for the detection of miR-21. The probe design was
240 relatively simple, and the assaying time was short (40 min) compared to other reported
241 methods for miRNA detection (Table S2).

242 The substitution of Cas12a with Cas14a can improve the sensitivity for miR-21
243 detection by 2.86 times (Fig. S1). This is because Cas14a can confer higher trans-
244 cleavage activity compared to Cas12a, by activating single-stranded DNA sequences.
245 One-step assay can shorten the analysis time and reduce the complexity of miRNA

246 detection. The feasibility of one-step assay that integrates the amplification process and
247 Cas12a cleavage was tested further. Positive samples resulted in lower fluorescence
248 using the one-step protocol compared to that using the two-step protocol (Fig. S2). The
249 one-step, mix-and-read assay can be optimized such as buffer condition of SDA and
250 Cas14a.

251 Differentiating among miRNAs is momentous for exploring the relationship between
252 human disease and miRNA biological functions. However, high similarity between
253 miRNA sequences and their short nature, make it a great challenge. To evaluate the
254 selectivity of the proposed method, the Cas14SDA system was exposed to different
255 miRNAs (miR-21-mis-1, miR-21-mis-3, miR-24, miR-141, miR-155, miR-192, miR-
256 378, let-7a). Among these miRNAs, the test group that included miR-21 had marked
257 fluorescence enhancement in contrast to the signal obtained with other interference
258 groups (with presence of other miRNAs) (Fig. 4C).

259 Following, the capacity of the Cas14SDA assay was tested for discriminating
260 miRNAs with single-base variation (Fig. 4C and D). The miR-21-mis-3 differed with
261 3-bases with respect to miR-21 and it yielded a signal within the background noise.
262 When the assay was carried out with miR-21-mis-1, which differs in 1-base with respect
263 to miR-21, it resulted in a fluorescence intensity half of that yielded by miR-21. The
264 linear template may not confer high capacity to resolve the base mutations just by the
265 recognition of DNA polymerase. Therefore a strategy to improve the discrimination
266 capacity of the assay for single-base variations in miRNA was attempted: we modified

the 3rd base from 3' of the template to incorporate one-base mismatch when hybridized with miR-21. In the design, microRNAs differing one-base compared to miR-21 would result in two base-mismatch when hybridized with the template. The incorporation of one-base mismatch slightly reduced the fluorescence of the assay response to miR-21. Remarkably, it cause an important reduction in the assay fluorescent response to single-base changed non-target microRNA (miR-21-mis-1(11)) (Fig. S3). Even the addition miR-21-mis-1(11) at 50 times the concentration of miR-21, the assay led to a fluorescence much lower than that in the presence of miR-21. These results indicate that the Cas14aSDA can be designed to discriminate miRNA family members differing with single-base via the optimization of the template.

3.4. Detection of circulating miR-21 in clinical samples

To further explore the potential of the Cas14SDA assay in detection of clinical samples and discriminate different expression levels of cholangiocarcinoma associated miRNAs, miR-21 was quantified in total RNA extracts from blood samples (Fig. 5). The samples containing total RNA were diluted to 100 ng/μL and used for their testing with the Cas14SDA assay. The fluorescence intensities are showed in Fig. 5A and Fig. S4A, and the samples were detected three times in parallel. These results indicated that miR-21 has different degrees of expression between healthy volunteers and patients with cholangiocarcinoma. The expression of miR-21 in the blood from cholangiocarcinoma patients was significantly higher than that from healthy volunteers. It is reported that miR-21 is overexpressed in cholangiocarcinoma[5,49]. In parallel,

the blood samples were analyzed by the RT-qPCR assay (Fig. 5B and Fig. S4B). The results of the Cas14SDA assay testing the eight samples were consistent with the results obtained from the RT-qPCR assay. The initial estimation of the Cas14SDA assay testing indicates its potential use for non-invade diagnosing cholangiocarcinoma.

4. Conclusions

A rapid, isothermal, and specific assay for detecting circulating miRNAs via integrating CRISPR-Cas14a with an isothermal amplification strategy has been developed. The assay termed, Cas14SDA, can detect miR-21 in one-test tube within 1 h, without needing reverse transcription process. Thus, the proposed assay is faster and simpler than the commonly used RT-qPCR method. CRISPR-Cas14a serves as the specific reporter of the SDA amplification of miRNAs, which makes it possible to identify miRNAs with similar sequences. This work has demonstrated that the Cas14SDA assay can detect miRNAs from blood samples and distinguish cholangiocarcinoma patients from healthy donors. Nevertheless, the potential use of the assay for early detection of cholangiocarcinoma occurrence should be tested with greater number of clinical samples. Considering its sensitivity, simplicity and rapidness, the Cas14SDA assay may facilitate the use of miRNAs for cholangiocarcinoma diagnosis.

Declaration of competing interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

https://ars.els-cdn.com/content/image/1-s2.0-S0003267022003348-mmc1.pdf

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Figure captions

Fig. 1. Schematic illustration of the Cas14SDA assay for detecting miRNA.

Fig. 2. Validation of the Cas14SDA assay for miR-21 detection. (A) Fluorescence analysis of miR-21 triggered SDA process; (B) Fluorescence analysis of activators triggered CRISPR-Cas14a activation; (C) Electrophoretic analysis of each step of the Cas14SDA assay.

Fig. 3. Optimization of the Cas14SDA assay. (A) Monitoring the fluorescence of the Cas14SDA assay in the presence and absence of miR-21. (B) The effect of the molar ratio of Cas14 to reporter on the detection performance of the Cas14SDA assay.

Fig. 4. Quantification and discrimination performance of the Cas14SDA assay for miR-21 detection. (A) Fluorescence curves of the Cas14SDA assay corresponding to the addition of the concentrations of miR-21 ranging from 0 to 10 nM (0, 500 fM, 1 pM, 5 pM, 10 pM, 50 pM, 100 pM, 500 pM, 1 nM, 5 nM, 10 nM); (B) Relationship between the concentration of total miR-21 and the fluorescence intensity; (C) Fluorescence response of the Cas14SDA assay towards different miRNAs (miR-21, miR-21-mis-1(8) (have one mismatch base at the 8th site from 5'), miR-21-mis-1(11) (have one mismatch base at the 11th site from 5'), miR-24, miR-141, miR-155, miR-192, miR-378, let-7a and miR-21-mis-3 (have three mismatch basics)) with a concentration of 10 nM. Statistical significances tested were obtained by two-tailed unpaired Student's t-test: ****, $P < 0.0001$. Inset Fluorescence response of the Cas14SDA assay towards miR-21 and miR-21-mis-1(11) using perfectly matched template and one base-mismatched

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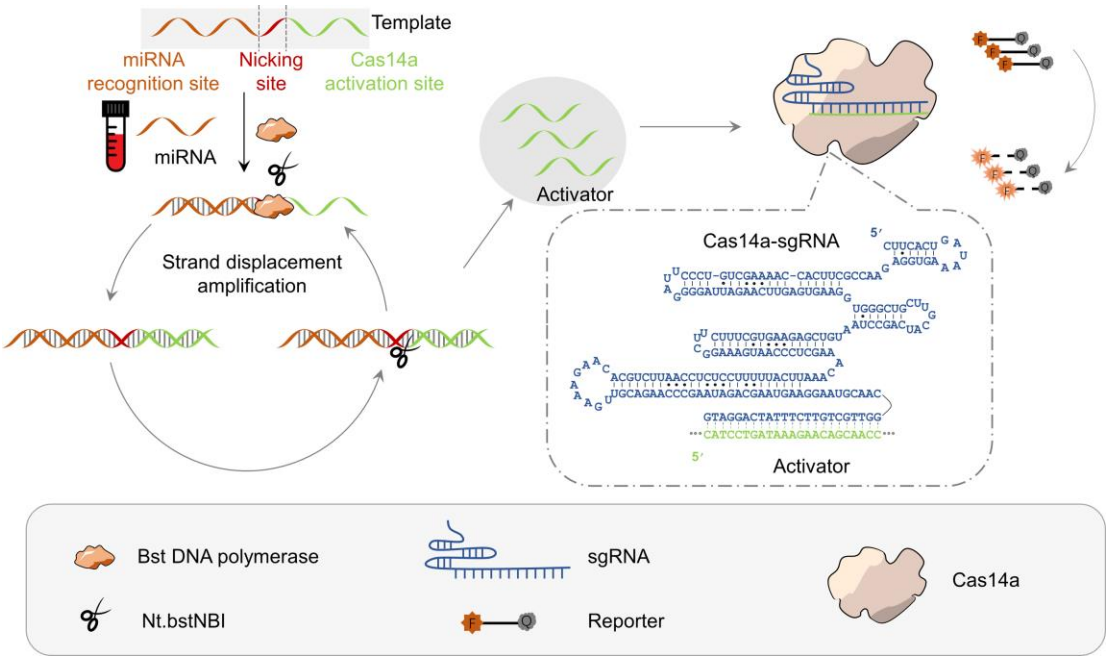
Fig. 5. Detection of miR-21 in clinical samples. (A) The fluorescence intensity of the Cas14SDA assay responding to blood samples from healthy donors and patients with cholangiocarcinoma; (B) The *CT* value of qPCR corresponding to blood samples from healthy donors and patients with cholangiocarcinoma.



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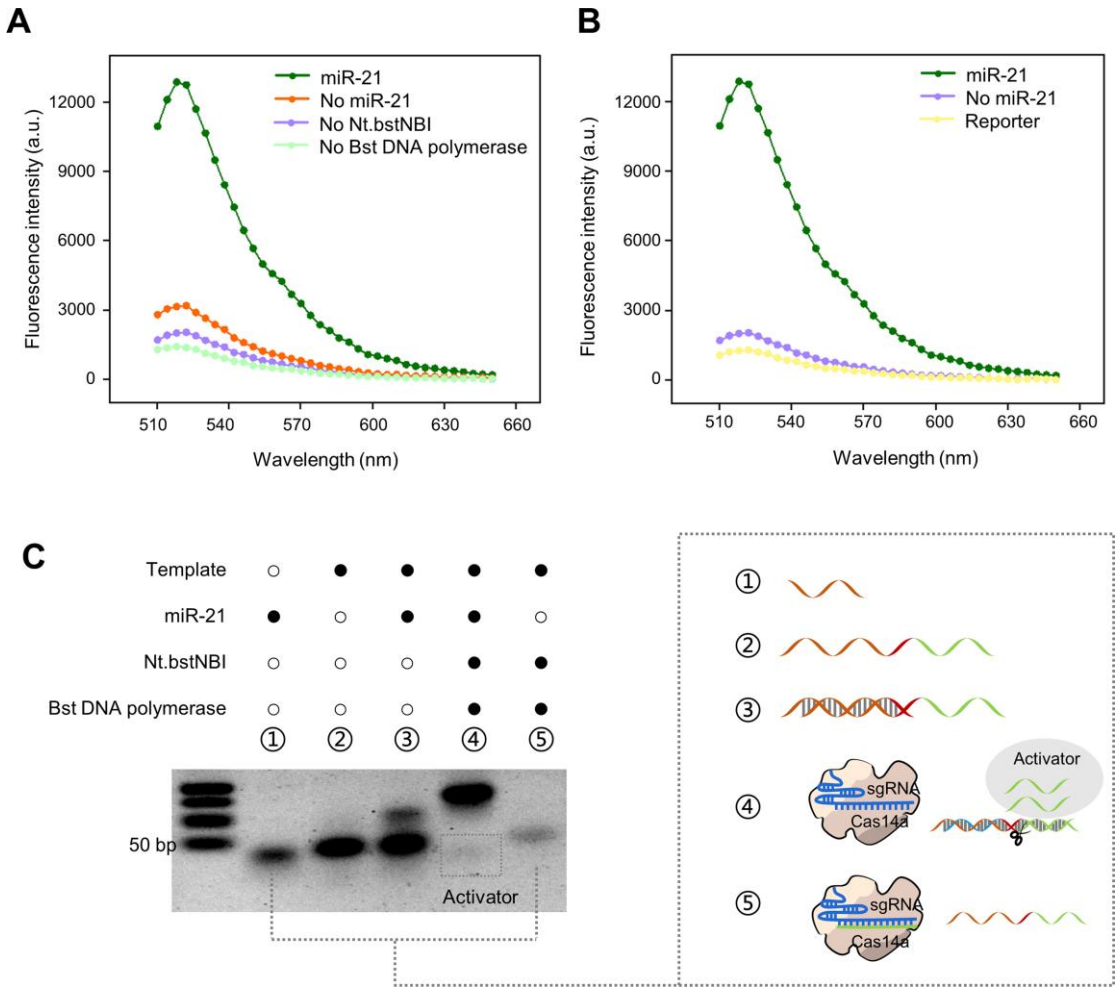

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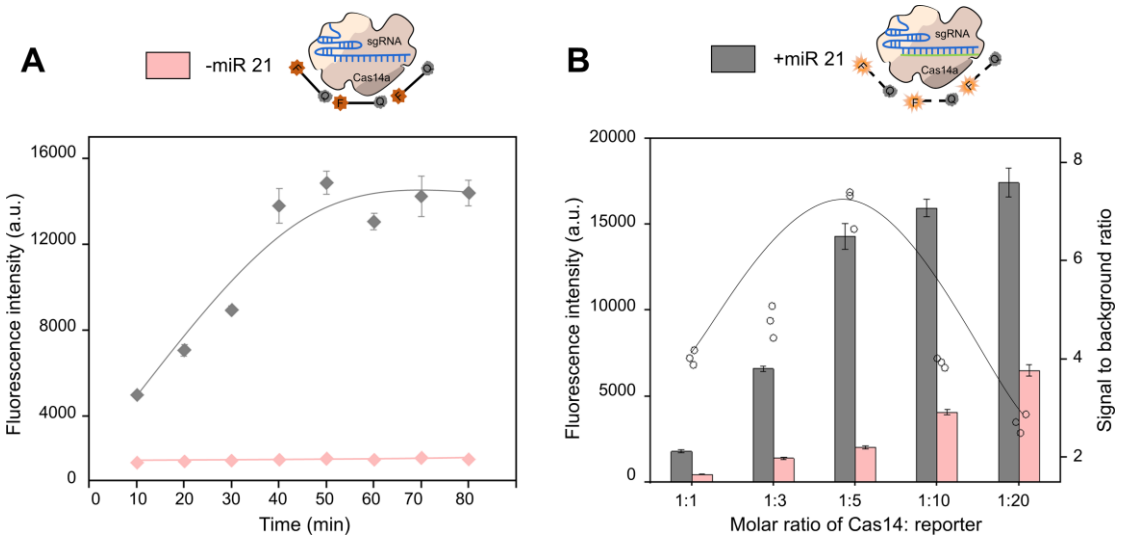

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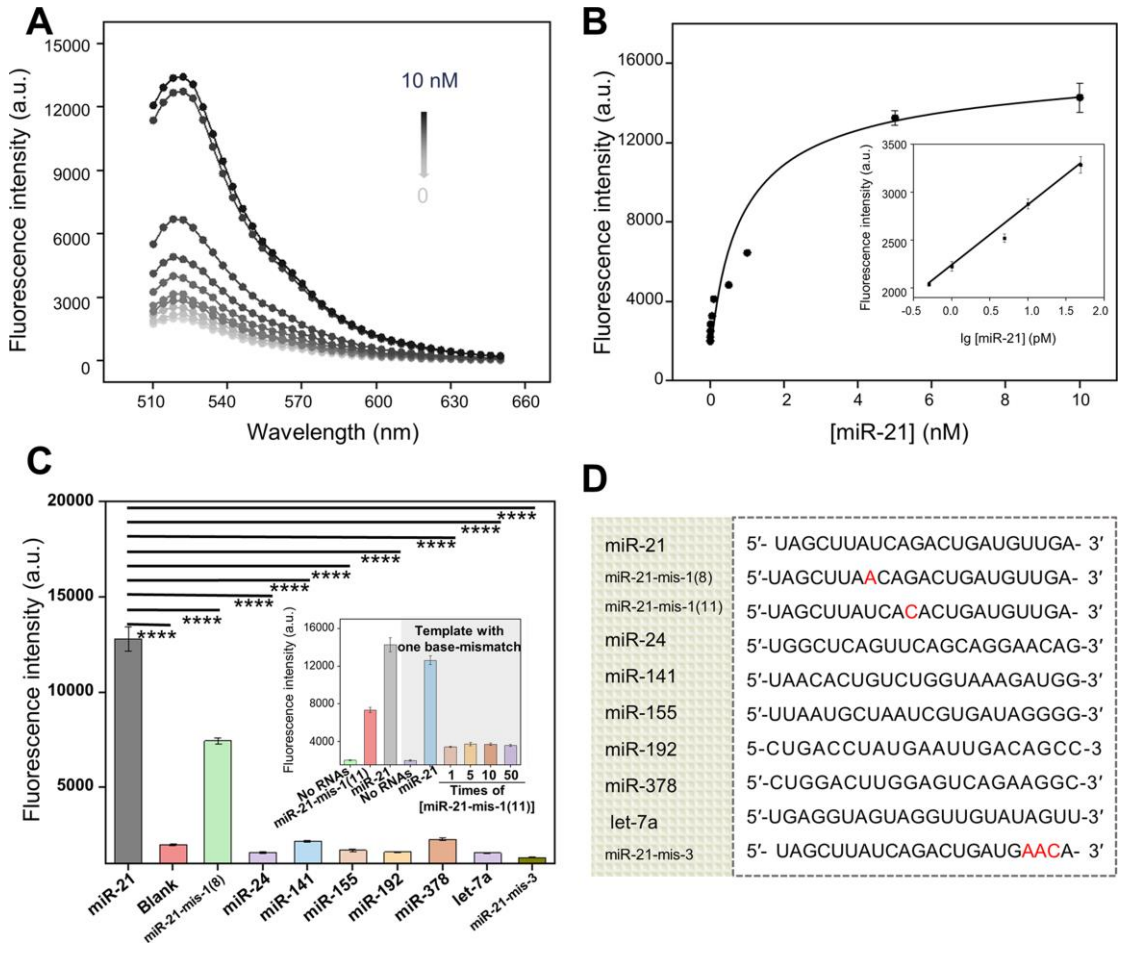

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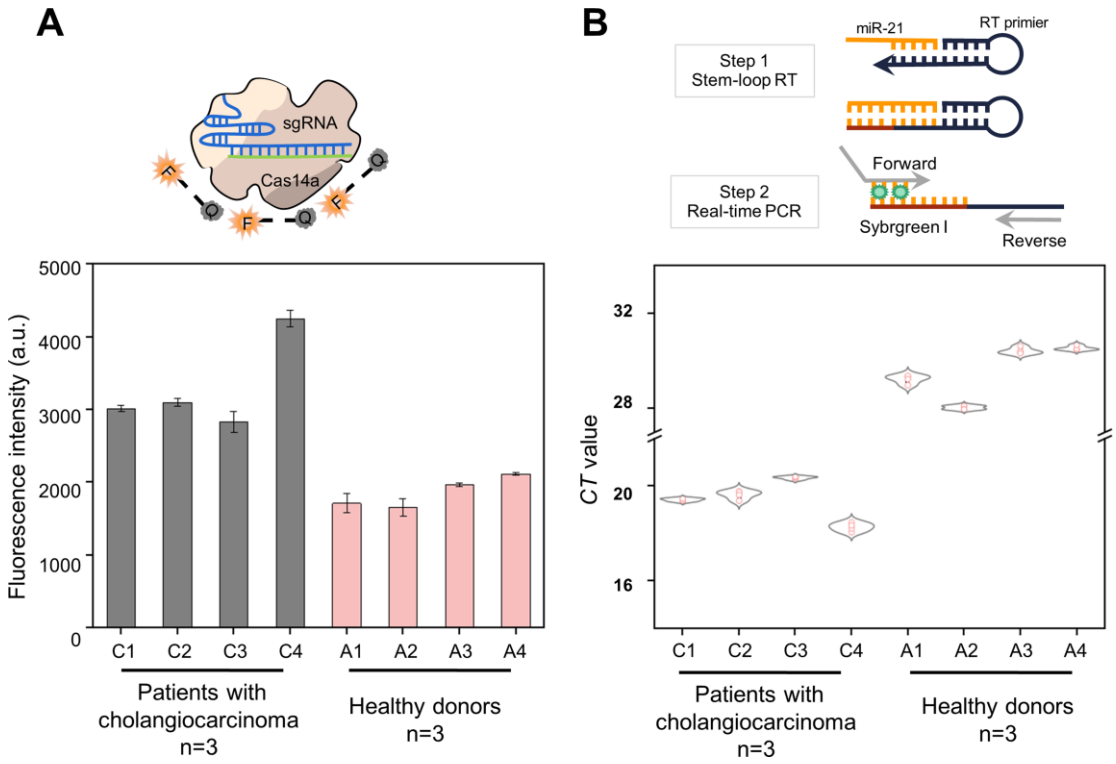

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