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Visual detection of SARS-CoV-2 with a CRISPR/Cas12b-based platform

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\textbf{A B S T R A C T}

The coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a global pandemic, highlighting the unprecedented demand for rapid and portable diagnostic methods. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) proteins-based platforms have been used for the detection of pathogens. However, in further applications and research, due to multiple steps needed, many methods showed an increased risk of cross-reactivity. The thermostable Cas12b enables the combination of isothermal amplification and CRISPR-mediated detection, which could decrease the risk of cross-contamination. In this study, we developed a portable and specific diagnostic method that combined the gold nanoparticle (AuNP) with thermal stable CRISPR/Cas12b-enhanced reverse transcription loop-mediated isothermal amplification (RT-LAMP), which is called SCAN, to distinguish the N gene of SARS-CoV-2 from flu gene. We validated our method using RNA from cells transfected by plasmids. We could easily distinguish the positive results by the naked eye based on the strong molar absorption coefficient of AuNP. Moreover, SCAN has the potential for high-throughput tests owing to its convenient operation. In sum, SCAN has broken the site and equipment restrictions of traditional detection methods and could be applied outside of hospitals and clinical laboratories, greatly expanding the test of COVID-19.

\textbf{1. Introduction}

Over the past two years, coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has emerged as a global pandemic \cite{1}. As of February 20, 2022, there have been over 422 million SARS-CoV-2-confirmed cases worldwide with over 5 million deaths \cite{2}. Although the vaccines and therapeutic agents have been developed, early detection of SARS-CoV-2 is still critical to prevent the infection and control the pandemic. Of note, real-time reverse transcription-polymerase chain reaction (RT-PCR) is still the gold standard of nucleic acid diagnosis due to its sensitivity and specificity \cite{3}. However, RT-PCR needs trained technicians and a thermal cycling controller, which has limited its onsite application.

Nowadays, the SARS-CoV-2 rapid antigen test has been applied widely all around the world, which is fast and convenient while with low sensitivity and specificity ($10^6$ copies/mL, 77.4–96.8% specificity) \cite{4}. Therefore, there is still an unprecedented need to develop detection methods that are rapid, convenient, sensitive, and portable to use at the point-of-care (POC).

As an alternative to the thermal cycling amplification, a plethora of isothermal amplification methods, such as recombinase polymerase amplification (RPA) \cite{5}, loop-mediated isothermal amplification (LAMP) \cite{6, 7}, and rolling circle amplification (RCA) \cite{8}, have been applied to detect the SARS-CoV-2 which could get rid of the need of expensive equipment. Recently, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein has

\begin{center}
\textbf{Abbreviations:} COVID-19, Corona Virus Disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RT-PCR, reverse transcription-polymerase chain reaction; POC, point of care; LAMP, loop-mediated isothermal amplification; RPA, recombinase polymerase amplification; RCA, rolling circle amplification; CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated; AuNP, Gold nanoparticle; TCEP, Tris(2-carboxyethyl) phosphine; DMEM, Dulbecco’s modified Eagle medium; TEM, transmission electron microscope; DEPC, Diethylpyrocarbonate.
\end{center}

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been applied to molecular diagnostics. These endonucleases guided by crRNA have the target-dependent cis- and trans-cleavage activities, resulting in a high-turnover signal amplification mechanism. Several Cas-based assays have been established, such as Cas12a-based DNA endonuclease-targeted CRISPR trans reporter (DETECTR) [9], Cas13a-based specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) [10], Cas12a-based 1-h low-cost multipurpose highly efficient system (HOLMES) [11]. These Cas-based methods were usually divided into two steps, target nucleic acid amplification and CRISPR recognition, which might bring a risk of cross-reactivity and false positive. Hereupon, numerous one-pot detection methods have been developed to cope with the inaccuracy caused by multistep reactions; Zhang’s group developed STOPCovid.v2 to detect SARS-CoV-2 with lateral-flow readout by combining RT-LAMP and CRISPR/Cas12b in one-pot [12]; J. S. Park et al. developed deCOVID to detect SARS-CoV-2 based on fluorescence which combined RT-RPA and CRISPR/Cas12a b in one-pot [13]; W. Feng et al. developed an integrated RT-RPA/CRISPR/Cas12a assay to detect SARS-CoV-2 with end-point visualization and fluorescence [14]. Most of these methods use fluorescence and microfluidic devices to display the results, which limit the wide application at POC.

The Cas12b which came from *Alicyclobacillus acidiphilus* has been reported to be more thermostable [15]. Compared with the previous methods based on Cas12a [16], the reaction temperature (31 °C–59 °C) of Cas12b protein is compatible with RT-LAMP to initiate the reaction in a homogeneous reaction system. This property could make the RT-LAMP and CRISPR/Cas12b detection be performed isothermally without sophisticated instruments and professional operators. Furthermore, the simplified operation process would make it possible to be an automatic platform.

Here, we developed a visual assay for the detection of SARS-CoV-2 N gene RNA by combining the gold nanoparticle (AuNP) with thermal stable CRISPR/Cas12b-enhanced RT-LAMP (SCAN). The visual detection was based on the strong molar absorption coefficient of AuNP [17]. AuNPs were pre-assembled with two different sulfhydryl DNA (DNA1 and DNA2). Since the sequence of linker-ssDNA was complementary to partial sequences of DNA1 and DNA2 at 5′ and 3′ ends respectively, the distance between the two AuNP probes was shortened resulting in a color change (Fig. 1). When the sample contained target RNA sequences, SARS-CoV-2 N gene RNA was reverse transcribed and amplified by RT-LAMP. The amplification product was then recognized by Cas12b/crRNA which could activate the trans-cleavage activity of Cas12b to cut linker-ssDNA non-specifically. Therefore, the AuNP probes remained mono-dispersed and red. In the absence of the target nucleic acid, the linker-ssDNA would not be cleaved by Cas12b, and the AuNP-DNA1/2 would be cross-linked by linker-ssDNA resulting in a color change from red to purple.
2. Materials and methods

2.1. Materials

AuNP was purchased from Suzhou Tanfeng Graphene Technology Co., Ltd. (Suzhou, Jiangsu, China). The crRNA (Table S1) was ordered from Sangon Biotech (Shanghai, China). NaCl was purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Tris(2-carboxyethyl)phosphine (TCEP) was obtained from Aladdin (Shanghai, China). AapCas12b was obtained from Magigen Biotechnology Co., Ltd. (Guangzhou, Guangdong, China). The crRNA (Table S1) was ordered from Bio-Lifesci (Guangzhou, Guangdong, China). Other nucleic acid buffer (2 M of NaCl, 200 mM of NaHPO₄·2H₂O and KCl were purchased from Beijing Chemical Works (Beijing, China). WarmStart® LAMP Kit (DNA & RNA), Isothermal Amplification Buffer Pack, WarmStart® RTx Reverse Transcriptase, Bst 2.0 WarmStart® DNA Polymerase, and MgSO₄ were obtained from New England Biolabs (Beijing, China). Thermo Fisher Scientific (Shanghai, China). COVID-19 RNA reference material and the RNA from transfected cells, 8.5 μL of target RNA (different concentrations of KCl (0, 25, 50, 100 mM) for 10 min at 37 °C. The assays were performed with serial dilutions of dsDNA, 400 nM reporter DNA, 1 × buffer, 31.25 nM AapCas12b, and 31.25 nM crRNA in a total volume of 25 μL. The real-time fluorescence was detected by a qPCR machine every 1.5 min at 60 °C.

2.5. The optimization of the CRISPR/Cas 12b detection by fluorescent assay

To optimize the Cas12b assay, the dsDNA was used to verify the feasibility of the Cas12b/crRNA detection. Firstly, Cas12b was pre-assembled with crRNA in one × buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 50 mM KCl, 2 mM MgSO₄, 0.1% Tween-20, pH 8.8) for 10 min at 37 °C. The assays were performed with serial dilutions of dsDNA, 400 nM reporter DNA, 1 × buffer, 31.25 nM AapCas12b, and 31.25 nM crRNA in a total volume of 25 μL. The real-time fluorescence was detected by a qPCR machine every 1.5 min at 60 °C.

2.6. Cell culture and RNA extraction

293 T cells (a human renal epithelial cell line, obtained from the American Type Culture Collection, No CRL-11268) was cultured with Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin in a complete humidity incubator with CO₂/air (5%/95%) at 37 °C. 293 T cells were seeded into 6-well plates at the density of 3 × 10⁵ cells/well and then transfected by a plasmid with the N gene of SARS-CoV-2 at a dose of 2 μg per well, the negative group was set to be transfected by the plasmid without the N gene of SARS-CoV-2 at the same dose. The RNA was then extracted using an RNA Purification Kit according to the manufacturer’s instructions and the concentration was measured by a micro UV spectrophotometer.

2.7. RT-LAMP and CRISPR detection

The amplification of SARS-CoV-2 RNA was performed with the WarmStart® LAMP Kit (DNA & RNA) according to the manufacturer’s instructions. Briefly, the reaction, contained 12.5 μL of 2 × WarmStart® LAMP Master Mix reaction premix, 2.5 μL of 10 × primers mixture, 0.5 μL of 50 × fluorescent dye, and 1 μL of target RNA (different concentrations of COVID-19 RNA reference material and the RNA from transfected cells), 8.5 μL of water, was performed using a real-time PCR machine at 65 °C. Then, 2.5 μL of the amplification product was used to activate the Cas12b for fluorescent or visual assay. The fluorescence assay was performed as described above. For visual detection, the reporter DNA was replaced by linker-ssDNA, and the premixed AuNP-DNA1/2 solution was added to the reaction system at the ratio of 50: 1: 1 (linker-ssDNA: AuNP-DNA1: AuNP-DNA2) when the Cas12b/crRNA detection finished (30 min, 60 °C).

2.8. Gel electrophoresis

The RT-LAMP amplification products were then tested and verified using 2% agarose gel electrophoresis. Images were photographed by a gel Imaging System.


2.9. SCAN assay

The SCAN reaction master mix consisted of the following components: 1 × Isothermal Amplification Buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 50 mM KCl, 2 mM MgSO₄, 0.1% Tween-20, pH 8.8), 1.4 mM dNTPs, 8 units of Bst 2.0 WarmStart® DNA Polymerase, 7.5 units of WarmStart® RTx Reverse Transcriptase, 100 nM Cas12b protein, 100 nM crRNA, 400 nM fluorescent reporter, 0.8 μM FIP/BIP primers, 0.2 μM F3/B3 primers, 0.2 μM LoopF/B primers and 8 mM MgSO₄. The detection was performed at 60 °C in a qPCR machine with fluorescent measurements every 1.5 min. For visual detection, the whole reaction was performed at 60 °C on a metal bath for 2 h, then the premixed AuNP-DNA1/2 solution was added to the reaction system as described above, and the phenomenon was recorded by a mobile phone.

2.10. High-throughput assay

A 96-well plate was used to detect the N gene of SARS-CoV-2. For each component reaction of SCAN, the 96-well plate was then incubated in a well plate incubator at 65 °C for 30 min (RT-LAMP reaction) and 60 °C for 30 min (CRISPR/Cas12b detection). For the SCAN assay, the 96-well plate was incubated in a well plate incubator at 60 °C for 120 min. Then the premixed AuNP-DNA1/2 solution was added to the 96-well plate after reaction in the visual detection group. The color change was observed and the absorbance of the mixture was detected at 520 nm and 560 nm using a microplate reader.

3. Results

3.1. The visual detection based on AuNPs

To demonstrate the utility of the AuNPs-based visual detection, the AuNPs were modified by two different sulfhydryl DNAs (v-DNA1 and v-DNA2), which could be cross-linked by a linker-ssDNA. The solution of AuNP-DNA1/2 was red in the absence of linker-ssDNA, and the color changed from red to purple in 5 min when the linker-ssDNA was mixed with pre-assembled AuNP-DNA1/2 due to the shortened distance between AuNPs (Supplementary Fig. 1A). The photograph of TEM has confirmed that the color change was caused by agglomerated AuNPs in the presence of linker-ssDNA (Supplementary Fig. 1B).

3.2. The optimization for CRISPR/Cas12b assay

To reduce the risk of cross-reactivity and increase the possibility of application at POC, the SCAN assay was designed to combine the RT-LAMP with CRISPR-mediated detection. It is necessary to optimize the reaction conditions which could satisfy the need for each reaction (Fig. 2A). The trans-cleavage activity of CRISPR/Cas12b was evaluated by a fluorescent assay using the synthetic dsDNA as the target. As shown in Fig. 2B, Cas12b could be activated by target dsDNA at concentrations of at least 1 nM and 10 nM dsDNA was chosen for the following optimization. To acquire stable and efficient reaction conditions, we next optimized the reaction buffer in which RT-LAMP and CRISPR-mediated detection could occur simultaneously. The isothermal amplification buffer of RT-LAMP was used as a basis for the optimization because it has a similar composition to the buffer for CRISPR/Cas12b detection. It was shown that 50 mM KCl was the most efficient concentration for the Cas12b assay system (Fig. 2C). For Mg²⁺, the best signal quality was obtained at 10 mM and 20 mM (Figs. 2D), and 10 mM MgSO₄ was chosen as the final reaction concentration considering costs. Since the most suitable temperature for Cas12b to maintain the highest trans-cleavage activity is from 31 to 59 °C according to the previous report [15], and the optimum reaction temperature for RT-LAMP is 60–65 °C, we then investigated the temperature of the CRISPR/Cas12b assay from 50 to 65 °C with an interval of 5 °C. Notably, the best trans-cleavage activity of Cas12b could be observed at 55 °C (Fig. 2E). In addition, the Cas12b at...
50 and 60 °C also showed strongly trans-cleavage activity, but dropped significantly at 65 °C. Considering the optimum temperature of RT-LAMP, we selected 60 °C as the final temperature for the further experiment.

### 3.3. Establishment of SCAN assay

The diluted COVID-19 RNA reference materials were used to validate the SCAN assay. We performed the assay step by step, containing RT-LAMP, CRISPR/Cas12b activation, and visual detection. First, flu virus RNA was used as the negative control to demonstrate the feasibility of RT-LAMP. The amplification curve of serially diluted SARS-CoV-2 N gene RNA from $4 \times 10^3$ copies/μL to $4 \times 10^9$ copies/μL rose sharply and plateaued in 20 min (Fig. 3A), and the amplification products were further confirmed by gel electrophoresis (Supplementary Fig. 2), indicating the SARS-CoV-2 RNA could be successfully amplified by RT-LAMP. We next tested if the amplification products could activate the trans-cleavage activity of Cas12b. As shown in Fig. 3B, the increased fluorescence could be observed in the presence of the amplification products, indicating the amplification products were specific. Finally, a difference in visual signal could be noticed between SARS-CoV-2 and flu virus (Fig. 3C).

![Image](image_url)
applications. As shown in Fig. 3D, the positive and negative samples were successfully discriminated by color change. The NTC and negative group turned to light purple color and the positive group ($4 \times 10^0$ to $4 \times 10^3$ copies/μL) maintained pink color. The ratio of A520/A560 at $4 \times 10^0$ copies/μL of target RNA was consistent with the visual results above (Fig. 3E). Collectively, these results indicated that the target RNA could be successfully detected by multistep SCAN.

### 3.4. The sensitivity of the SCAN assay

Since all component reactions in the SCAN assay have been verified and the reaction conditions have been optimized, we combined the RT-LAMP and CRISPR/Cas12b detection in one tube to simplify the workflow. First, the sensitivity and reliability of the one-step SCAN assay were evaluated. The results showed that 40 copies/μL of COVID-19 RNA reference material could be detected in 90 min by fluorescent assay (Fig. 4A). In contrast, 400 copies/μL could be distinguished against the negative control with a visual assay (Fig. 4B). We also evaluated the sensitivity using a well plate incubator (Fig. 4C). The 96-well plate results correlated well with those observed by the naked eye in tubes, briefly, 400, 1000, and 4000 copies/μL RNA showed an obviously pink color and the other groups turned to purple color (Fig. 4D and E). Collectively, these results showed that the RNA of SARS-CoV-2 could be detected specifically using the one-step SCAN assay.

### 3.5. Detection of RNA from transfected cells using SCAN assay

We have confirmed that the COVID-19 RNA reference material could be successfully detected by the SCAN assay. Then we evaluated the clinical feasibility of the SCAN assay by using the cells transfected by a plasmid with the N gene of SARS-CoV-2 to simulate the clinical samples. First, the RNA was detected by a multistep SCAN assay that we performed RT-LAMP, CRISPR/Cas12b detection, and visual detection separately to confirm the feasibility (Supplementary Fig. 3A). Consistent with the fluorescence results (Supplementary Fig. 3B), the presence of purple was observed by the naked eye in tubes (Supplementary Fig. 3C), indicating that the RNA from cells could be detected using a multistep SCAN assay. The color change in the 96-well plate (Supplementary Fig. 3D) and the increase of A520/A560 (Supplementary Fig. 3E) also suggested no cross-reactivity with the negative control group.

Next, we performed the one-step SCAN assay directly to detect SARS-CoV-2 RNA extracted from transfected cells, which were serially diluted.
The fluorescent results (Fig. 5A) and visual detection results showed that our assay could distinguish positive samples when the concentration of RNA was more than 1 ng/μL (Fig. 5B). Finally, to demonstrate the feasibility of the assay for high-throughput assay and clinical application, we employed the assay to detect the RNA samples in the well plate. We observed a notable difference in color from pink to purple and the ratio of A520 to A560 between the RNA concentration of more than 1 ng/μL and other groups (Fig. 5C, Supplementary Fig. 4). Since the high-throughput reaction could be performed using a metal bath or a well plate incubator, it suggested the potential POC application.

4. Conclusion

In this study, we have developed a SCAN assay to detect SARS-CoV-2 in both visual-based and fluorescence-based platforms. The RT-LAMP and Cas12b detection are combined in one step owing to the heat-resistant activity of Cas12b. SCAN assay can satisfy several requirements of POC diagnosis. First, the procedure of SCAN is simple, that the RT-LAMP and CRISPR/Cas12b detection were performed in one tube to simplify the whole operation. Second, the SCAN assay is portable with inexpensive equipment, and the whole assay only relies on a metal bath. Furthermore, the results of the SCAN can be easily distinguished by naked eye due to the strong molar absorption coefficient of AuNP. Most important of all, the SCAN assay has a high-throughput potential to detect enormous amounts of virus samples simultaneously since the assay is isothermal and easy to be performed. To test the performance of SCAN in clinical settings, we used the extracted total RNA from cells transfected by SARS-CoV-2 to simulate the real extracted RNA, which showed good specificity and sensitivity. Given many RNA extraction-free methods have been developed to combine with RT-LAMP to detect SARS-CoV-2 [19,20], we expect the SCAN assay could be useful for diagnosis of COVID-19 at POC tests. In short, this method which is based on CRISPR/Cas12b and AuNP has the potential to assist in the rapid diagnosis and screening of patients with COVID-19.

Author statement

Yaqin Zhang: Investigation, Methodology, Visualization, Validation, Formal analysis, Writing - Original Draft. Xiangyu Quan: Methodology, Validation, Investigation. Yingchun Li: Methodology, Validation, Investigation. Hangyu Guo: Validation, Investigation. Fange Kong: Methodology, Validation, Supervision, Writing - Review & Editing. Jiali Li: Validation, Data Curation. Lirong Teng: Methodology, Visualization. Jiasi Wang: Conceptualization, Visualization, Supervision, Writing - Review & Editing, Di Wang: Conceptualization, Visualization, Writing - Review & Editing, Funding acquisition.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2022.124093.
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