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Multiplexed lateral flow assay integrated with orthogonal CRISPR-Cas system for SARS-CoV-2 detection

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1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic has posed great threat to the global healthcare systems and human lives over the world [1–3]. To control the disease transmission, reliable and efficient diagnostic technologies for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) highlighted its urgency [4]. As a single-stranded RNA (ssRNA) virus, reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) is the golden standard [5,6]. Whereas the high false negative rate derived from the sampling as well as requirement of well-trained personnel and expensive instrument remained to be improved. Moreover, the long turnaround time also made it impractical for popularity in resource-limited settings [7,8]. Therefore, the development of accurate and field-deployable SARS-CoV-2 detection methods is in an urgent unmet need.

As a complement to RT-PCR, the isothermal amplification-based platform without the requirement for a thermal cycler offers better accessibility [9]. The loop-mediated isothermal amplification (LAMP) [10] and recombinase polymerase amplification (RPA) [11] are the most widely exploited techniques. On the other hand, the development of clustered regularly interspaced short palindromic repeats (CRISPR) technology has opened a new era for nucleic acids detection [12–14]. The CRISPR-associated (Cas) protein is able to unleash the nuclease activity to cleave substrates indiscriminately after target binding, which endows it great sensitivity in nucleic acid detection [15]. By coupling the isothermal amplification with CRISPR system, various nucleic acids detection methods have been proposed [16–19]. Typically, by combining the trans-cleavage ability of Cas12a with RPA, Doudna et al. created DETECTR for human papillomavirus detection [20]. Zhang group, on the other hand, utilized the RNases property of Cas13a, established the SHERLOCK platform [21]. Recently, our group proposed Cas12a mediated colorimetric assay for SARS-CoV-2 detection [22]. In the effort to avoid the reliance on fluorescence equipment and achieve better POCT feasibility, lateral flow assays are further combined with

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proposed CRISPR platforms for visual readout [23–25]. Albeit the significant progress in developing field-deployable lateral flow strips for SARS-CoV-2 detection, the readout capability is usually constrained to 1 gene per test, which severely impacts the accuracy and efficiency of the results [26–28]. During the diagnosis of COVID-19, multiplexed gene detection are recommended (for example, ORF1ab and N genes) to avoid the false negative or positive results caused by gene degradation or amplification errors [29,30]. Thus, the development of multiplexed and portable platform for SARS-CoV-2 detection will be of great help to facilitate the improvement of POCT reliabilities.

Isothermal amplifications through employing two primer sets can realize multiplexed gene targets amplification. By coupling with nanoparticle-based lateral flow assay, both the targets can be visualized on the strips [31–34]. Whereas the non-specific amplification and aerosol pollution are intrinsically prone to cause false positive results. When coupling with CRISPRP strategy, under the dual variations inspecting from the isothermal amplification and sgRNA guided activation process, the false positive events can be effectively avoided and hence improving the specificity and accuracy significantly [35–38]. By combining with RT-RPA, Zhou group developed a CRISPR-Cas9 mediated dual-gene detection platform through making a trade-off between sensitivity and POCT potential and the results can be observed in a single strip test [39]. Relying on the specific target activation and substrate cleavage preferences of Cas enzymes, Zhang et al. developed SHERLOCKv2 with four-channel signal readout [40]. Notably, although the orthogonal CRISPR-Cas strategy has been proposed, the multiplexed results can only be obtained through fluorescent instruments.

Herein, an orthogonal CRISPR-Cas-mediated multiplexed lateral flow assay (designated as OC-MLFA) was proposed for SARS-CoV-2 genome detection. It has been reported that Cas12a can be activated by DNA and then exhibit indiscriminate single-stranded DNase activity [41]. On the other hand, Cas13a showed collateral RNase activity towards ssRNA substrates once binding with RNA target [42]. Utilizing these activation and cleavage preferences, we could detect ORF1ab and N genes of SARS-CoV-2 genome simultaneously in the same reaction solution with orthogonal Cas proteins. The orthogonal system will yield two-independent-channel signals without any interfere with each other. The preamplification products from multiplexed RT-RPA can then be analyzed directly with the Cas12a/Cas13a system without any purification step. Lateral flow strips with two target lines were designed to accommodate the dual-channel readout CRISPR system. The interference between Cas12a and Cas13a channels was effectively eliminated via the rational substrates design, which was further validated by both native PAGE, fluorescence, and lateral flow assay. Clinic SARS-CoV-2 samples were analyzed to verify the reliability of the established method. With great accuracy and efficiency, the OC-MLFA platform made a promising alternative for field-deployable POCT application.

2. Experimental section

2.1. Multiplexed RT-RPA preamplification of ORF1ab and N genes

Multiplexed RT-RPA for simultaneous SARS-CoV-2 ORF1ab and N gene was realized using a commercial RPA kit. In the presence of dual primer sets (ORF-F, ORF-R, N-F, N-R, 400 nM each), 1 µM RNase inhibitor (0.8 U/µL), 1 µM ProtoScript® II Reverse Transcriptase (4 U/µL), 10 µM NTP mixture (2 mM), 1 µM T7 RNA Polymerase (1 U/µL), 1 µM DTT (2 mM) and 29.4 µM primer sets (ORF-F, ORF-R, N-F, N-R, 400 nM each), 10 µL RNase free water was added to the RPA Basic kit. Then 1 µL different concentration of SARS-CoV-2 standard substance was added. At last, 2.5 µL MgOAc (280 mM) was added to initiate the amplification process. After incubation at 37 °C for 20 min, the yielding products were further analyzed with orthogonal CRISPR-Cas system. In terms of pseudovirus sample, RNA was extracted according to the RNA extraction experimental procedure before subjected to the RT-RPA process same as the SARS-CoV-2 RNA standard sample. The oligonucleotides details were listed in Table S1.

Fig. 1A was the schematic illustration of the OC-MLFA strategy. The procedure was typically divided into 3 steps: preamplification, orthogonal CRISPR activation and lateral flow strips visualization. To realize the orthogonal CRISPR-Cas output, the ORF1ab and N genes were selected as the targets of Cas12a and Cas13a, respectively. Two pairs of RPA primers were designed for preamplification. The SARS-CoV-2 ssRNA genome was extracted from the virus particles and then underwent multiplexed RT-RPA process to get the dsDNA products. As for the ORF1ab region, the products contained overall four functional regions (Fig. S1): a forward primer, protospacer adjacent motif (PAM), sgRNA-ORF1ab binding region and reverse primer. The primer regions were essential for amplification, while the PAM and RNA binding regions were indispensable for Cas12a activation [43]. As Cas13a activation did not require PAM, there were only three regions contained in the N target region. And a T7 promoter was included in the primer region for subsequent transcription, which would turn the dsDNA into ssRNA target to activate Cas13a.

After isothermal preamplification, the yielding products were utilized for orthogonal CRISPR activation. Cas12a was demonstrated to hydrolyze dsDNA in a nonspecific manner. To couple with the lateral flow assay, a novel half-blocking dsDNA substrate was designed for Cas12a for the first time, which was modified with digoxin (Dig), biotin and FAM groups in the strands’ terminals, respectively. After activation, Cas12a cleaved the single strand sequence off the substrate, thus producing double stranded Dig/FAM products. As Cas13a selectively hydrolyzed ssRNA substrate, biotin and TAMRA were modified at the ssRNA substrate terminals. Using the substrate specific cleavage preferences, Cas12a and Cas13a were able to respond independently after activation.

Lateral flow strips with two target lines (T lines) and a control line (C line) were designed to accommodate the orthogonal CRISPR output signals. As illustrated in Fig. 1B, the flow direction was from the sample pad to the absorbent pad. Anti-rat FAM antibody conjugated AuNPs and
anti-rabbit TAMRA antibody conjugated AuNPs were pre-embedded on the conjugate pad. Streptavidin, anti-digoxin antibody and anti-rabbit secondary antibody were embedded on the C, T1 and T2 lines, respectively. The above orthogonal CRISPR reaction products were dropped onto the sample pad, where the substrates can bind antibody conjugated AuNPs. Intact substrates will be hold at C line via the biotin-streptavidin interaction. Cleaved dsDNA substrates with Dig/FAM will stay at the T1 line due to the recognition of anti-digoxin antibody. The cleaved ssRNA substrate with TAMRA modification will be bound at T2 line along with the AuNPs. Eventually, the red band can be visualized owing to the AuNPs accumulation. Only C line was shown when both the DNA and RNA substrates were intact (negative). C line and T1 line will be visualized if only the half-blocking DNA substrate was cleaved (ORF1ab positive, N negative). C line and T2 line meant the Cas13a was activated (ORF1ab negative, N positive). If the T1 and T2 lines appeared simultaneously, both the ORF1ab and N genes were positive. The C line can act as an indicator of strips quality and be a way for validation of strips’ results. If C line failed to appear after incubation with intact DNA and RNA substrates, the results were invalid. Thus, the diagnosis results of SARS-CoV-2 could be estimated from the strips directly.

3.2. Verification of the RT-RPA integrated orthogonal CRISPR-Cas system

As a proof of concept, the corresponding target regions of ORF1ab and N genes were employed to characterize the specific collateral cleavage property of the Cas proteins. Fig. 2A illustrated the Cas12a mediated. ssDNA substrate cleavage process after activation by ORF1ab targets. The PAGE results in Fig. 2B demonstrated that in the presence of dsDNA target, the ssDNA substrates were degraded completely (lane 3). Whereas, the ssRNA substrate remained intact (lane 6), indicating that the Cas12a selectively cleaved ssDNA after activation. It was worth noting that the target band in lane 3 (Fig. 2E) disappeared due to the degradation of activated Cas13a. The dsDNA target for Cas12a maintained (lane 3, Fig. 2B) because the dsDNA cannot be hydrolyzed by Cas12a. The increased FAM signal for fluorescence measurement (Fig. 2C) also confirmed the ssDNA cleavage preference for Cas12a. We then attempted to investigate the substrate preference of Cas13a with the two kinds of substrates (Fig. 2D). As shown in Fig. 2E, only the ssRNA substrate band disappeared in the PAGE image (lane 3), which can be ascribed to the completely degradation by Cas13a. The increased fluorescent signal from Cy5 also verified the ssRNA cleavage after Cas13a activation (Fig. 2F). The PAGE and fluorescence results above confirmed our hypothesis that the specific substrate preferences of the Cas proteins rendered the orthogonal CRISPR system adequate ability to eliminate the cross-interference and thus providing individual two-channel signal readouts.

Doudna et al. demonstrated that Cas12a cleaved ssDNA other than dsDNA substrates once binding with targets [20]. Thus inspired, we designed the half-blocking dsDNA substrate for the OC-MLFA system (Fig. 2G). As per our hypothesis, the single stranded part would be cleaved while the double strand region remained intact. Fluorescence measurement was conducted to validate the feasibility of the half-blocking substrates. The two strands were modified with Cy5 and FAM/BHQ1, respectively. We found that without target activation, neither the fluorescence signals at 520 nm (FAM) nor the 660 nm (Cy5) could be detected. In the presence of 10 nM target, only the FAM signal increased due to the Cas12a mediated cleavage (Fig. 2H), validating only the single stranded segment modified with FAM was cleaved. The decreased Cy5 signal in Fig. 2H may be due to the increased BHQ1 quenching efficiency after FAM cleavage. The cleavage site preference of Cas12a towards the half-blocking substrate confirmed its capability to couple with the subsequent lateral flow strips.

The RT-RPA efficiency was also evaluated via the Cas proteins mediated fluorescent signals utilizing SARS-CoV-2 RNA standard samples. The virus RNA genome underwent multiplexed RT-RPA in the presence of both ORF1ab and N primer pairs before subjected to subsequent fluorescent analysis. The orthogonal CRISPR-Cas system containing Cas12a and Cas13a simultaneously were employed herein to characterize the RT-RPA products, which have been verified to yield individual two-channel fluorescent signals towards substrates including half blocking dsDNA and ssRNA substrates (Fig. 2I and J). The
Multiplexed RT-RPA feasibility was first validated by native PAGE. We can observe both the dsDNA amplicons for ORF1ab and N genes in lane 7 (Fig. S2) in the presence of two sets of primers and SARS-CoV-2 RNA. Fluorescence measurement was then conducted. As illustrated in Fig. 2 K and L, after the multiplexed isothermal amplification, both ORF1ab and N genes amplicons yielded significantly increased and concentration-dependent fluorescence signals via orthogonal Cas12a/Cas13a activation. On the other hand, the signals before amplification stayed low and irrelevant with copy numbers. While the ORF1ab gene amplicon can activate Cas12a directly, the Cy5 signals resulting from ssRNA degradation in Fig. 2 L demonstrated that the RT-RPA products of N gene were effectively transcribed into ssRNA target with the guidance of T7 promoter prior to activating Cas13a.

3.3. Feasibility evaluation of the proposed OC-MLFA

To realize the goal of multiplexed visual readout, lateral flow strips with two target lines were designed to accommodate the orthogonal CRISPR-Cas system. Intact half-blocking dsDNA and ssRNA substrates accumulated at the first line (C line). Cleavage of the substrate would result in the visualization of second (T1 line) and third lines (T2 line). Firstly, the feasibility of the designed strips was tested with orthogonal CRISPR-Cas system triggered with different targets (Fig. 3A). In the absence of SARS-CoV-2 genome targets, only C lines appeared. The presence of ORF1ab target yielded C and T1 lines, while C and T2 lines were seen for N target. And we observed the whole three lines if ORF1ab and N gene targets were present simultaneously. Results above validated that the T1 and T2 lines in our designed lateral flow strips were specific for ORF1ab and N genes without any cross-interference.

We then evaluated the lateral flow results of RT-RPA amplicons. SARS-CoV-2 RNA standard or pseudovirus amplicons from multiplexed RT-RPA were subjected to lateral flow strips directly without orthogonal CRISPR-Cas system. We could infer from Fig. 3 B that neither of the amplification products would lead to target lines appearance. The results validated that only after the Cas mediated substrates cleavage could the strips lines be visualized.

At last, the feasibility of RT-RPA integrated orthogonal CRISPR-Cas detection system was investigated with the lateral flow assay. The isothermal amplicons of SARS-CoV-2 RNA standard or pseudovirus sample (Fig. 3 C) were added to the CRISPR-Cas system for orthogonal substrates cleavage. Similar to the results with pure target strands in Fig. 3A, the three lines of strips can only be visualized under the circumstance of both ORF1ab and N gene primer pairs were present. And no cross-interference was observed between the two genes. Notably, although the nonspecific amplification or primer dimer interference was inevitable for RT-RPA, we did not find any false positive results for all
The RT-RPA followed by CRISPR-Cas evaluation. SARS-CoV-2 RNA and pseudovirus standard samples were employed here. The samples were amplified through RT-RPA in the presence of different primers both 100 nM. (B) RT-RPA results without CRISPR cleavage. SARS-CoV-2 RNA strip intensity and heat map analysis. (A) Orthogonal CRISPR-Cas mediated multiplexed targets detection with single strip colorimetric readout, the sequence alignment of the virus genomes was analyzed in Fig. S1. In terms of MERS-CoV, we could not find any significant similarity to the selected SARS-CoV-2 target regions among the whole MERS-CoV gene length. SARS-CoV had several variations in both the primer binding and sgRNA binding regions. SARS-CoV and MERS-CoV pseudovirus standards were employed herein. We can conclude in Fig. 4D that for both the ORF1ab and N genes, SARS-CoV-2 yielded significantly stronger T lines than SARS-CoV and MERS-CoV. And both T1 and T2 lines were visualized on these strips. Notably, the whole detection was finished within 1 h (20 min for RT-RPA, 30 min for CRISPR-Cas cleavage, 3 min for strip visualization (Fig. S4)). Additionally, 50 clinical samples with different Ct values were also tested with OC-MLFA. As shown in Fig. S5, our method was sensitive enough to detect the samples with Ct values over 30, which endowed great reliability and usability of OC-MLFA for practical clinical diagnosis. As lower target concentrations will render higher Ct values but weaker strip line intensities. We can observe in Fig. S5 that strip line intensities for lower Ct values were normally stronger than those for higher Ct values. Notably, during the clinical samples analysis, the OC-MLFA results showed 76 out of 76 consistencies with the RT-PCR results, presented in Table S3. The multiplexed gene detection ability endowed the orthogonal CRISPR-Cas two-channel readouts and lateral flow strips with two target lines can effectively avoid false negative or positive results commonly encountered in single gene detection system, but stronger strip line intensities. We can observe in Fig. S5 that strip line intensities for lower Ct values were normally stronger than those for higher Ct values. Notably, during the clinical samples analysis, the OC-MLFA results showed 76 out of 76 consistencies with the RT-PCR methods (Fig. S6).

3.5. Clinical sample analysis

The analytical performance was then investigated against different concentrations of SARS-CoV-2 pseudovirus standard samples, which contained the target segments of ORF1ab and N gene regions used in our design. The extracted RNA genome was subjected to RT-RPA prior to OC-MLFA. As revealed of the strips in Fig. 4A, consistent with our expectation, both the color intensity of T1 (Fig. 4B) and T2 lines (Fig. 4C) increased accordingly with the pseudovirus numbers from 0 to 1000 copies, with PROBIT \((p) = -3.338 + 6.594 \times \log\text{(Copy Numbers)}\) for the probit regression analysis. We can achieve the limit of detection down to 10 copies per reaction (30 µL). For simultaneously dual-gene detection, the OC-MLFA exhibited better performance compared with recent reported Cas-based LFAs (Table S2). This great sensitivity can be ascribed to the combination of inherent high amplification efficiency from RT-RPA and collateral cleavage from orthogonal CRISPR-Cas.

Next, the specificity of our proposed OC-MLFA was investigated. Severe Acute Respiratory Syndrome (SARS-CoV) and Middle East Respiratory Syndrome (MERS-CoV) virus genomes were chosen because they had ~82% and 67% similarity to that of SARS-CoV-2, respectively [44]. The sequence alignment of the virus genomes was analyzed in Fig. S1. In terms of MERS-CoV, we could not find any significant similarity to the selected SARS-CoV-2 target regions among the whole MERS-CoV gene length. SARS-CoV had several variations in both the primer binding and sgRNA binding regions. SARS-CoV and MERS-CoV pseudovirus standards were employed herein. We can conclude in the tested samples. The great accuracy can be ascribed to the dual checking process during the sgRNA-mediated binding and activation of Cas12a/13a. All these results confirmed the feasibility and reliability of the designed OC-MLFA platform.

### 3.4. Analytical performance of OC-MLFA

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### 3.5. Clinical sample analysis

Given the validated performance, the OC-MLFA platform was further utilized to detect clinical nasopharyngeal swab samples. To make a direct comparison between our OC-MLFA method with the established Chinese clinical RT-PCR test, ORF1ab and N test results were both evaluated for the final diagnosis. Briefly, the samples were identified positive if both ORF1ab and N genes were positive. The results were negative when both genes were negative. If only one of genes was positive, the result was identified as suspicious, which should be tested again. To ensure the reliability, a total of 26 di-identified clinical nasopharyngeal swab samples, including 6 pseudovirus standard-spiked positive samples, were analyzed with our proposed OC-MLFA as well as RT-PCR. Fig. 5 illustrated that all the 6 positive samples were identified accurately. And both T1 and T2 lines were visualized on these strips. Notably, the whole detection was finished within 1 h (20 min for RT-RPA, 30 min for CRISPR-Cas cleavage, 3 min for strip visualization (Fig. S4)). Additionally, 50 clinical samples with different Ct values were also tested with OC-MLFA. As shown in Fig. S5, our method was sensitive enough to detect the samples with Ct values over 30, which endowed great reliability and usability of OC-MLFA for practical clinical diagnosis. As lower target concentrations will render higher Ct values but weaker strip line intensities. We can observe in Fig. S5 that strip line intensities for lower Ct values were normally stronger than those for higher Ct values. Notably, during the clinical samples analysis, the OC-MLFA results showed 76 out of 76 consistencies with the RT-PCR methods (Fig. S6).

### 4. Conclusions

In summary, lateral flow assay for dual genes visualization based on orthogonal CRISPR-Cas responsive system for SARS-CoV-2 detection has been developed. The proposed OC-MLFA platform exhibited several exclusive and intrinsic features feasible for promising field-deployable POCT application: (a) The multiplexed gene detection ability endowed by the orthogonal CRISPR-Cas two-channel readouts and lateral flow strips with two target lines can effectively avoid false negative or positive results commonly encountered in single gene detection system, significantly increasing the reliability during the COVID-19 diagnosis; (b) the great specificity validated with human coronaviruses and relevant human respiratory pathogens ensured the accuracy of our method; (c) both multiplexed RT-RPA and Cas12a/Cas13a mediated orthogonal signal readout can be finished at 37 °C, which offered great operation convenience for POCT application; (d) the efficient amplifications from RT-RPA and Cas12a/Cas13a mediated trans- cleavage process brought the sensitivity of the lateral flow visualization assay to 10 copies of viral genome sequence per test, ensuring our method great potential for sensitive COVID-19 diagnosis. While the orthogonal CRISPR-Cas strategy has been proposed for multiplexed target detection, the optical instruments were usually incorporated for signal readout. The OC-MLFA platform established herein enabled rapid and facile diagnosis without requirement for well-trained personnel and sophisticated instrumentation. Whereas, considering the RNA extraction requirements during practical detections, facile sample preparation procedure may need to be investigated for field-deployable POCT. Through integration the multiplexed targets detection with single strip colorimetric readout, the orthogonal CRISPR-Cas mediated multiplexed lateral flow assay made a promising alternative for SARS-CoV-2 detection. Moreover, the OC-
The MLFA system can also be compatible for other pathogens genes detection after rational nucleic acids design.

**CRediT authorship contribution statement**

**Gaoxing Su**: Resources, Formal analysis, Writing – review & editing, Project administration. **Min Zhu**: Conceptualization, Investigation, Visualization, Writing – review & editing. **Diyuan Li**: Methodology, Investigation, Validation. **Mengting Xu**: Resources, Writing – review & editing. **Yuedong Zhu**: Writing – review & editing. **Yan Zhang**: Writing – review & editing. **Hongyan Zhu**: Writing – review & editing. **Feng Li**: Resources, Formal analysis, Writing – review & editing, Project administration. **Yanyan Yu**: Conceptualization, Writing – original draft, Writing – review & editing, Supervision, 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.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2022.132537.

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