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Exploiting the orthogonal CRISPR-Cas12a/Cas13a *trans*-cleavage for dual-gene virus detection using a handheld device

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ABSTRACT

Although CRISPR-Cas12a and CRISPR-Cas13a systems work individually effective on gene detection, their multiplex detection capability is limited due to the lack of specific probe cleavage mechanism. Herein we present a high-efficient dual-gene diagnostic technique based on the orthogonal DNA/RNA collateral cleavage mechanism of Cas12a/Cas13a system. In this design, dual-gene amplified products from the multiplex recombinase polymerase amplification (RPA) were simultaneously detected by Cas12a and Cas13a assay in a single tube. The resulting orthogonal DNA/RNA collateral cleavage can specifically illuminate two spectral differentiated DNA and RNA probes, respectively. By integrating with the smartphone-based fluorescence readout, a portable detection platform is achieved. As a proof-of-concept, reliable dual-gene detection of SARS-CoV-2 and African Swine fever virus (ASFV) were demonstrated, exhibiting 100% sensitivity and specificity for clinical samples analysis (32 swab specimens for SARS-CoV-2 and 35 ASFV suspected swine blood samples). This developed portable dual-gene detection platform can provide accurate point-of-care screening of infectious diseases in resources-limited settings.

1. Introduction

Reliable point-of-care testing (POCT) diagnostics are urgently needed to respond to infectious disease outbreaks, especially in the context of furious spreading of the COVID-19 pandemic. The recently developed CRISPR tools are regarded as a promising technology for POCT (Chertow, 2018; Dai et al., 2020; Yue et al., 2021a; Zuo et al., 2017). The CRISPR tools exhibit some unprecedented advantages, such as rapid and accurate gene recognition (Jinek et al., 2012), reaction at physiological temperature (Chen et al., 2018; East-Seletsky et al., 2017; Jinek et al., 2012; Li et al., 2018b), and robust signal amplification mechanism (Chen et al., 2018; East-Seletsky et al., 2017; Li et al., 2018a; Swarts and Jinek, 2019). CRISPR-based diagnostics were originally developed by employing Cas9 (Pardee et al., 2016), the first discovered gene editing tool (Hsu et al., 2014; Jinek et al., 2012; Ran et al., 2015). These methods are based on either Cas9-mediated specific DNA/RNA binding (Azhar et al., 2021; Pardee et al., 2016; Wang et al., 2020; Yang et al., 2018; Zhang et al., 2018), or cleavage (Huang et al., 2018; Zhou et al., 2018), or nicking (Wang et al., 2019) reaction and subsequent signal transduction through fluorescence (Huang et al., 2018), electrochemistry (Balderston et al., 2021), or colorimetric detection (Pardee et al., 2016; Wang et al., 2020). More recently, *trans*-cleavage mechanism was consecutively discovered in CRISPR/Cas12 (Chen et al., 2018; Li et al., 2018a; Swarts and Jinek, 2019) and CRISPR/Cas13 systems (Abudayyeh et al., 2016; Shmakov et al., 2015), prompting the establishment of a new type of CRISPR diagnostic method (Ackerman et al., 2020; Arizti-Sanz et al., 2020; Broughton et al., 2020; Chen et al., 2021; Dai et al., 2019; Ding et al., 2020; Fozouni et al., 2021; Gootenberg et al. 2017, 2018; Li et al. 2018b, 2021a; Liang et al., 2019; Maji et al., 2019; Patchsung et al., 2020; Shi et al., 2021; Xiong et al., 2019). This *trans*-cleavage mechanism is quite attractive because its activation is strictly derived from *cis*-recognition, that is, the pairing of target DNA/RNA and crRNA (Chen et al., 2018; East-Seletsky et al., 2016; Li et al., 2018a; Liu et al., 2017). In addition, this cis-recognition triggered *trans*-cleavage presents a multiple turnover behavior. For example, the CRISPR/Cas13a system has been proven to achieve at least 10^4 turnovers per target RNA recognition (Chen et al., 2018; Tambe et al., 2018). For Cas12a system, its *trans*-cleavage efficiency was considered to be

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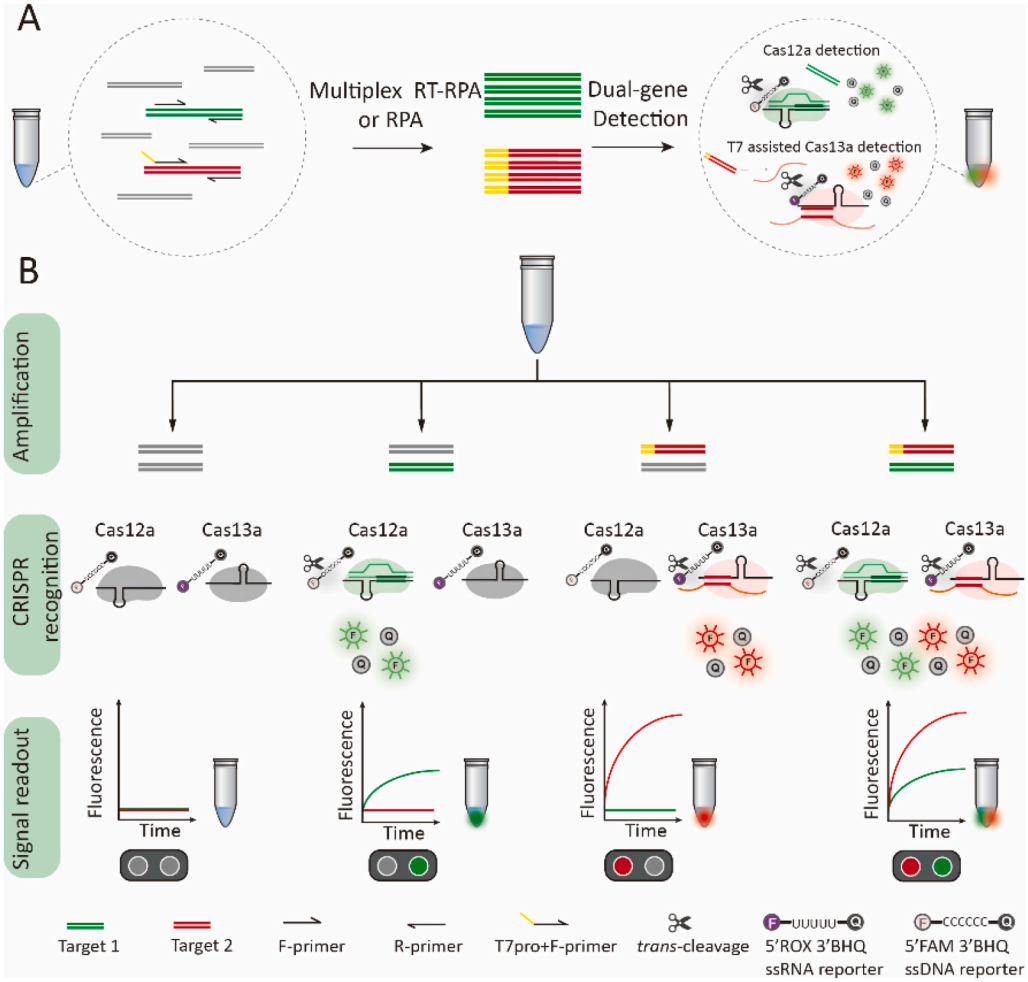


Fig. 1. Schematic of single-tube orthogonal Cas12a/Cas13a detection. (A) Two targets are isothermally amplified by multiplexed RT-RPA or RPA. Target 1 DNA amplicons are directly recognized by Cas12a-crRNA complexes. Target 2 DNA amplicons are converted into RNA by T7 transcription and recognized by Cas13a-crRNA complexes. Orthogonal cleavage of DNA and RNA reporters by target-activated Cas12a/Cas13a induces two-colored fluorescence emission, which can be detected using a standard fluorometer or observed directly by naked-eye. **(B)** Schematic illustration of orthogonal Cas12a/Cas13a detection platform and for dual-gene detection. The carton shows four detection situations: In absence of target 1 and target 2; Only in the presence of target 1; Only in the presence of target 2; In presence of both target 1 and target 2.

lower than Cas13a, but it has now been improved to a comparable level through optimization of reaction conditions (Hsieh et al., 2020; Yue et al., 2021b).

Although specifically derived, both CRISPR/Cas12a and Cas13 systems exhibit non-specific chaotic *trans*-cleavage mechanism. Such mechanism allows a universal probe system design for versatile nucleic acid detection. For example, in a Cas12a-based assay, a short single-strand DNA (ssDNA) *trans*-cleavage substrate that dual-labeled with a quenched fluorescent reporter, can be used in the homogeneous fluorescent detection for all target DNAs (Chen et al., 2018; Li et al., 2018a). In the Cas13a assay, such a universal *trans*-cleavage substrate can be a single-stranded poly (U) RNA probe (East-Seletsky et al., 2017). Despite the advantages of simple design and cost effectiveness, the indiscriminate probe cleavage mechanism also limits its multiplex detection capability. Multiplex detection is particularly important for molecular diagnosis. Simultaneous detection of multiple genes is an effective means to improve the detection accuracy, due to the high risk of false-negative results in a single-gene testing. For example, in SARS-CoV-2 detection, partial gene degradation, gene copy number differences, amplification errors, and virus variation all may lead to false-negative results if only single-gene testing adopted (Peñarrubia et al., 2020). In fact, in molecular diagnostic applications, multiplex detection, especially dual-gene detection, is widely adopted in gold-standard PCR method (Artesi et al., 2020; Lee et al., 2010; Li et al., 2021b).

CRISPR-based diagnostics with multiplex detection capability are rarely reported, although some attempts have been carried out. For example, we have reported a test strip technology for dual-gene SARS-CoV-2 detection based on the CRISPR/Cas9 system (Xiong et al., 2021). However, such a technology cannot be transplanted to the Cas12 or Cas 13a system, which is more sensitive than the Cas9 system. Efforts have also been devoted to the development of new Cas effectors with preferred substrate cleavage capabilities. For example, LwaCas13a, CcaCas13b, LbaCas13a, and PsmCas13b have been explored to present AU, UC, AC, and GA cleavage preference, respectively (Gootenberg et al., 2018). However, the detection signal crosstalk still exists, resulting in the lack of the absolute orthogonal detection capabilities. When detecting two targets with varied concentrations, signal leakage may lead to the confusion of detection results. Besides, the readout of this multiplexed detection platform relies on specialized laboratory fluorescent reader which limits its implementation outside of laboratories.

Another straightforward strategy is geometrically-multiplexed detection. For example, the CARMEN (combinatorial arrayed reactions for multiplexed evaluation of nucleic acids) integrated SHERLOCK with self-organizing and miniaturized microfluidic technology, enables scalable and highly-multiplexed detection (Ackerman et al., 2020). However, its widespread implementation is limited by the requirement of advanced microscopy and skilled lab personnel. Besides, Richard and colleagues designed an electrochemical microfluidic biosensor by separating immobilization areas into subsections to realize parallel detection of multiple targets (Bruch et al., 2021). But this separation-based approach requires complicated chip fabrication and may introduce the risk of cross-reactivity by diffusion between the different sub-regions.

Therefore, improvements in CRIPSR-based multiplexed detection are still needed to facilitate its potential utility to address point-of-care

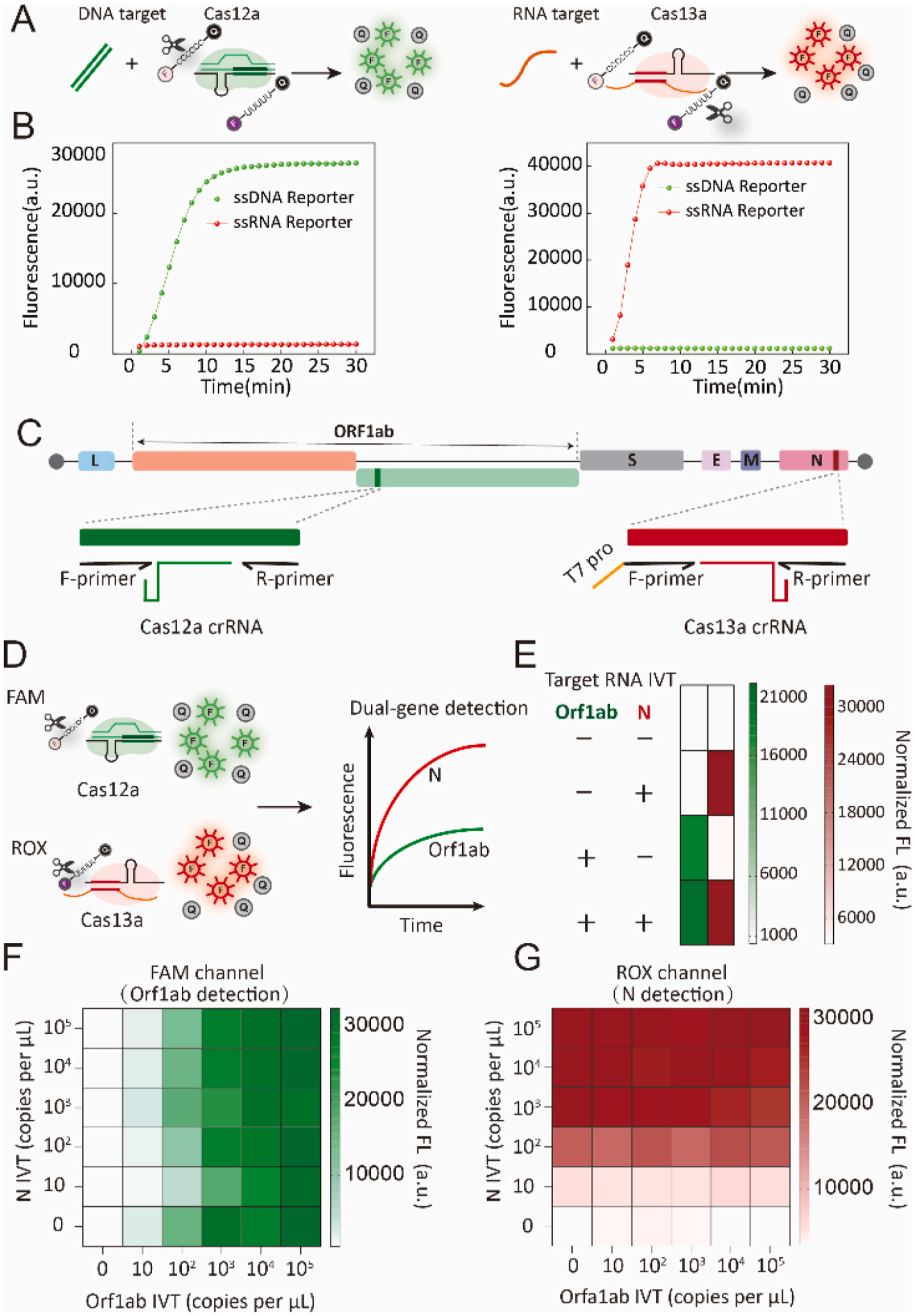


Fig. 2. Establishment of single-tube orthogonal Cas12a/Cas13a detection. (A) Schematic of evaluation of reporter preferences of Cas12a and Cas13a assay. **(B)** Cas12a only cleaves ssDNA reporters when activated by synthetic DNA target and Cas13a only cleaves ssRNA reporters when activated by synthetic RNA target. **(C)** Schematic of the SARS-CoV-2 genome map showing the selected Orf1ab gene and N gene regions for multiplexed RT-RPA and corresponding Cas12a/Cas13a spacer location. **(D)** Schematic of orthogonal detection of Orf1ab and N gene with LbCas12a and LbuCas13a. **(E)** Validation of multiplexed RT-RPA and the orthogonal detection of Orf1ab and N gene with LbCas12a and LbuCas13a. The presence of each target RNA (10^5 copies per μL) was confirmed by the fluorescence heat-map analysis. **(F)** Fluorescence heat-map analysis of detecting Orf1ab gene at orthogonal concentration ratios with N gene. Normalized fluorescence data was calculated by subtracting the initial fluorescence of every sample from the endpoint fluorescence to allow for comparisons between different conditions. **(G)** Fluorescence heat-map analysis of detecting N gene at orthogonal concentration ratios with Orfa1b gene.

diagnostic needs. 1) The orthogonal *trans*-cleavage characteristics of Cas enzymes needs to be further explored to achieve cross-talk free detection. 2) Imparting CRISPR-based multiplexed detection with point-of-care properties could fulfill the need for a test that provides rapid and accurate results in the control of pandemics.

In this work, we have developed a dual-gene detection technology by exploiting the orthogonal cleavage activity from two heterologous CRISPR-Cas12a and CRISPR-Cas13a systems. It is well known that CRISPR-Cas12a and CRISPR-Cas13a systems exhibit excellent orthogonal *trans*-cleavage capabilities for DNA and RNA probes, respectively. Thus, we developed a dual-CRISPR reaction at a single test tube by combining the Cas12a assay and the T7 transcription-mediated Cas13a assay for simultaneously detect multiplex recombinase polymerase amplification (RPA) products. To facilitate the POCT application, we designed a handheld, smartphone-based fluorescence imaging device. The test result can be judged by the naked eye with a sensitivity of 8 copies/μL. For proof-of-concept, reliable dual-gene detection of SARS-CoV-2 (O and N gene) and African Swine fever virus (ASFV) (*p72* and house-keeping ACTB gene) were demonstrated, exhibiting 100% sensitivity and specificity on clinical samples (32 swab specimens for SARS-CoV-2 and 35 ASFV suspected swine blood samples). We anticipate that this developed portable dual-gene detection platform can offer more accurate point-of-care screening of infectious pathogens in settings with limited resources.

2. Material and methods

For experimental details, please refer to the supplementary information.

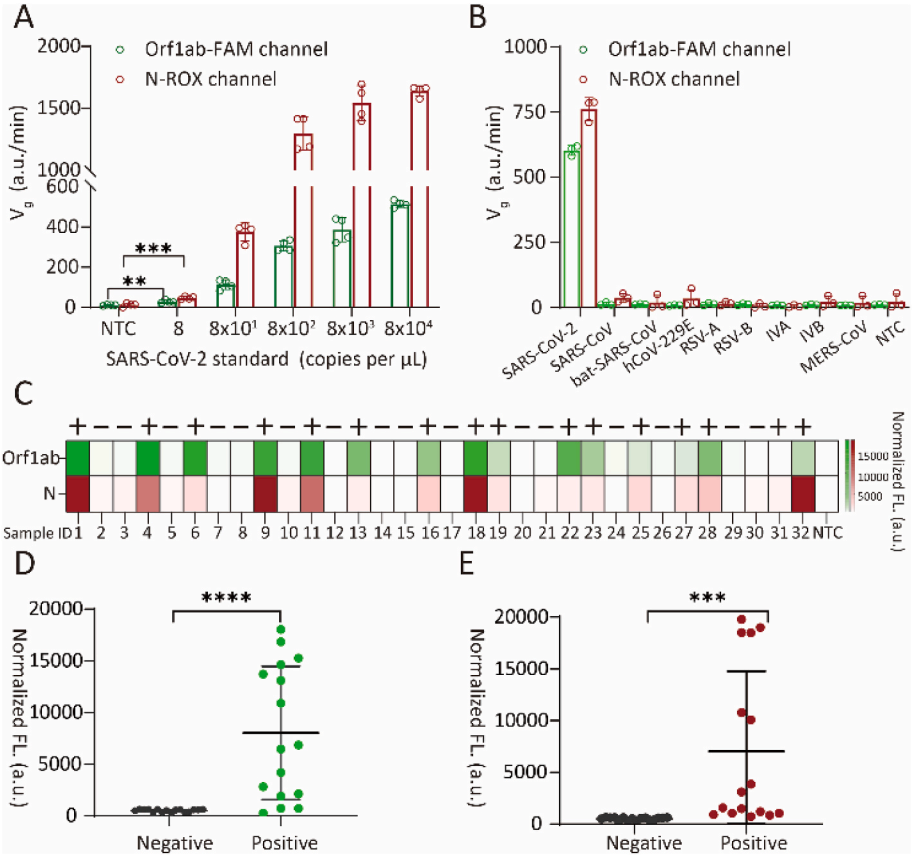


Fig. 3. Evaluation of sensitivity, specificity and diagnostic performance of the orthogonal Cas12a/Cas13a detection on SARS-CoV-2. (A) Determination of the limit of detection of the orthogonal Cas12a/Cas13a detection on SARS-CoV-2 standards RNA. Vg represents fluorescence growth rate = △F/△t (△F = the change of the fluorescence intensity before reaching plateau, △t = 30 min). Values represent mean ± s.e.m. from three replicates (two-tailed Student's test; **, $p < 0.01$; ***, $p < 0.001$). **(B)** The specificity of the orthogonal Cas12a/Cas13a detection on the analysis of SARS-CoV-2 RNA against other coronaviruses (SARS-CoV, bat-SL-CoVZC45, MERS-CoV, and hCoV-229E) and other human respiratory virus (RSV-A, RSV-B, IVA, and IVB). Values represent mean ± s.e.m. from three replicates. **(C)** Heat map showing the results of SARS-CoV-2 detection in 32 suspected COVID-19 nasopharyngeal swab clinical samples using the orthogonal Cas12a/Cas13a detection. Signals values represent in the heat map are the normalized fluorescence in two detection channels of each sample detection results. **(D and E)** Scatter plot analysis showing the normalized fluorescence values of 16 negative clinical samples and 16 positive clinical samples detected by the orthogonal Cas12a/Cas13a detection in FAM and ROX detection channels, respectively (two-tailed Student's test; ***, $p < 0.001$; ****, $p < 0.0001$).

3. Results and discussion

3.1. Construction of a dual-gene assay by orthogonal Cas12a/Cas13a detection

Both Cas12a and Cas13a systems work individually effective on gene detection, but their multiplex detection capability is limited. This is due to the *cis*-recognition triggered *trans*-cleavage activity is non-specific, meaning that different target DNA or RNA cannot be signal-distinguished in a single-tube test. It is well known that Cas13a is an RNA-guided RNase and Cas12a is an RNA-guided DNase. We envision that these two heterologous CRISPR systems with orthogonal DNA/RNA cleavage characteristic can be exploited to develop a simultaneous dual-gene detection method.

Because both Cas12a assay and Cas13a assay can work at physiological temperature(Arizti-Sanz et al., 2020; Broughton et al., 2020; Fozouni et al., 2021), this single-tube combined reaction will be temperature compatible. In addition, T7 transcription-mediated Cas13a assay has been successfully proven to be used for DNA detection (Ackerman et al., 2020; Gootenberg et al. 2017, 2018), so both Cas12a assay and Cas13a assay can be combined in one tube for direct and indirect DNA recognition, respectively. To design such a dual-gene CRISPR assay, two target genes can be pre-amplified by performing multiplex reverse transcription-recombinase polymerase amplification (RT-RPA) (for RNA targets) or multiplex RPA (for DNA targets) with conventional primer and T7 promotor tagged primer pairs (Fig. 1A). DNA products amplified by conventional primer pairs can be recognized by Cas12a system and DNA products amplified by T7 promoter tagged primer pairs can be recognized by T7 transcription-mediated Cas13a assay. FAM–CCCCCC–BHQ1 probe is designed for Cas12a assay and ROX-UUUUU-BHQ2 probe is designed for Cas13a assay. The orthogonal DNA/RNA *trans*-cleavage triggered by Cas12a assay and T7 transcription-mediated Cas13a assay can produce four potential detection situations (Fig. 1B): i. in absence of both target genes, both probe signals cannot be detected; ii. in presence of one of target genes, FAM or ROX signals can be detected; iii. in presence of both target genes, both FAM or ROX signals can be simultaneously detected.

3.2. Establishment of the single-tube orthogonal Cas12a/Cas13a detection

Because Cas13a is an RNA-guided RNase and Cas12a is an RNA-guided DNase, we want to confirm that whether they can exhibit the expected orthogonal *trans*-cleavage mechanism. We first verified the cleavage preferences on ssDNA and ssRNA reporters in Cas12a and Cas13a assay, using dsDNA target or ssRNA target separately (Fig. 2A). In single Cas12a or Cas13a assay, both ssDNA reporter and ssRNA reporter are added. Results show that only ssDNA reporters were cleaved by *trans*-activated Cas12a and only ssRNA reporters were cleaved by *trans*-activated Cas13a. Thus, in the presence of the corresponding targets, both Cas12a and Cas13a systems exhibit excellent orthogonal collateral cleavage of ssDNA and ssRNA reporters respectively (Fig. 2B).

Having demonstrated that the orthogonal collateral cleavage of Cas13a and Cas12a assay, another key to realizing multiplex detection is the establishment of well reaction compatibility for both Cas12a and Cas13a assays. Hence, we implement simultaneous orthogonal detection by analyzing the O and N genes of SARS-CoV-2 (Fig. 2C). First, several parameters were observed in order to boost the combined Cas12a/Cas13a detection efficiency. We selected the Cas12a homolog from *Lachnospiraceae bacterium* (LbCas12a) and Cas13a homolog from *Leptotrichia buccalis* (LbuCas13a) as they have demonstrated robust *trans*-cleavage activity and high sensitivity relative to other Cas12a and Cas13a homologs (Chen et al., 2018; Li et al. 2018a, 2018b; Tambe et al., 2018). A preliminary evaluation of the combined Cas13a and Cas12a assay showed that the Cas12a assay was very inefficient (Supplementary Fig. S1). After a thorough screening, we have observed some key factors:

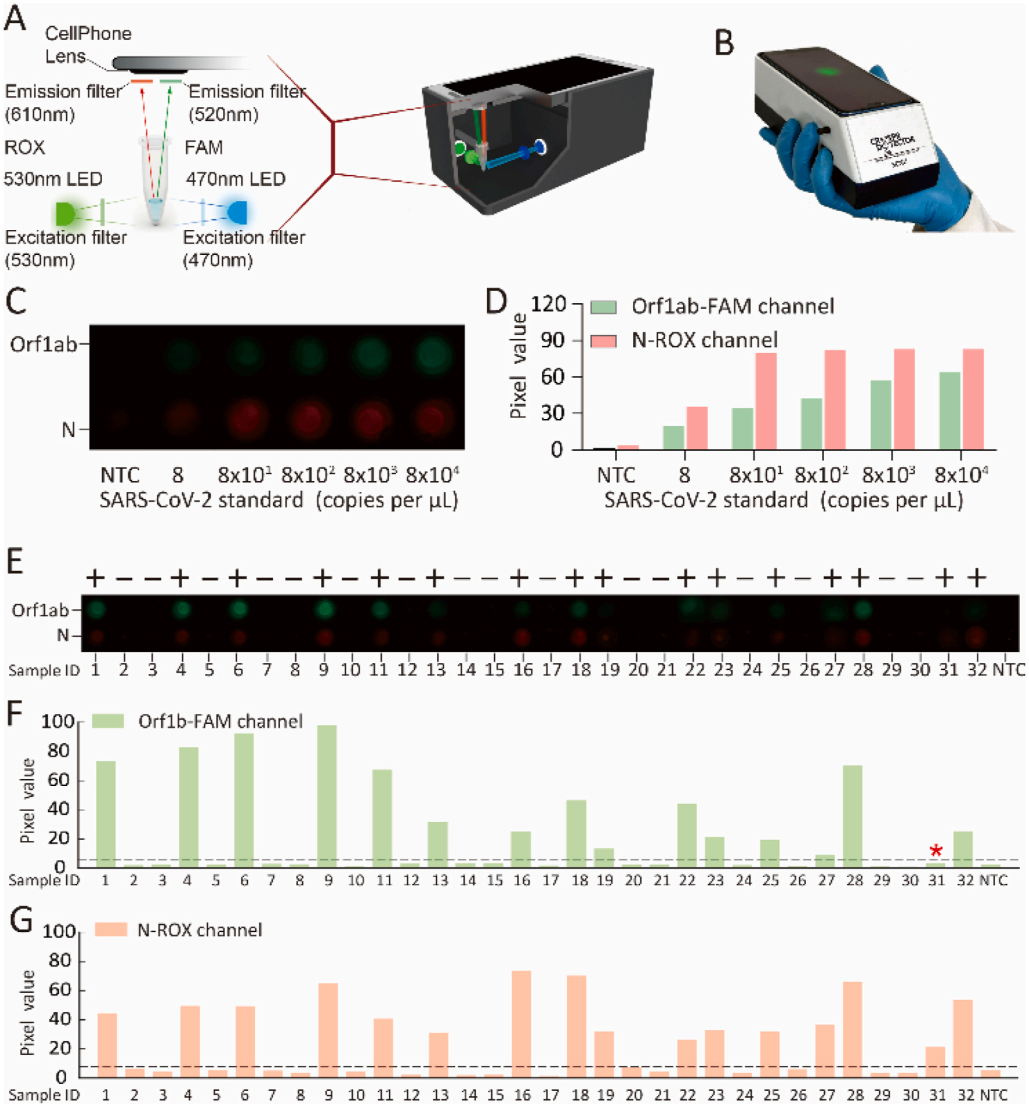


Fig. 4. Design of a portable dual-channel detection device integrated with smartphone. (A) Schematic of dual-channel detection device showing LED transilluminators, excitation filters and emission filters for visualization of FAM and ROX fluorescence signals and a smartphone for image capture. **(B)** Picture of the assembled portable device. **(C)** Sensitivity evaluation by imaging FAM and ROX signal generated from orthogonal Cas12a/Cas13a detection of the gradient-diluted Orf1ab and N genes of SARS-CoV-2. **(D)** Collection and analysis of pixel intensity in two channels from images taken with the smartphone in **C**. **(E)** FAM and ROX signal images capture by smartphone based on the detection of 32 suspected COVID-19 nasopharyngeal swab clinical samples. **(F–G)** Collection and analysis of pixel intensity in two channels from images taken with the smartphone in E. Threshold line represents the mean plus 3 standard deviations of pixel intensity from a non-target control.

i. High concentrations of rNTPs used in the T7 transcription reaction had a significant inhibitory effect on the Cas12a assay, but this inhibitory effect disappeared when the concentration of rNTPs was reduced to 1–1.5 mM (Supplementary Fig. S2); ii. The buffer component is also very crucial. For such a hybrid CRISPR assay, among the four buffers we tested (NEB 1.1, 2.1, 3.1, and cut smart), we determined NEB1.1 as the optimal reaction buffer for orthogonal CRISPR-Cas12a/Cas13a detection system. (Supplementary Fig. S3); iii. In addition, we also found that lowering the concentration of other Cas13a assay components, such as T7 RNA polymerase, Cas13a enzyme, and crRNA, is beneficial to obtain a more balanced detection signal and reduce the background signal (Supplementary Fig. S4, material and methods).

After optimizing the Cas12a/Cas13a detection parameters with synthetic O and N gene templates, we then evaluated the orthogonal detection capabilities of Cas12a and Cas13a assays for analyzing multiplex RT-RPA products in a single-tube system (Supplementary Fig. S5). As shown in Fig. 2D and E, all four types of O and N gene testing situation (both are present or missing and one of the two is present) have obtained the expected test results. No crosstalk signal indicates that excellent orthogonal detection capabilities are obtained even under the single-tube test conditions.

Because the above test uses the same O and N RNA concentration, we wonder whether such a system still works well with different O and N RNA concentration ratios. Such a test is essential to evaluate the possibility of signal leakage. At varied ratios of these two target RNAs, the orthogonal detection system specifically produced fluorescence that responded only to respective target RNAs and there is no signal disturbance when mixed with 10,000-fold non-target RNA, thereby enabling orthogonal simultaneous detection of two genes (Fig. 2F and G). These results demonstrated that Cas12a and Cas13a can be reacted simultaneously in a single tube with excellent orthogonal performance, whether using synthetic targets or amplified products.

3.3. Evaluation of sensitivity, specificity and diagnostic performance of the orthogonal Cas12a/Cas13a detection on SARS-CoV-2

With the serial dilutions of the SARS-CoV-2 standard RNA (including full length N gene, full length E gene and Orf1ab gene fragment), we demonstrated that the orthogonal LbCas12a and LbuCas13a assay can simultaneously detect as low as 8 copies/μL of Orf1ab and N genes, respectively (Fig. 3A). Since only 5 μL samples are added in each reaction, the obtained sensitivity is equivalent to 40 copies per reaction. To confirm its analysis specificity, we performed the orthogonal Cas12a/Cas13a detection using RNA input from other coronaviruses, including SARS-CoV, bat-SL-CoVZC45, MERS-CoV, and hCoV-229E, which exhibited no cross-reactivity. It is also showed that there is no cross-reactivity for detecting other human respiratory virus, i.e., respiratory syncytial virus-A (RSV-A), respiratory syncytial virus-B (RSV-B),

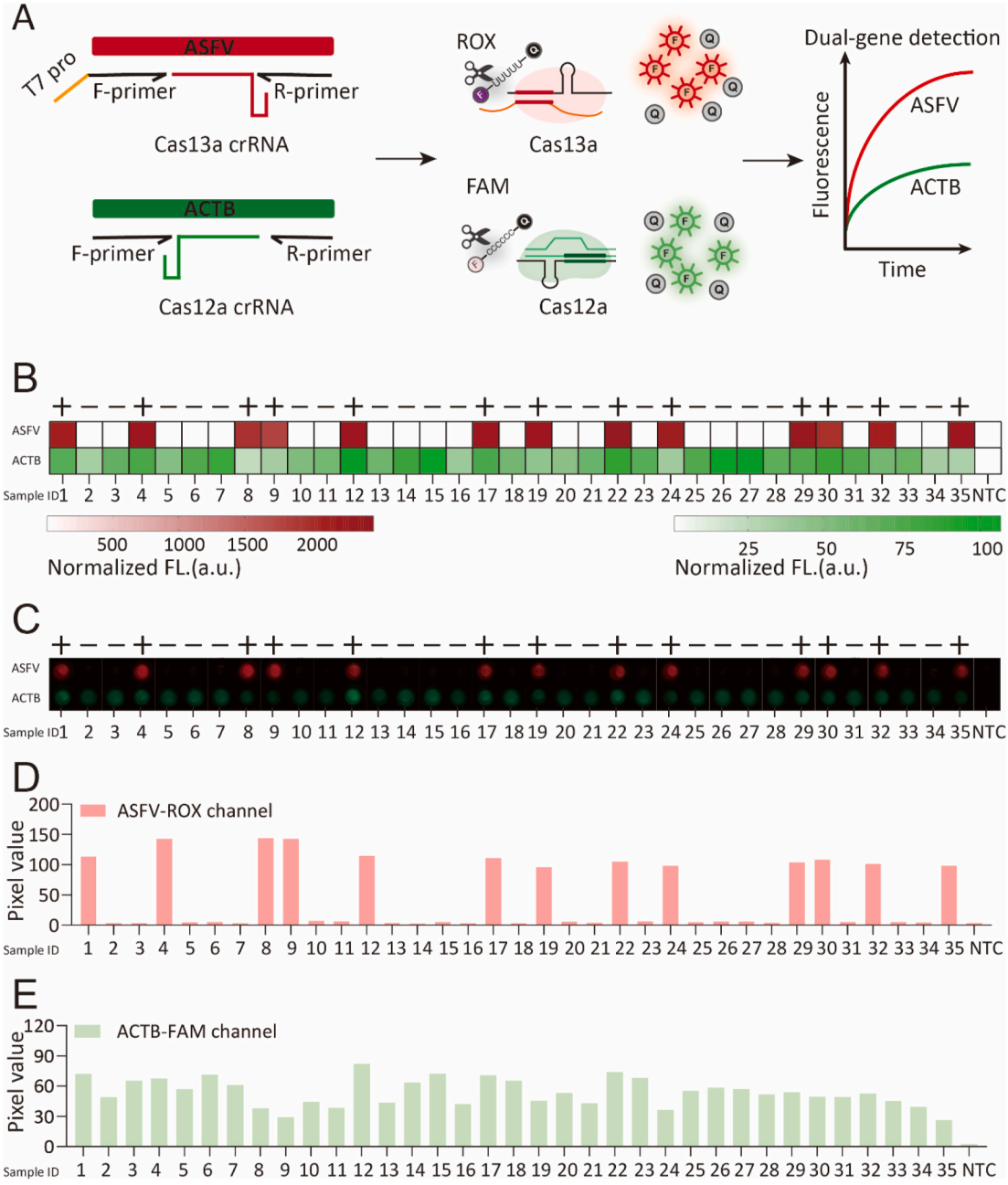


Fig. 5. Reliable dual-gene detection of ASFV p72 gene and ACTB gene through the orthogonal Cas12a/Cas13a multiplex detection. (A) Schematic of the orthogonal detection of ASFV *p72* and ACTB gene with LbuCas13a and LbCas12a. **(B)** Heat map analysis shows the results of ASFV detection in 35 ASFV suspected swine blood samples using the orthogonal Cas12a/Cas13a detection. Signal values represented in the heat map are the normalized fluorescence in two detection channels of each sample detection results. **(C)** Images capture by smartphone show the FAM and ROX signal for ASFV detection in 35 ASFV suspected swine blood samples. **(D and E)** Collection and analysis of pixel intensity in two channels from images taken with the smartphone in Fig. 5C.

influenza A virus (IVA), and influenza B virus (IVB) (Fig. 3B).

The clinical performance of the orthogonal dual-gene detection platform on viral RNA extracts has been validated by testing 32 nasopharyngeal swab clinical samples (Fig. 3C, D, and E). Among 16 RT-qPCR-positive samples (Ct values ranging from 25.2 to 37.4), we were able to detect both O and N genes in 15 samples except for sample 31#, and all RT-qPCR approved negative samples were undetectable (Supplementary Figs. S6 and 7, Supplementary Note 2). Notably, we found that in current assay sample 31# (Ct of N gene is 36.03 and Ct of Orf1ab gene is 37.4) was identified as COVID-19 positive by N gene, but failed to detect O-gene. Collectively, the clinical sensitivity of our multiplexed detection with the determined LoD is 100% for the detection of either the Orf1ab or N gene of SARS-CoV-2, and 93.75% for the detection of both genes based on the 36 clinical samples analysis. We noticed that the negative sample should be identified with a Ct threshold above 38 in the CFDA approved RT-qPCR kits. This means that the sensitivity of the developed dual-gene method is very close to the gold standard RT-qPCR.

3.4. Orthogonal Cas12a/Cas13a detection using a handheld device

To simplify signal readout and data interpretation, and demonstrate that the use of the orthogonal Cas12a/Cas13a multiplexed detection in point-of-care settings, we designed a handheld device integrated with smartphone-based fluorescence imaging (Fig. 4A and B). The FAM-based ssDNA reporter and ROX-based ssRNA reporter could be efficiently excited by a blue and green LED light source, respectively. Then the FAM

and ROX emissions can be simultaneously monitored through the smartphone integrated device when using different combinations of colored emission filters. The two-colored in-tube fluorescence signals could be easily visualized directly by the naked-eyes. To further reduce user bias in the determination of results of this in-tube fluorescence readout, we also use cellphone camera to image the illuminated reaction tubes in two-colored fluorescence channels and calculate the pixel intensity, respectively. We set a detection threshold at mean plus 3-fold standard deviations of pixel intensity from non-target control analysis. A sample would be determined as positive if the fluorescence pixel intensity is higher than the detection threshold, otherwise, the sample would be negative.

To determine the LoD of the orthogonal Cas12a/Cas13a detection using the portable device, we tested serially diluted SARS-CoV-2 RNA and collected fluorescence from these two channels after 60-min reaction. It is found that equivalent sensitivity is achieved when compared to standard fluorescence detectors (Fig. 4C and D). We next evaluated its performance for detecting these clinical samples analyzed in Fig. 3C, to make a comparison between the standard fluorimeter and our portable device. We imaged each in-tube sample reaction at 60 min post incubation in two fluoresce channels (Fig. 4E). Encouragingly, consistent detection results can be achieved when compared with the standard fluorescence detector (Fig. 4F and G). These results demonstrated that this dual-channel fluorescence readout only requires minimal laboratory infrastructures and thus eliminates the need for specialists to interpreting results with guaranteed high sensitivity and accuracy.

3.5. Reliable dual-gene detection of ASFV

African swine fever (ASF) is an acute, virulent, highly contagious disease caused by the ASFV. ASF has a mortality rate up to 100%. The outbreak and spread of ASF since 2017 have brought serious economic damage to the global swine industry (Mazur-Panasiuk et al., 2019). At present, there is no specific treatment or effective vaccine to combat this animal disease. Therefore, the development of a rapid, accurate and sensitive diagnostic method is essential for the prevention and control of ASF. PCR, the current gold standard for ASFV confirmation, usually only target the single capsid protein gene, *p72* (Aguero et al., 2003; Bastos et al., 2003). In order to avoid false-negative results and improve detection accuracy, an internal control such as ACTB which is expressed consistently in various mammalian matrices including blood could be incorporated (Tignon et al., 2011). The realization of simultaneous detection of ASFV and an internal control gene will greatly enhance detection accuracy and testing capacity.

To demonstrate the adaptability of our handheld platform to other pathogens, here we further expanded the capability of the orthogonal Cas12a/Cas13a multiplex detection for simultaneous detection of ASFV *p72* gene and house-keeping ACTB gene. First, we designed two pairs of RPA primers to amplify ASFV p72 and ACTB, respectively (Supplementary Figs. S8 and 9). Then ASFV amplicons amplified by conventional primer pairs can be recognized by Cas12a system and ACTB amplicons amplified by T7 tagged primer pairs can be recognized by T7 transcription-assisted Cas13a assay (Fig. 5A). Using both fluorescent readouts monitored by standard laboratorial fluorimeter and visual in-tube fluorescent readout by this handheld device, we correctly identified 13 ASFV-positive samples and 22 ASFV-negative samples from 35 ASFV suspected swine blood samples (Fig. 5B and C). The positive predictive agreement and negative predictive agreement of orthogonal Cas12a/Cas13a detection relative to the PCR assay were both 100% (Supplementary Figs. S10 and 11, Supplementary Note 3). Similar to SARS-CoV-2 detection, pixel intensity was also calculated from two detection channels of the images captured by the smartphone (Fig. 5D and E). It is found that whether by direct visual readout or fluorescence detection, results are consistent with that of standard fluorescence detectors. These results revealed the flexibility and practicality of orthogonal Cas12a/Cas13a detection platform by imparting it with point-of-care characteristics when implemented with this multiplexed in-tube readout integrated with the smartphone and low-cost optics.

4. Conclusion

This orthogonal Cas12a/Cas13a detection system exhibits some promising advantages, including multiplexed detection capability, low requirements of sophisticated instruments and specialists, and enhanced testing accuracy, thus offers opportunity for the development of an accurate, user-friendly, inexpensive platform towards point-of-care testing application of CRISPR-based diagnostics.

Besides, we also expect additional improvements for fully translating the current detection device to a widely available point-of-care platform. First, a nucleic acids extraction-free protocol for sample progressing will be required for simplify operating processes and shorten the overall sample-to-answer turnaround time. Second, ideally, experiment optimization should be developed to combine the pre-amplification and Cas12a/Cas13a detection into one-pot, closed-tube reaction. Last, in order to further minimize artificial bias, a smartphone app should be developed to enhance the data progressing capability. Besides, the discovery of new Cas effector with orthogonal cleavage preferences and integration with other approaches such as microfluidic can provide opportunity to achieve multiplexed identification of more than two targets. In summary, these refinements could greatly enhance the sustainable use of this CRISPR-based multiplex detection and eventually transfer it from the laboratory benchtop to clinical or practical use.

CRediT authorship contribution statement

Tian Tian: conceived the concept, designed and performed all the experiments, writing original draft. **Zhiqiang Qiu:** designed and manufactured the handheld device. **Yongzhong Jiang:** conducted clinical sample analysis. **Debin Zhu:** assisted in data analysis. **Xiaoming Zhou:** conceived the primary idea, wrote and reviewed the manuscript.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2021.113701.

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