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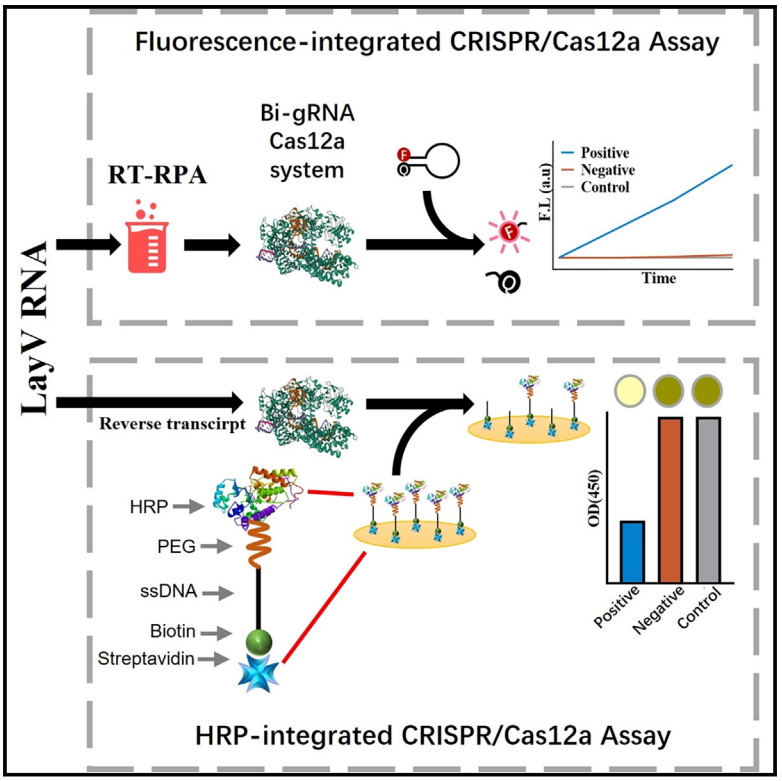
HRP-integrated CRISPR-Cas12a biosensor for rapid point-of-care detection of Langya henipavirus

Graphical abstract



Highlights

- Cas12a biosensor developed here to fill the gap of rapid diagnostics for LayV
- The RPA-fluorescence-Cas12a system achieves rapid and ultra-sensitive LayV detection
- The amplification-free HRP-Cas12a system enables sensitive point-of-care detection
- HRP-Cas12a system enables broad point-of-care pathogen diagnostics

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In brief

Natural sciences; Biological sciences; Immunological methods; Microbiology.

Wu et al., 2024, iScience 27, 111466
December 20, 2024
https://doi.org/10.1016/j.isci.2024.111466

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HRP-integrated CRISPR-Cas12a biosensor for rapid point-of-care detection of Langya henipavirus

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https://doi.org/10.1016/j.isci.2024.111466

SUMMARY

Global pandemic has emphasized the needs for advanced pathogen diagnosis in dealing with newly emerged infectious threats, including the Langya henipavirus (LayV). LayV, as an emerging zoonotic pathogen, has potential to cause pandemic, but lacks of rapid diagnostic tools, particularly at point-of-care level. Here, we leveraged the merits of CRISPR-Cas12a biosensing and established a highly sensitive LayV detection method. By integrating CRISPR-Cas12a with RPA, 10 copies/μL ultra-sensitive LayV RNA detection has been achieved at room temperature within 30 min. More importantly, this study developed a special horseradish peroxidase (HRP)-single-stranded DNA (ssDNA) reporter enabling CRISPR-Cas12a to detect LayV RNA without pre-amplification, achieving a sensitivity of 1,200 copies/μL detectable by the naked eye. These explorations can serve as accelerator for CRISPR-Cas biosensing toward rapid response for newly emerged biological threats, and also provide a method to realize simple, precise, and amplicon-free point-of-care pathogen screening for resource limited or underdevelopment regions.

INTRODUCTION

Newly emerged pathogenic microbes pose increasing challenges for global public health systems due to prolonged periods required for identifying infectious sources, potentially increasing mortality and morbidity.[1,2] For example, the COVID-19 pandemic, caused by the SARS-CoV-2, has resulted in over 770 million confirmed cases and 6.95 million deaths worldwide.[2] Recently, another infectious threat, the zoonotic pathogen Langya henipavirus (LayV) initially identified in China, has posed additional biological risks to humans.[3] Classified as a high-priority pathogen by the WHO, this new single-stranded negative RNA virus of the henipavirus genus (*Paramyxoviridae* family) causes severe symptoms in humans, has an extensive host reservoir, possesses potential for zoonotic transmission, and limits treatment options.[4–7] LayV's genome comprises an 18.4 kb single-stranded, negative-sense RNA encoding six major proteins—nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), G protein, and large polymerase (LP)—along with three accessory proteins: V, W, and C.[8] The N protein, encapsulating the genome RNA, is the most conserved structural protein of henipaviruses and serves as a prominent target for diagnostic purposes.[9] At present, detection methods for henipaviruses encompass virus isolation and serological procedures, both of which require a BSL-4 laboratory setting, as well as molecular biology identifications, including PCR-based methods.[10] However, current methods significantly limit the diagnostic options and speed for detecting the presence or spread of these viruses. This limitation is particularly acute for pathways originating from known wildlife reservoirs with limited diagnostic resource.[7] In addition, the symptomatic similarities between LayV and SARS-CoV-2 infections also pose additional challenges on preventing newly emerged virus from existed diagnosis.

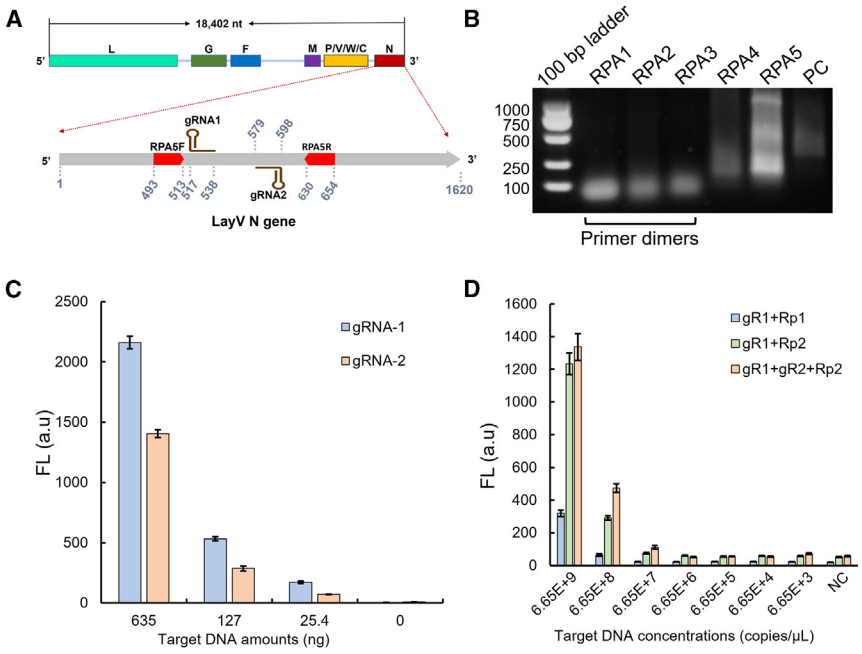

Figure 1. Identification of LayV genome target and reaction condition for CRISPR-Cas12a-based detection

(A) Schematic for the selection of genomic target loci for the RPA primers and its corresponding Cas12a gRNAs.

(B) The gel electrophoresis result for verifying RPA reaction using different RPA primer sets.

(C) The difference of Cas12a RNP *trans*-cleavage efficiency with the use of different gRNA sequences (n = 3).

(D) Investigation of Cas12a activation efficiency with different combinations of gRNAs (gR) and reporters (Rp) (n = 3). Data are presented as mean ± SD.

Conventional reverse-transcription PCR (RT-PCR), the gold standard for RNA detection, has continuously demonstrated its unparalleled value in combating viral infections and controlling their spread. Recently, LayV detection methods based on quantitative RT-PCR (RT-qPCR) targeting the L gene were reported.[11,12] These methods offer ideal specificity and sensitivity; however, they face limitations in providing rapid viral molecular diagnosis without a centralized laboratory, which is crucial for controlling early-stage virus dissemination. Due to the need for dedicate thermal cycling equipment, highly trained specialist, and also temperature-sensitive reagents, it has relatively higher cost and not suitable for rapid response to newly emerged threat with large-scale deployments.[13] Antigen-based tests, especially the rapid antigen test (RAT) that works similarly to the sandwich enzyme-linked immunosorbent assay (ELISA) on a lateral flow strip, were widely used as deployable point-of-care screening methods for at-home self-testing. Despite its limitations in sensitivity and specificity, the high demand for RAT during the pandemic underscored its value in pathogen control due to its rapid, user-friendly, and affordable screening capabilities. Therefore, combining the strengths of existing methods into novel, applicable molecular diagnostic approaches is crucial for enhancing public health protection against new viral threats. CRISPR-Cas-based biosensing technology, derived from prokaryotic adaptive immune systems, has attracted attention for developing rapid, deployable, and user-friendly point-of-care testing methods with PCR-level specificity and sensitivity. CRISPR-Cas-based diagnostic platforms, successfully detecting pathogens like Zika virus, dengue virus,[14] and SARS-CoV-2,[15] offer attomolar sensitivity (~1 copy/μL) and can genotype and discriminate single nucleotide polymorphisms (SNPs).[16–18] Despite its success in virus screening, CRISPR's ability to detect the newly emerged LayV and enable naked-eye diagnosis remains unexplored, posing a significant challenge for point-of-care clinical applications.

In this study, we developed two CRISPR-Cas12a-based methods for detecting the N-gene of LayV, each tailored to a different diagnostic scenario. Both methods employed dual-guidance RNA (gRNA) signal boosting strategy and hairpin-structured reporters to enhance the Cas12a *trans*-cleavage reaction, improving detection sensitivity and speed. Initially, we integrated CRISPR-Cas12a with recombinase polymerase amplification (RPA) for pre-amplification, enhancing the final fluorescent signal detection. This method can detect as few as 1 copy/μL for DNA and 10 copies/μL for RNA at room temperature, making it ideal for laboratories with a simple plate reader. To avoid nucleic acid pre-amplification and reduce the risk of amplicon contamination, we developed a horseradish peroxidase (HRP)-modified single-stranded DNA (ssDNA)-linked reporter to provide extra signal amplification. This approach also enables the final signal to be read colorimetrically, allowing for naked-eye detection of viral nucleic acids at the point-of-care. This secondary method provides rapid, simple, amplicon-free detection of LayV, achieving sensitivities of 80 copies/μL for DNA and 1,200 copies/μL for RNA within 20 min at room temperature (~25°C). These CRISPR-Cas12a-based LayV detection methods demonstrate the versatility and feasibility of CRISPR-Cas in addressing the diagnostic needs posed by emerging pathogens. Additionally, these biosensing systems can be easily adapted to meet various diagnostic needs in a scalable and cost-effective way.

RESULTS

Identification of LayV genome target and reaction condition for CRISPR-Cas12a-based detection

The henipavirus genome encodes several structural proteins, among which the N gene is conserved and commonly used as a target for molecular diagnosis of henipavirus. PCR diagnostic methods targeting the henipavirus N gene have demonstrated satisfactory specificity.[7,19] Consequently, the N gene was selected for RPA primer screening, incorporating a unique "5′-TTTV" sequence in the amplicon, essential for designing Cas12a gRNAs (Figure 1A). Five sets of RPA

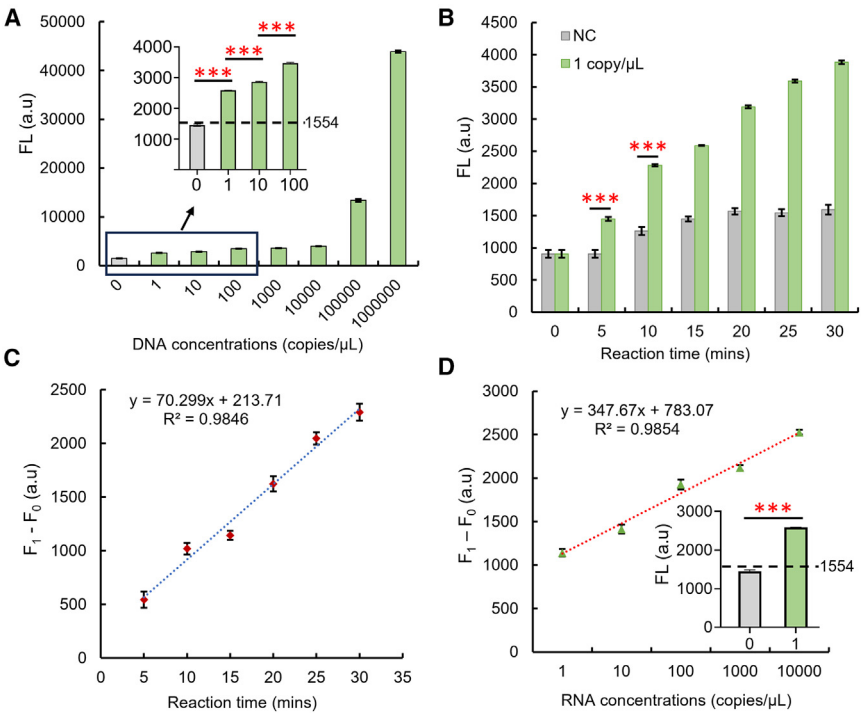

Figure 2. Combination of RPA with CRISPR-Cas12a for nucleic acid detection

(A) The system performance in response to the presence of target DNA at different concentrations, from 1 to 1,000,000 copies/μL (n = 3). The p values between 0 copies/μL and 1 copy/μL, 1 copy/μL and 10 copies/μL, and 10 copies/μL and 100 copies/μL are 1×10^{-6}, 2×10^{-5}, and 3×10^{-6} respectively.

(B) The detectable signal pattern in response to ultra-low DNA at 1 copy/μL, indicating a potential for rapid detection (n = 3). The p values between 1 copy/μL and NC at 5 min and 10 min are 1.4×10^{-4} and 1×10^{-5}, respectively.

(C) Linear correlation between reaction time to the increased fluorescence intensity levels (n = 3).

(D) Quantitative detection of DNA with a 4-logs linear range from 1 to 10,000 copies/μL, and sensitivity at 1 copy/μL (n = 3). The p values between 0 copies/μL and 1 copy/μL is 1×10^{-6}. Data are presented as mean ± SD. ***: $p < 0.001$, two-tailed t test. The dashed lines represent the threshold, and signals above it are considered positive.

reaction primers were designed and tested (Table S1), but only primer sets 4 and 5 yielded the expected amplicons (Figure 1B). The amplicon from primer set 5 showed higher band intensity on gel electrophoresis compared to that from primer set 4, indicating greater amplification efficiency (Figure 1B). Hence, primer set 5 was selected for further studies. Two gRNAs targeting either the *cis*- or *trans*-strand of the N gene (Figure 1A and Table S1) were combined with Cas12a protein to form the functional Cas12a ribonucleoprotein (RNP). Enzymatic activity tests on N gene fragment-inserted DNA plasmids demonstrated positive *trans*-cleavage activation by both gRNA-1 and gRNA-2 (Figure S1). As the quantity of target DNA increased, so did the intensity of Cas12a *trans*-cleavage activation (Figure 1C), demonstrating the effectiveness of the designed gRNAs in amplifying the signal for targeted LayV nucleic acid detection.

As the reaction time for CRISPR-Cas12a increased, the overall fluorescence intensity from the cleavage reporters also continuously rose (Figure S2). Similarly, increasing the concentration of reporters in the CRISPR-Cas12a reaction mixture also raised the signal intensity (Figure S3). The structure of fluorescence-quenched reporters can affect the *trans*-cleavage efficiency of Cas12a RNP.[20,21] Accordingly, two different reporter formats were tested in combination with two gRNAs for the CRISPR-Cas12a reaction (see Table S1 for reporter details). The results showed that under identical conditions, hairpin reporter 2 (Rp2) produced significantly higher reaction intensity compared to ssDNA reporter 1 (Rp1). Moreover, combining two gRNAs with reporter 2 enhanced the detectability of varying target sequence concentrations (Figure 1D).

Nucleic acid detection with combination of RPA reaction and CRISPR-Cas12a

With the functionally tested RPA primers and Cas12a gRNA design, the RPA and CRISPR-Cas12a reaction were then combined to response to the same nucleic acid target. To evaluate the combined system's performance, synthesized LayV N-gene DNA fragments ranging from 0 to 1×10^6 copies/μL were first used as targets. These were amplified via RPA for 15 min at room temperature, and the amplicons were then mixed with the CRISPR-Cas12a reaction mixture to generate fluorescence signals over 5–60 min at room temperature. The results showed that the combined RPA+CRISPR-Cas12a nucleic acid detection system could detect target DNA with a sensitivity of 1 copy/μL, producing fluorescence values above the threshold. Furthermore, a positive correlation was observed between the target DNA concentration and fluorescence signal intensity, suggesting potential for quantitative detection (Figure 2A). Notably, signals higher than the threshold were observed with 1 copy/μL of target DNA after just 5 min of CRISPR-Cas12a reaction, and these signal values were also significantly higher than the negative control. These differences increased as the reaction time extended up to 1 h (Figures 2B and S4). This demonstrated the potentials to realize rapid and ultra-high sensitivity nucleic acid detection. Further tests revealed a linear correlation between reaction time and the difference in fluorescence intensity of positive to negative reactions ($F_1 - F_0$) (Figure 2C), suggesting that device sensitivity in measuring fluorescence also affects detection time. Importantly, as target DNA concentration increased, the final signal from the RPA+CRISPR-Cas12a system showed a linear correlation with the detected fluorescence intensity (Figure 2D). This system provides a four-order

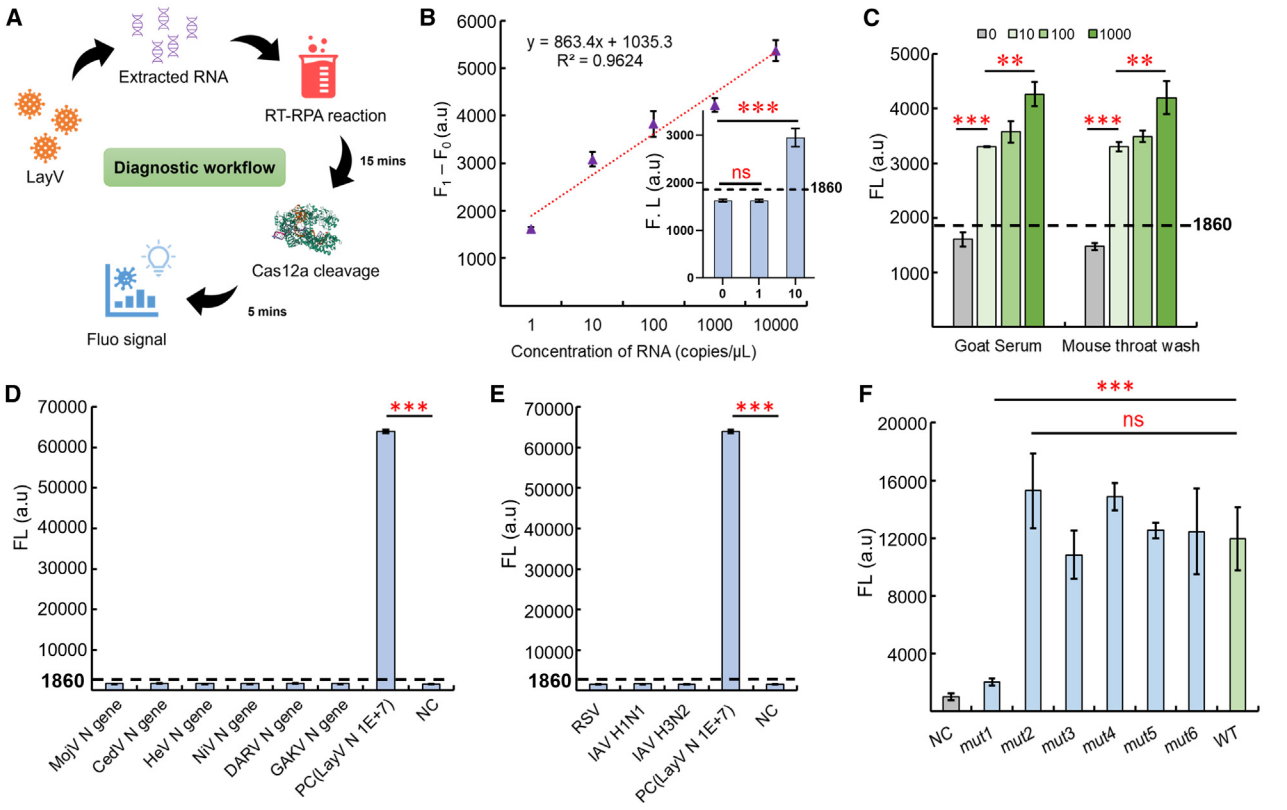

Figure 3. RT-RPA+CRISPR-Cas12a combined system for LayV RNA detection

(A) Schematic of LayV RNA detection with the RT-RPA+CRISPR-Cas12a combined nucleic acid detection system.

(B) Quantitative detection of LayV RNA with a 4-logs linear range from 1 to 10,000 copies/μL, and tested sensitivity at 10 copies/μL ($n = 3$). The p values between 1 copy/μL and 0 copies/μL, and between 10 copies/μL and 0 copies/μL are 0.928 and 3×10^{-4}, respectively.

(C) Detection of LayV RNA from complex animal samples ($n = 3$). In serum and throat wash samples, the p values for reactions between 10 copies/μL and 0 copies/μL are 2.2×10^{-5} and 8×10^{-6}, respectively; the p values for reactions between 10 copies/μL and 1,000 copies/μL are 1.7×10^{-3} and 7×10^{-3}, respectively.

(D) System specificity for RNA interferences ($n = 3$). The p value between PC and NC is 2.9×10^{-9}. MojV represents Mojiang virus, CedV represents Cedar virus, HedV represents Hendra virus, NiV represents Nipah virus, DARV represents Daeryong virus, and GAKV represents Gamak virus, NC represents negative control.

(E) System specificity for DNA interferences ($n = 3$). The p value between PC and NC is 2.9×10^{-9}. RSV represents respiratory syncytial virus, IAV represents influenza A virus, PC represents positive control, and NC represents negative control.

(F) Single nucleotide mutation detection with targeted mismatch nucleotide at various loci of Cas12a gRNA sequence ($n = 3$). "mut" is the abbreviation for mutation, and WT represents wild type. The p values between mut 1 to mut 6 and WT are 0.001, 0.163, 0.523, 0.099, 0.679, and 0.826, respectively. Data are presented as mean ± SD. ns: $p > 0.05$, **: $p < 0.01$, ***: $p < 0.001$, two-tailed t test. The dashed lines represent the threshold, and signals above it are considered positive.

magnitude linear range from 1 to 10,000 copies/μL, enabling quantitative detection of targeted DNA fragments with a sensitivity of 1 copy/μL (Figure 2D).

RT-RPA+CRISPR-Cas12a combined system for LayV RNA detection

By integrating reverse transcription with the RPA reaction, a standard LayV detection protocol using the RT-RPA+CRISPR-Cas12a combined biosensing system can be established, named fluorescence-integrated CRISPR-Cas12a. After the viral RNA extraction and reverse transcription, the entire detection process can be completed in 20 min at room temperature, eliminating the need for a thermocycler (Figure 3A). The results showed that this system can detect LayV RNA, achieving a sensitivity of 10 copies/μL and a linear range from 1 to 10,000 copies/μL (Figure 3B). To validate the system's ability to screen LayV RNA from various clinical samples, LayV RNA was spiked into goat serum and mouse throat wash samples at concentrations ranging from 0 to 1,000 copies/μL. The data indicated that the system maintained a sensitivity of 10 copies/μL in clinical samples. Additionally, we observed that the linearity was not as good as that seen in Figure 3B, which used pure RNA, especially in the mouse throat wash samples. This is likely because the LayV RNA was directly mixed with serum and throat wash after preparation without any RNA protectant, leading to varying degrees of RNA degradation. Despite this, we still observed a positive correlation between target RNA concentration and the detectable signal in these complex biological samples, ranging from 10 to 1,000 copies/μL (Figure 3C). This suggests that the method is suitable for direct clinical applications and demonstrates that interference from complex biological matrices has minimal impact on performance.

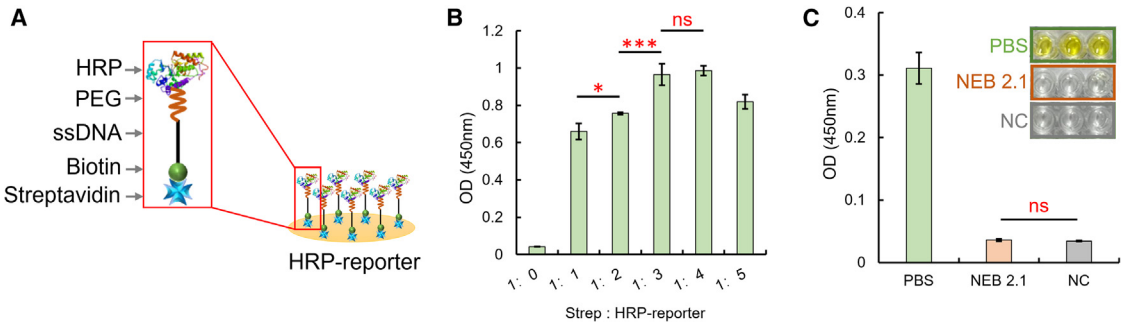

Figure 4. Development of amplification-free colorimetric CRISPR-Cas12a biosensing system

(A) Schematic of formation of HRP-based probe interface on high-binding 96-well plate.

(B) Optimization of HRP-reporter concentrations for surface fixation onto 96-well plate ($n = 3$). The p value between the 1:1 and 1:2 reactions is 0.018, between the 1:2 and 1:3 reactions is 0.003, and between the 1:2 and 1:3 reactions is 0.607. Strep represents streptavidin.

(C) Negative effect of buffering system on HRP enzymatic activity ($n = 3$). The p value between the NEB 2.1 and NC reactions is 0.279. NEB 2.1 represents NEBuffer 2.1. Data are presented as mean ± SD. NC represents negative control. ns: $p > 0.05$, *: $p < 0.05$, ***: $p < 0.001$, two-tailed t test.

Moreover, the system's specificity was investigated, demonstrating exceptional ability to differentiate targeted LayV RNA from other closely related interfering viral RNA sequences (Figure 3D). This high specificity was also confirmed against various viral cDNA interferences (Figure 3E). Subsequently, the RNA detection system's ability to handle single nucleotide mutations was evaluated. The targeted LayV RNA sequence was intentionally modified with various single nucleotide mismatches to test the CRISPR-Cas12a RNP targeting the wild-type LayV N gene (Figure S5). When these single mismatched RNA targets were tested, significant signal intensity changes were observed only when the mismatch (or mutation) occurred within the first three nucleotides of the gRNA's 5′ binding region (Figure 3F). This demonstrated the system's potential to differentiate closely related viral strains, contingent upon the use of properly designed gRNAs targeting the mutation region.

Development of amplification-free colorimetric CRISPR-Cas12a biosensing system

Although the aforementioned developed biosensing system exhibited high sensitivity and specificity for viral RNA detection under isothermal condition within 30 min, it shares limitations common to other isothermal amplification-assisted CRISPR-Cas12a biosensors. The reliance on fluorescence signals necessitates the use of a sensitive fluorescence spectrometer for signal measurement. Importantly, the abundant amplicons generated during isothermal amplification can lead to contamination, a significant concern for field-deployable diagnostic scenarios that lack centralized laboratory facilities and well-trained specialists. To extend our nucleic acid detection system's utility for field-deployable viral screenings, especially for a rapid response to newly emerging infectious threats, we designed a ready-to-use HRP-based colorimetric signal reporting probe for CRISPR-Cas12a-based LayV detection.

To optimize the conditions for binding the HRP-reporter to the 96-well plate surface, various streptavidin to HRP-reporter ratios were evaluated. A ratio of 1:3 was found to be optimal (Figure 4B) and was used in all subsequent tests. Although NEBuffer 2.1 offers optimal conditions for CRISPR-Cas12a *trans*-cleavage, it is detrimental to the HRP colorimetric reaction. It significantly suppresses HRP enzymatic activity and reduces colorimetric signal intensity (Figure 4C), potentially compromising system sensitivity.[22] Therefore, washing the 96-well plate with PBS buffer before adding the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate is crucial to maintaining optimal HRP detection performance.

Detection of the LayV with amplification-free CRISPR-Cas12a colorimetric biosensor

In a typical CRISPR-Cas12a *trans*-cleavage reaction,[23,24] replacing the fluorescence-quenched reporter with our colorimetric HRP-reporter eliminates the need for isothermal amplification processes like RPA. The HRP enzymatic activity functions as additional signal amplification. Consequently, this modification liberates the system from potential amplicon-related issues in nucleic acid diagnostics.[25] This also allows the CRISPR-Cas12a system to independently detect changes in target nucleic acid concentrations using a common absorbance reader, or even visually, under a simplified diagnostic protocol (Figure 5A).

With a 10-fold serial dilution of the target, this biosensor can produce positive OD450 values below the threshold (0.3) with 200 copies/μL (~330 aM) of targeted plasmid containing the LayV N-gene sequence (Figure 5B) and 2,000 copies/μL (~3.3 fM) of target RNA after reverse transcription (Figure 5C). The specificity of the HRP-integrated CRISPR-Cas12a system was verified, showing positive OD450 values below the threshold only in response to the presence of LayV target sequences (Figure 5D). Although this system trades some sensitivity compared to those with isothermal amplification, it may be more suitable for future point-of-care test development, particularly for rapid responses to newly emerging biological threats and large-scale prescreening in resource-limited areas.

To further explore the limit of detection (LoD) and quantitative capabilities of this colorimetric system for LayV detection, DNA targets ranging from 20 to 200 copies/μL and RNA targets ranging from 200 to 2,000 copies/μL were selected for detailed evaluation. This analysis revealed that the LoD of the colorimetric

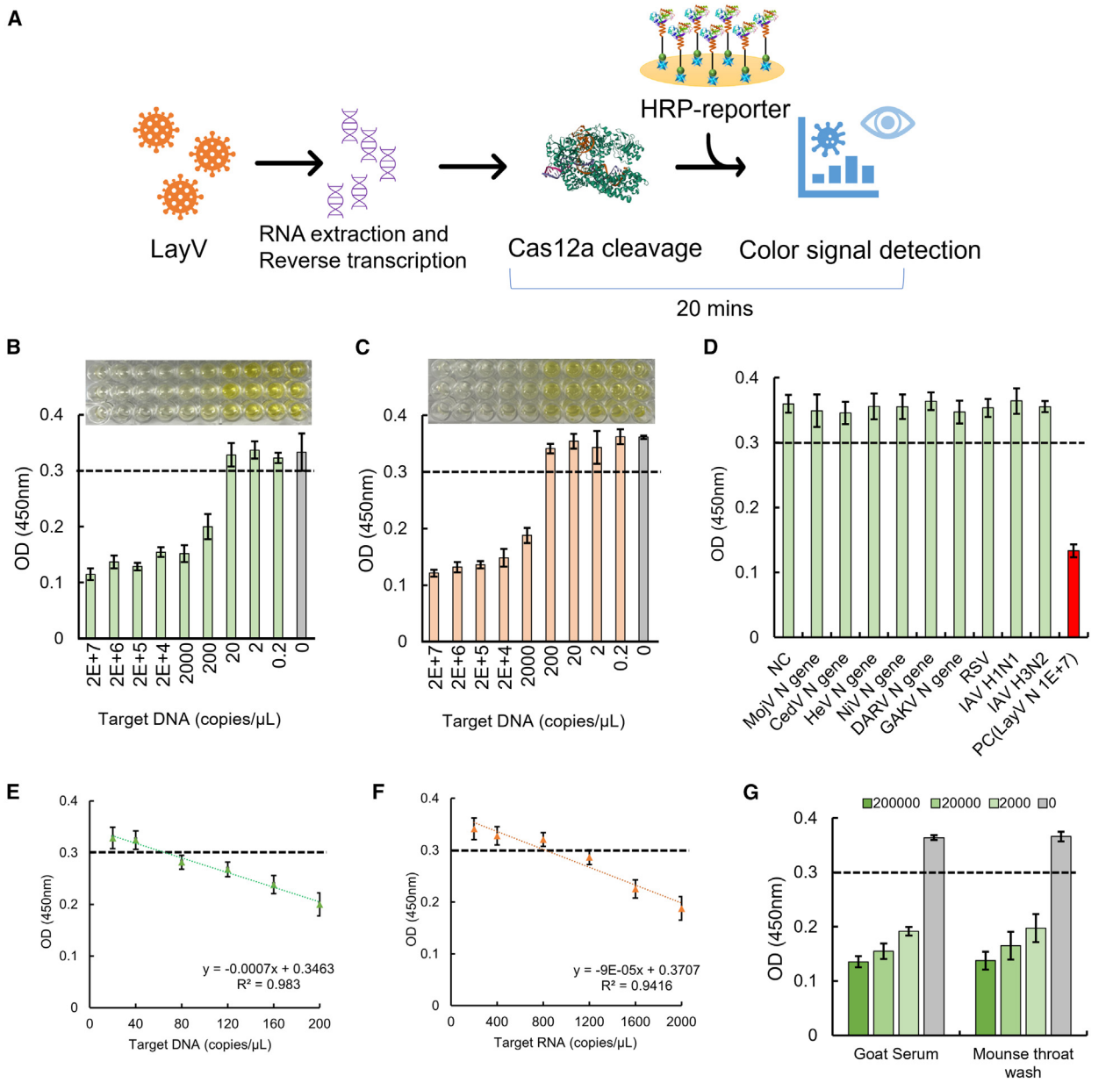

Figure 5. Detection of the LayV virus with amplification-free CRISPR-Cas12a colorimetric biosensor

(A) Schematic of the amplification-free CRISPR-Cas12a colorimetric biosensor for virus detection.

(B) Detection of DNA target with developed colorimetric biosensor, results of naked-eye observation and color intensity signal acquired by plate reader (n = 3).

(C) Detection of RNA target with developed colorimetric biosensor, results of naked-eye observation and color intensity signal acquired by plate reader (n = 3).

(D) System specificity test for the developed colorimetric biosensor. Both DNA and RNA interferences are applied (n = 3). NC represents negative control, MojV represents Mojiang virus, CedV represents Cedar virus, HedV represents Hendra virus, NiV represents Nipah virus, DARV represents Daeryong virus, and GAKV represents Gamak virus, RSV represents respiratory syncytial virus, IAV represents influenza A virus, PC represents positive control.

(E) Quantitative detection of target DNA with linear range from 20 to 200 copies/μL (n = 3).

(F) Quantitative detection of target RNA with linear range from 20 to 2,000 copies/μL (n = 3).

(G) Detection of targeted LayV RNA from complex biological samples (n = 3). Data are presented as mean ± SD. The dashed lines represent the threshold, and OD450 values below it are considered positive.

system was 80 copies/μL for DNA and 1,200 copies/μL for RNA, both producing OD450 values below the threshold. A linear correlation was observed for DNA detection between 20 and 200 copies/μL and for RNA detection between 200 and 2,000 copies/μL (Figures 5E and 5F). Additionally, LayV viral RNA spiked into goat serum and mouse throat wash samples was used to evaluate the HRP-integrated CRISPR-Cas12a system's performance with actual clinical specimens. The results

confirmed the system's ability to detect target RNA at concentrations of 2,000 copies/μL (~3.3 fM) in these complex samples, with a negatively correlated colorimetric change observed from 2,000 to 200,000 copies/μL (Figure 5G).

Additionally, we compared a conventional RT-PCR LayV detection method with our developed CRISPR-Cas12a LayV biosensors (Figure S6). The data show that the fluorescence-based system is 10^5 times (1×10^6 versus 10) more sensitive than conventional RT-PCR (Figures 2A and S6), and the HRP-integrated amplification-free colorimetric method is 833 times (1×10^6 versus 1,200) more sensitive when detecting LayV RNA targets (Figures 5C and S6).

Test system universality for the amplification-free colorimetric CRISPR-Cas12a biosensor

To demonstrate the inherent advantages of CRISPR-Cas-based biosensing technology for detecting new nucleic acid targets, we reprogrammed the gRNA in the HRP-integrated CRISPR-Cas12a biosensor to target the NP genes of influenza A virus (IAV) H1N1 and H3N2 strains, as well as the 16s gene of the *Porphyromonas gingivalis* (Pg) W83 strain. Proof-of-concept results showed that by replacing the original gRNA targeting LayV, our system could be adapted for detecting other viruses, exhibiting a similar negative correlation between target concentrations and colorimetric signal intensities (Figure S7).

DISCUSSION

The catastrophic impact of the COVID-19 pandemic highlighted the need for more effective and precise diagnostic methods in early-stage infectious disease control. The emergence of the zoonotic LayV further underscored this need within public healthcare systems. Although LayV has not yet resulted in large-scale infections, henipaviruses have historically caused widespread infections and fatalities in both humans and livestock.[4,5] In addition, the potential wildlife natural reservoirs and transmission pathway through saliva or respiratory secretions also indicated the need for a decentralized, end-user friendly, and fast point-of-care virus detection method. Recently, CRISPR-Cas biosensing possesses unparalleled precision in identifying distinct DNA and RNA sequences. Certain orthologues, such as Cas13[26] and Cas12a,[27] have demonstrated highly efficient non-specific enzymatic activities (*trans*-cleavage) following the recognition of specific target sequences. This unique functionality has been utilized in nucleic acid detection, achieving remarkable sensitivity and specificity.[28] Importantly, the ability to easily switch detection targets by simply replacing the gRNA makes CRISPR-Cas biosensing an ideal choice for rapidly responding to emerging infectious diseases.

In this study, CRISPR-Cas12a-based biosensing technology has been reprogrammed to detect the newly emerged LayV. We developed two approaches: a pre-amplification integrated CRISPR-Cas12a fluorescence biosensing system and an amplification-free colorimetric CRISPR-Cas12a system for potential point-of-care testing. To maximize the overall CRISPR-Cas12a *trans*-cleavage efficiency, the developed biosensing systems had implemented two additional enhancement strategies, including the use of 22nt hairpin ssDNA reporter design[20,21] and multiple gRNAs on a single target gene.[29] By combining these two strategies, the fluorescence based LayV detection system can reveal the presence of 10 copies/μL target RNA within 20 min (5-min CRISPR-Cas12a following 15-min RPA reaction). This performance is comparable to other reported CRISPR-Cas12a nucleic acid biosensing systems.[14,18,30–32] Importantly, by substituting the standard fluorescence-quenched ssDNA reporter with a specially designed HRP-reporter, we developed a rapid virus screening method suitable for resource-limited settings. Leveraging the highly efficient catalytic reaction of HRP,[20] which enhances the colorimetric signal, we eliminated the need for RPA pre-amplification. The system sensitivity is approximately 8,300 times (6.65×10^5 versus 80) higher than that of the pre-amplification-free fluorescent Cas12a assay when detecting DNA target (Figures 1D and 5B), with even faster detection time. Although the HRP-integrated CRISPR-Cas12a reaction is somewhat less sensitive compared to the fluorescence-integrated CRISPR-Cas12a reaction and a LayV qPCR method that reported a LoD of 10 copies/μL for DNA,[12] research has shown that high copy numbers of the LayV genome can be detected in respiratory samples from infected patients, especially those with pneumonia, reaching up to 7.64 ± 0.98 log10 copies/mL.[3] Similarly, a study on Nipah virus, which is closely related to LayV, revealed high titers of the virus in the respiratory tracts of early-stage patients.[33] Furthermore, as demonstrated in Figure 5G, the HRP-integrated CRISPR-Cas12a reaction showed good stability when detecting LayV RNA in serum and throat wash samples. Thus, these data suggest that the HRP-integrated CRISPR-Cas12a reaction can achieve the sensitivity required to detect LayV in clinical samples from infected patients.

Notably, eliminating nucleic acid amplification simplifies the experimental procedure and reduces equipment requirements. Additionally, it eliminates the risk of aerosol contamination from amplicons, a major source of false positives in subsequent detections.[34] These advantages enable untrained end-users to directly use our system without the need for a laboratory environment.

In a previous study, HRP was immobilized on magnetic beads via ssDNA.[22] This approach involved the activation of Cas enzymes, leading to the cleavage of ssDNA and subsequent release parts of HRP into the liquid phase. After removing the magnetic beads and adding the TMB substrate to the liquid phase, the released HRP catalyzed the TMB degradation, resulting in a colorimetric reaction. However, while effective, this method posed limitations in terms of reaction kinetics and system sensitivity. In this study, we took a divergent approach by innovatively anchoring the HRP-reporter complex directly within the reaction wells (Figure 4A). Activated Cas12a enzymes *trans*-cleave, releasing partial HRP into the liquid phase and thereby reducing the quantity of anchored HRP-reporter complexes in the wells. Following removal of the liquid phase, color development occurred through the degradation of TMB by residual HRP within the wells (Figure 5). Since the Cas reaction solution suppresses the enzymatic activity of HRP (Figure 4C), removing the liquid phase facilitates the subsequent HRP-mediated TMB degradation, enhancing detection sensitivity. Additionally, using

hairpin ssDNA reporters and multiple gRNAs as enhancement strategies improved DNA detection sensitivity by 75-fold compared to the previous study (from 6×10^3 copies/μL [10 fM] to 80 copies/μL). Our HRP-integrated CRISPR-Cas12a reaction completes within 20 min after RNA reverse transcription, faster than the previously reported method involving beads.[22] In conclusion, with a simple change to the gRNAs of CRISPR-Cas12a system, it has been directed to target the LayV specific genes for detection, with two different approaches developed here for different clinical diagnostic scenarios. The fluorescence-integrated CRISPR-Cas12a method can detect as few as 10 copies/μL of target RNA within 20 min using RPA pre-amplification, making it ideal for precise laboratory identifications. Alternatively, the HRP-integrated method requires no pre-amplification and can detect 1,200 copies/μL of target RNA within 20 min following reverse transcription, visible to the naked eye, making it ideal for point-of-care screening in resource-limited areas. The CRISPR-Cas12a-based LayV detection methods developed in this study offer a highly sensitive and versatile solution for LayV diagnostics and have the potential for rapid, sensitive, user-friendly, and affordable point-of-care responses to newly emerging pathogenic threats.

Limitations of the study

Like most RNA target detection methods, the approaches in this study also require RNA extraction and reverse transcription, which extends the detection time. Additionally, the RPA pre-amplification step in the fluorescence-integrated CRISPR-Cas12a method introduces the risk of aerosol contamination. Although careful laboratory practices and the scientific use of nucleic acid removal agents can effectively mitigate this risk, further optimization is needed to meet the simplicity and convenience required for point-of-care test. For example, exploring RNA-targeting Cas orthologues, adopting a ''one-pot'' strategy to reduce aerosol contamination risk, or employing methods to enhance Cas enzyme activity to eliminate reliance on pre-amplification. Although the HRP-integrated CRISPR-Cas12a method is simple, does not rely on pre-amplification, and has a sensitivity comparable to reported LayV qPCR,[11] further sensitivity enhancement is necessary to improve the accuracy of this method. This includes optimizing Cas reaction components and conditions, as well as exploring Cas orthologues with stronger *trans*-cleavage activity.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yi Li (yi.li@cqmu.edu.cn).

Materials availability

This study did not generate any unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (grant numbers 32060024 and 32000088) and the Natural Science Foundation of Hunan Province (grant number 2022JJ40050).

AUTHOR CONTRIBUTIONS

Conceptualization, X.W., N.P., and Y.L.; methodology, X.W. and R.X.; investigation, X.H., L.Z., F.S., and H.L.; formal analysis, X.W., R.X., and D.Y.; writing – original draft, X.W. and Y.L.; writing – review & editing, X.W., R.X., and Y.L.; visualization, X.W. and R.X.; supervision, N.P., and Y.L.; funding acquisition, X.W. and R.X.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Mice
  - Microbe strains
- METHOD DETAILS
  - Preparation of nucleic acid sequences
  - RNA detection with RT-PCR assay
  - RPA preamplification
  - Fluorescence-integrated CRISPR/Cas12a assay
  - Synthesis of HRP-reporter conjugates
  - HRP-integrated CIRSPR/Cas12a assay
  - Detection of LayV from clinical samples
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.111466.

Received: September 4, 2024
Revised: October 15, 2024
Accepted: November 21, 2024
Published: November 23, 2024

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Porphyromonas gingivalis: strain: W83	ATCC	ATCC BAA-308
Influenza A virus: H1N1 subtype	Lad stored	N/A
Influenza A virus: H3N2 subtype	Lad stored	N/A
Human Respiratory Syncytial Virus: strain: A2	ATCC	ATCC VR-1540
Chemicals, peptides, and recombinant proteins		
2× M5 HiPer Pfu PCR MasterMix	Mei5	Cat.# MF162
10× NEBuffer 2.1	NEB	Cat.#B7202
LbCas12a protein	NEB	Cat.#M0653T
DTT	Aladdin Scientific	CAS: 3483-12-3
HRP	Aladdin Scientific	CAS: 9003-99-0
Azido-PEG4-NHS ester	Aladdin Scientific	CAS: 944251-24-5
TMB	Aladdin Scientific	CAS: 54827-17-7
Streptavidin	Beyotime	Cat.#P5084
TRIzol	Invitrogen	Cat.# 15596026CN
RNase-free DNase I	NEB	Cat.#M0303
Critical commercial assays		
PrimeScript™ Double Strand cDNA Synthesis Kit	TaKaRa	Cat.#: 6111A
TwistAmp® Basic Kit	TwisDx Inc.	Cat.#: TABAS03KIT
HiScribe™ T7 High Yield RNA Synthesis Kit	NEB	Cat.#: E2040S
Experimental models: Cell lines		
Dog: MDCK cells	ATCC	Cat.#: CCL-34
Human: HEp-2 cells	ATCC	Cat.#: CCL-23
Experimental models: Organisms/strains		
Mouse: C57BL/6	Chongqing Medical University	N/A
Oligonucleotides		
See Table S1 for the sequences of DNA and RNA oligos	Synthesized by Tsingke Biotech	N/A
Recombinant DNA		
LayV-N-pUC19 plasmid	Synthesized by Tsingke Biotech	GenBank: OM101128.1
Mojiang virus-N-pUC19 plasmid	Synthesized by Tsingke Biotech	GenBank: NC_025352.1
Cedar virus-N-pUC19 plasmid	Synthesized by Tsingke Biotech	GenBank: NC_025351.1
Hendra virus-N-pUC19 plasmid	Synthesized by Tsingke Biotech	GenBank: NC_001906.3
Nipah virus-N-pUC19 plasmid	Synthesized by Tsingke Biotech	GenBank: NC_002728.1
Daeryong virus-N-pUC19 plasmid	Synthesized by Tsingke Biotech	GenBank: MZ574408
Gamak virus-N-pUC19 plasmid	Synthesized by Tsingke Biotech	GenBank: MZ574409
cDNA of IAV H1N1	This paper	N/A
cDNA of IAV H3N2	This paper	N/A
cDNA of RSV A2	This paper	N/A
Software and algorithms		
Primer Premier 6 software	Premier Biosoft	https://www.premierbiosoft.com/primerdesign/index.html
GraphPad Prism version 8	GraphPad Software Inc.	https://www.graphpad.com/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice

The animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Chongqing Medical University (no. 2019653). Twelve female C57BL/6 mice, aged 6–8 weeks, were obtained from the Experimental Animal Center of Chongqing Medical University for the collection of throat wash fluid. Mice were anesthetized by intraperitoneal injection of 4% sodium pentobarbital, after which 200 μL of sterile PBS was gently injected into the throat using a 1 mL syringe. The PBS was allowed to mix with secretions for a few seconds and then carefully aspirated back into the syringe. The collected throat wash fluid was used in subsequent experiments to simulate clinical samples. All mice were treated humanely in accordance with ethical guidelines.

Microbe strains

The Pg W83 strain (ATCC BAA-308) were purchased from ATCC and stored in the lab. The W83 strain was cultured anaerobically at 37°C for 2 to 3 days in brain heart infusion (BHI) broth, with the medium supplemented by 5 mg/L hemin and 5 mg/L menadione.

IAV was cultured in Madin-Darby Canine Kidney (MDCK) cells (ATCC CCL-34). MDCK cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO_2. The virus was adsorbed onto cells at 80–90% confluence for about an hour. The cells were then incubated at 37°C in a 5% CO_2 atmosphere with serum-free medium containing 2 μg/mL of trypsin to facilitate viral replication. After 2–4 days, the virus was harvested upon observing cytopathic effects (CPE). After centrifugation at 4000 × g for 15 min, the supernatant was collected, aliquoted, and stored at −80°C with a final concentration of 10% glycerol.

Human Respiratory Syncytial Virus (RSV) was cultured in HEp-2 cells (ATCC CCL-23). HEp-2 cells were cultured in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere with 5% CO_2. When the cells reached 80–90% confluence, virus adsorption was carried out at 37°C for 1–2 h. Afterward, the cells were incubated at 37°C in a 5% CO_2 atmosphere. The virus was harvested when 80% of the cells displayed cytopathic effects (CPE), centrifuged at 4000 × g for 15 min, the supernatant was collected, aliquoted, and stored at −80°C with 10% glycerol.

METHOD DETAILS

Preparation of nucleic acid sequences

To establish LayV nucleic acid detection, the N gene of LayV was synthesized. Subsequently, cDNAs corresponding to RSV and IAV H1N1 and H3N2, preserved in our experimental repository, along with synthesized N genes of six distinct Paramyxoviruses—Mojiang, Cedar, Hendra, Nipah, Daeryong, and Gamak viruses—were prepared to investigate the system's specificity. All the above sequences were synthesized by Tsingke Biotech (Beijing, China), cloned into the pUC19 vector, and stored at −80°C for future use.

Furthermore, cDNA of IAV H1N1 and H3N2, along with DNA from the Pg W83 strain, were used to confirm the HRP-integrated CRISPR/Cas12a biosensor's universality by replacing the LayV N-targeting gRNA with ones targeting the H1N1 and H3N2 NP genes, and Pg 16s gene. Detailed sequences information is listed in Table S1.

To test whether our detection methods can detect LayV RNA, we synthesized LayV RNA *in vitro* using the HiScribe T7 High Yield RNA Synthesis Kit (NEB, Cat.#E2040S) following the manufacturer's protocol. The reaction mixture consisted of 2 μL 10× Reaction Buffer, 2 μL each of 100 mM ATP, GTP, UTP, and CTP, 1 μL of 0.1 M dithiothreitol (DTT), 2 μL of T7 RNA Polymerase Mix, 1 μg of LayV N gene sequence with a 5′ T7 promoter, and nuclease-free water to a final volume of 20 μL. The mixture was gently mixed and incubated at 37°C for 2 h. To remove the DNA template after transcription, 70 μL of nuclease-free water, 10 μL of 10× DNase I Buffer, and 2 μL of RNase-free DNase I (NEB, Cat.#M0303) were added to each reaction. RNA was then purified using TRIzol (Invitrogen, Cat.# 15596026CN), and the RNA concentration was quantified using the Qubit 4 Fluorometer (Thermo Fisher Scientific Inc., Massachusetts, USA).

RNA detection with RT-PCR assay

RT-PCR was employed as the standard methodology for assessing the detection assays. PCR primers (Table S1) were designed using Primer Premier 6 software (Premier Biosoft). cDNA synthesis was performed with the PrimeScript Double Strand cDNA Synthesis Kit (TaKaRa, Cat.# 6111A), following the manufacturer's guidelines. Briefly, the 1st strand cDNA synthesis involves a reaction with mixture 1, which contains 1 μL dNTP, 2 μL random primer, RNA, and RNase-free ddH_2O to a final volume of 10 μL. After incubating at 65°C for 5 min, place on ice, then combine mixture 1 with mixture 2, which contains 4 μL 5× 1st Strand Synthesis Buffer, 1 μL RNase Inhibitor, 1 μL PrimeScript RTase, and 4 μL RNase-free ddH_2O, and react at 42°C for 1 h. This is followed by a 2-min incubation on ice. Store the resulting cDNA at −20°C for use in Cas12a or PCR assays.

The PCR reaction mixture included 12.5 μL of 2× M5 HiPer Pfu PCR MasterMix (Mei5, Cat.# MF162), 10 pmol of each primer, 1 μL of cDNA as the template, and double-distilled water (ddH_2O), totaling 25 μL. The PCR cycling parameters included an initial denaturation step for 5 min at 94°C, followed by 32 cycles of denaturation at 94°C for 25 s, annealing for 25 s, extension at 72°C for 20 s, and a final extension step at 72°C for 10 min.

RPA preamplification

The primers used for RPA were the same as those used in the PCR assays. RPA reactions were conducted following the manufacture instruction of the TwistAmp Basic Kit (TwisDx Inc., Cat.# TABAS03KIT). Briefly, the reaction mixture consisted of 24 pmol of each primer, 29.5 μL of Primer Free Rehydration buffer, cDNA or N-pUC19 plasmid, and water, for a total volume of 47.5 μL. Subsequently, 2.5 μL of 280 mM MgOAc was introduced to initiate the reaction. After incubating for 30 min at room temperature (25°C), the products were either analyzed by gel electrophoresis for amplicon identification or used to trigger the subsequent CRISPR/Cas12a assay.

Fluorescence-integrated CRISPR/Cas12a assay

The selection of the gRNA sequences (Table S1) was predicated on identifying the protospacer adjacent motif (PAM) present in analogous sequences within the N gene. The Cas12a reaction mixture was prepared by mixing 10 μL of 10× NEBuffer 2.1 (NEB, Cat.#B7202), 6 pmol of LbCas12a protein (NEB, Cat.#M0653T), 6 pmol of gRNA, 20 pmol of reporter ssDNA (ssDNA modified with 5′-Texas Red moieties and 3′-BHQ2 moieties), 1 μmol of DTT (Aladdin Scientific, CAS: 3483-12-3), 10 μL of trigger DNA or cDNA (at varying concentrations to test sensitivity) and sufficient ddH_2O to reach a total volume of 100 μL. The thoroughly mixed reaction mixture was incubated at room temperature for 5 to 60 min. Fluorescence intensity was measured using the EnSight Multimode Plate Reader (PerkinElmer, Massachusetts, USA), with an excitation wavelength of 570 nm and an emission wavelength of 615 nm.

Synthesis of HRP-reporter conjugates

The click chemistry method was used to synthesize HRP-reporter conjugates.[22] Briefly, 0.05 μmol HRP (Aladdin Scientific, CAS: 9003-99-0) was dissolved in 1 mL of 0.1 M $NaHCO_3$, creating a 50 μM solution. Subsequently, 50 mmol azido-PEG4-NHS ester (Aladdin Scientific, CAS: 944251-24-5) was added to react with HRP for 2 h at room temperature. After a sufficient reaction, azide-functionalized HRP was purified using 30-kDa MWCO spin filters three times, centrifuging each time at 4,000 rcf for 5 min. Next, 200 μL of 10 μM azide-functionalized HRP and 2 nmol of ssDNA3, modified with Dibenzocyclooctyne (DBCO) and Biotin (Table S1), were mixed and incubated for 15 h at room temperature to form HRP-reporter conjugates via DBCO-azide bonding. The resulting HRP-reporter conjugates were purified three times using 30 kDa MWCO spin filters, centrifuging at 4,000 rcf for 5 min each time.

HRP-integrated CIRSPR/Cas12a assay

The principle of HRP-integrated CRISPR/Cas12a assay: The HRP-reporter is coated onto reaction wells through a biotin-avidin interaction to form the final probe interface (Figure 4A). Subsequently, the prepared Cas12a reaction mixture is introduced into the reaction wells. When the probe interface encounters activated CRISPR/Cas12a—triggered by the presence of target nucleic acids—the *trans*-cleavage activity continuously cleaves the ssDNA linker of the HRP-reporter, releasing HRP into the liquid phase and reducing its quantity on the wells. Higher concentrations of target nucleic acids are expected to activate more CRISPR/Cas12a resulting in more HRP proteins being removed from the probe interface. Eventually, after discarding the liquid phase and adding the 3,3′,5,5′-Tetramethylbenzidine (TMB) colorimetric substrate (Aladdin Scientific, CAS: 54827-17-7), which the HRP enzyme digests, an enhanced colorimetric signal is produced. As higher concentrations of target nucleic acid leads to less HRP remaining on the 96-well plate surface, a negative correlation is expected between target concentration and colorimetric intensity.

Preparation of HRP-reporter coated plate: First, streptavidin (Beyotime Biotechnology, Cat.#P5084) was reconstituted to a concentration of 1 ng/μL in 0.05 M carbonate buffer (pH 9.6). 100 μL of streptavidin solution (containing about 1.9 pmol streptavidin) was added to each well of a high-binding 96-well plate and incubated overnight at 4°C to ensure fixation. The wells were then emptied and the plate was washed three times with 200 μL of 0.02 M PBS containing 0.05% (v/v) Tween 20, each wash lasting 5 min. Subsequently, 100 μL of 2% (w/v) bovine serum albumin (BSA) solution was added to block the wells, followed by incubation for 2 h at room temperature. Afterward, the wells were emptied and the plate was washed three times using the same protocol as previously described. Theoretically, streptavidin can bind four biotinylated HRP-reporter molecules simultaneously. However, steric hindrance may prevent all four HRP-reporters from binding to the same streptavidin molecule in practice. Therefore, varying amounts of HRP-reporters (0–9.5 pmol), from 0 to 5 times the molar excess of streptavidin, were added to each well to determine their maximum binding capacity. The plate was then incubated at room temperature for 2 h to allow for the immobilization of biotinylated HRP-reporters through biotin-streptavidin interaction. Then, three thorough wash cycles were performed. The prepared HRP-reporter-coated plates were stored at 4°C for future use.

To evaluate the sensitivity of the HRP-integrated CRISPR/Cas12a reaction, the Cas12a reaction mixture consisted of 0.2–2×10^7 copies/μL of target DNA or cDNA, 10 μL of 10× NEBuffer 2.1, 6 pmol of Cas12a protein, 6 pmol of gRNA, 1 μmol of DTT, and ddH_2O to a final volume of 100 μL. After thorough mixing, the mixture was transferred into the HRP-reporter-coated wells. The reaction was carried out at room temperature for 5 min, enabling the CRISPR/Cas12a to cleave the immobilized HRP-reporter in the wells. Subsequently, the liquid was aspirated from the wells, followed by three wash cycles. Then, 50 μL of TMB substrate was added to each well and incubated at 37°C for 15 min. Subsequently, 50 μL of 2 M H_2SO_4 was added to each well to stop the reaction. The optical density at 450 nm (OD450) was measured using an ELISA reader.

Detection of LayV from clinical samples

To thoroughly assess the CRISPR/Cas12a assays' performance in detecting target RNA in clinical samples, gradient-diluted target RNA was mixed with 250 μL of goat serum or murine throat wash fluid, simulating clinical specimens. Following sample preparation, total RNA was extracted from these simulated clinical samples using TRIzol. The extracted RNA was then reverse transcribed to form target cDNA, which was used directly in the Fluorescence-integrated CRISPR/Cas12a-based system and the HRP-integrated CRISPR/Cas12a nucleic acid detection system.

QUANTIFICATION AND STATISTICAL ANALYSIS

All experiments in this study were performed in triplicate. Differences in fluorescence and OD450 were statistically analyzed using two-tailed t-test, performed with GraphPad Prism version 8 (GraphPad Software Inc.). Statistical significance was defined as ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$.

In fluorescence CRISPR/Cas12a assays under the same reaction conditions, the threshold was set as the mean of the negative control (NC) plus 3 times the standard deviation (SD). Reactions producing values higher than this threshold were considered positive, while those below were considered negative. In HRP-integrated CRISPR/Cas12a assays, the threshold was defined as the mean OD450 of the NC minus 3 times the SD. Reactions with OD450 values below the threshold were considered positive, while those above were considered negative.