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# Portable amplification-free digital droplet CRISPR/Cas12a platform for one-pot multiplexed viruses detection with attomolar sensitivity

Zhirun Ji [a,b], Yiran Zhao [a,b], Juncan Huang [c], Yanke Shan [a,b], Jiahao Li [d], Qinghua Huang [e,*], Minghui Wu [a,b,**], Fei Liu [a,b,***]

[a] Joint International Research Laboratory of Animal Health and Food Safety of Ministry of Education, Single Molecule Biochemistry & Biomedicine Laboratory (Sinmolab), College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, 210095, China
[b] Sanya Institute of Nanjing Agricultural University, Sanya, 572000, China
[c] Beijing University of Technology, Beijing, 100124, China
[d] Key Laboratory of Veterinary Biological Engineering and Technology, Ministry of Agriculture, Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Nanjing, 210014, China
[e] Ruichang Academy of Nuclear Physics Application, Jiangxi, 332200, China

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ABSTRACT

High-throughput, sensitive, and field-deployable nucleic acid detection is essential for timely pathogen surveillance. Here, we developed a novel amplification-free and portable platform based on an instrument-free polydisperse digital droplet CRISPR/Cas12a (DD-Cas12a) assay, enabling the simultaneous detection of three synthetic nucleic acid targets in a single reaction. The platform achieved attomolar-level sensitivity (0.15 fM) without pre-amplification, generated detection results within 40 min, and maintained high specificity even in the presence of a 10-fold excess of non-target nucleic acids. The DD-Cas12a was further applied to detect one DNA virus, porcine circovirus type 2 (PCV2), and two RNA viruses, porcine epidemic diarrhea virus (PEDV) and porcine reproductive and respiratory syndrome virus (PRRSV). The detection limits for PCV2, PEDV, and PRRSV were 126.0, 673.5, and 584.5 fg/μL, respectively, corresponding to sensitivity comparable to qPCR for PCV2 and 10-fold higher sensitivity for PEDV and PRRSV. To improve analytical throughput and objectivity, we established an Algorithm-Driven DD-Cas12a (ADC) platform for automated image analysis and result decoding. The custom-designed miniaturized portable instrument was developed for signal acquisition and field deployment. By combining the DD-Cas12a assay with the ADC platform and the portable instrument, we established a portable, digital, amplification-free, and ultrasensitive system for multiplex pathogen detection, providing a powerful tool for diagnostics, biosurveillance, and environmental monitoring.

## 1. Introduction

Rapid and accurate pathogen detection plays an essential role in disease surveillance, clinical diagnosis, and outbreak control (Feng et al., 2020; Liu et al., 2020). In both human and animal health, as well as in food safety and environmental monitoring, timely identification of pathogenic agents enables effective intervention measures, limits transmission, and reduces economic losses (Ceresini et al., 2024; Meliana et al., 2024; Tong et al., 2024). Conventional nucleic acid diagnostics primarily rely on quantitative polymerase chain reaction (qPCR), a method hampered by requirements for sophisticated thermal cycling, extended assay times, and skilled personnel (Blairon et al., 2021; Lima et al., 2022; Ma et al., 2021; Vogels et al., 2020). Isothermal amplification technologies, such as recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), and rolling circle amplification (RCA), have been widely adopted for pathogen detection without thermal cycles (Gadkar et al., 2018; Jeong et al., 2016; Zhang et al., 2024). While these methods facilitate rapid analysis,

* Corresponding author.
** Corresponding author. Joint International Research Laboratory of Animal Health and Food Safety of Ministry of Education, Single Molecule Biochemistry & Biomedicine Laboratory (Sinmolab), College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, 210095, China.
*** Corresponding author. Joint International Research Laboratory of Animal Health and Food Safety of Ministry of Education, Single Molecule Biochemistry & Biomedicine Laboratory (Sinmolab), College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, 210095, China.
*E-mail addresses:* 172739971@qq.com (Q. Huang), minghuiwu@njau.edu.cn (M. Wu), feiliu24@njau.edu.cn (F. Liu).

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typically yielding results within 10-30 min, their broader clinical implementation is frequently constrained by inadequate analytical sensitivity and an elevated risk of cross-contamination (Saki et al., 2021; Srivastava and Prasad, 2023; Wang et al., 2025). Moreover, multiplex detection based on these isothermal amplification technologies typically requires rigorous optimization to avoid cross-reactivity and signal interference, severely limiting their practical applicability. In the global swine industry, the high prevalence of co-infections scenarios urgently demands reliable simultaneous detection of critical epidemic pathogens. Representative targets include the RNA viruses of porcine epidemic diarrhea virus (PEDV) and porcine reproductive and respiratory syndrome virus (PRRSV), as well as the DNA virus of porcine circovirus type 2 (PCV2). Existing diagnostic strategies for these pathogens primarily rely on multiplex qPCR (Feng et al., 2024; Wang et al., 2024; Ruan et al., 2023) and isothermal techniques, such as LAMP (Areekit et al., 2022; Hong et al., 2023; Zhou et al., 2020) and RPA (Sun et al., 2025; Ye et al., 2024; Xia et al., 2022). However, their reliance on target amplification makes them suffer from inherent quantification bias and elevated risks of cross-contamination. Therefore, there is an urgent imperative to develop an advanced isothermal, amplification-free, and highly specific sensing approach for the simultaneous quantification of mixed RNA and DNA targets, aiming to overcome these analytical bottlenecks above.

Among the emerging diagnostic technologies, the clustered regularly interspaced short palindromic repeats and CRISPR-associated protein (CRISPR-Cas) system has emerged as a powerful tool for specific nucleic acid detection (Chen et al., 2018; Li et al., 2018a,b; Lu et al., 2022). Currently, the majority of CRISPR-Cas diagnostic (CRISPR-Dx) techniques necessitate pre-amplification of the target nucleic acid to be as sensitive as qPCR (Ding et al., 2020a,b; Pang et al., 2020; Zhang et al., 2021). However, a major challenge is that the incompatibility of reaction conditions between nucleic acid amplification and CRISPR-based detection, which necessitates their separation into distinct steps (Broughton et al., 2020). Consequently, early CRISPR-Dx platforms, such as DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) and One-Hour Low-cost Multipurpose Highly Efficient System (HOLMES), were designed as two-step, sequential workflows involving an initial amplification step followed by the manual addition of CRISPR components (Chen et al., 2018; Li et al., 2018a,b; Wang et al., 2021; Yin et al., 2020). This segmented approach increases operational complexity, prolongs the total assay time, and elevates the risk of aerosol-mediated nucleic acid contamination (Ding et al., 2020a,b; Hu et al., 2023; Kellner et al., 2019). To address this problems, different improvements have been made to achieve one-pot assays, such as light-activated CRISPR-Cas systems (Chen et al., 2022; Hu et al., 2022), phase separation methods (Uno et al., 2023), and suboptimal protospacer adjacent motif (PAM) sequences (Lu et al., 2022). However, these approaches not only require extra reagents and strict target sequence screening, but also suffer from suppressed Cas enzyme activity, which leads to reduced reaction efficiency and prolonged assay times. Alternatively, strategies based on physical isolation compartmentalize amplification and CRISPR detection within distinct microfluidic structures, including centrifugal devices, pressure-driven channels, and capillary networks (Yin et al., 2024; Zhang et al., 2025). Although promising in principle, these systems are hindered by the requirements for sophisticated microchannel designs, precise instrumentation, and high manufacturing costs, ultimately limiting their practical deployment and broader adoption.

Among microfluidic technologies, microdroplet-based assays represent a specialized platform for amplification-free nucleic acid detection. By splitting traditional bulk reactions into individual microreactors, they substantially reduce the interference caused by background signals and matrix inhibitors (Xu et al., 2025; Xue et al., 2023). This compartmentalization effectively increases molecular crowding within these droplets, significantly amplifying the reaction signal and even enabling single-molecule detection without amplification (Tian et al., 2021; Yue et al., 2021). While droplet-based CRISPR detection has been demonstrated, existing systems typically depend on specialized microfluidic apparatus to generate uniform droplets, limiting their accessibility (Liu et al., 2022; Shang et al., 2024). Although a few CRISPR-based droplet detection methods have been reported for multiplexed assays, they typically rely on multi-enzyme strategies, which increase assay complexity and cost (Cheng et al., 2025; Wan et al., 2024). Furthermore, the analysis of droplets currently depends on semi-automated software, such as ImageJ, introducing subjectivity and low throughput. Consequently, advancing the practical application of CRISPR-droplet technology requires the development of simplified multiplexing strategies integrated with automated, objective image analysis.

Here, we report a microfluidics-free, amplification-free polydisperse digital droplet CRISPR/Cas12a (DD-Cas12a) platform for the simultaneous, multiplexed digital detection of three pathogenic nucleic acids. To enable multiplexed digital detection, droplets were encoded with spectrally distinct fluorescent reporters, corresponding to the specific synthetic nucleic acid targets. The automated Algorithm-Driven DD-Cas12a (ADC) platform was established to replace manual analysis, enabling rapid and objective signal interpretation. A miniaturized instrument was developed for portable signal acquisition and on-site detection. By combining the DD-Cas12a assay with the ADC platform and the miniaturized instrument, we established an integrated system for portable, digital, amplification-free, and ultrasensitive multiplexed pathogen detection, with significant potential for applications in diagnostics and environmental monitoring.

## 2. Experimental methods

### 2.1. Chemicals and materials

All the DNA and RNA sequences (Table S1) were synthesized and purified by Sangon Biotech, diluted with RNase-free water. ABIL EM 180 was purchased from Evonik Industries. Isopropyl palmitate was obtained from Macklin Biochemical. HiScript II Q RT SuperMix for qPCR was bought from Vazyme. EasyPure Simple Viral DNA/RNA Kit was purchased from TransGen Biotech. The 10x NEBuffer2.1 was purchased from New England Biolabs. The Cas12a protein was from laboratory expression and purification. DI-water used in the experiment was processed with a Milli-Q system.

### 2.2. Bulk CRISPR/Cas12a assay

The volume of the reaction mixture for the CRISPR-Cas12a assay was 100 μl, which contained 50 nM Cas12a-crRNA, 1x NEBuffer 2.1 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 100 μg/mL BSA, pH 7.9), 500 nM ssDNA reporter labelled with Atto 425 and BHQ1 (AQ) or 6-FAM and BHQ1 (FQ) or TAMRA and BHQ2 (TQ) and corresponding to PCV2, PEDV, and PRRSV target templates, respectively. The real-time fluorescence signal was recorded every 30 s under the Tecan microplate reader.

### 2.3. Digital droplet CRISPR/Cas12a assay

The reaction mixture for DD-Cas12a assays was identical to that used in the bulk fluorescence-based assays. To generate droplets, 5 μL of the respective target reaction mixtures and 100 μL of oil phase (comprising 90% isopropyl palmitate and 10% Abil EM 180, v/v) were vortexed separately for 5 s, yielding polydisperse emulsions. Following vortexing, the droplet emulsions containing different targets were pooled and incubated for 30 min. Subsequently, the emulsion was deposited onto a glass slide, and droplet images were acquired using a Nikon fluorescence inverted microscope equipped with a 10x objective lens. Fluorescence excitation was performed at wavelengths specific to each target: 475 nm for PCV2, 515 nm for PEDV, and 595 nm for PRRSV. The entire DD-Cas12a detection process, from droplet generation to signal acquisition, was completed within 40 min.

### 2.4. Cell culture and DNA/RNA extraction

PK-15, Vero, and Marc145 cell lines were grown in Dulbecco's modified Eagle medium (Gibco) and supplemented with 5% FBS (Excell Bio) at 37 °C in a humid atmosphere containing 5% $CO_2$. DNA and RNA was extracted from the cell lines mentioned above using EasyPure Simple Viral DNA/RNA Kit according to the manufacturer's protocols, and the extracted RNA was subsequently reverse transcribed into cDNA using HiScript II Q RT SuperMix for qPCR experiments.

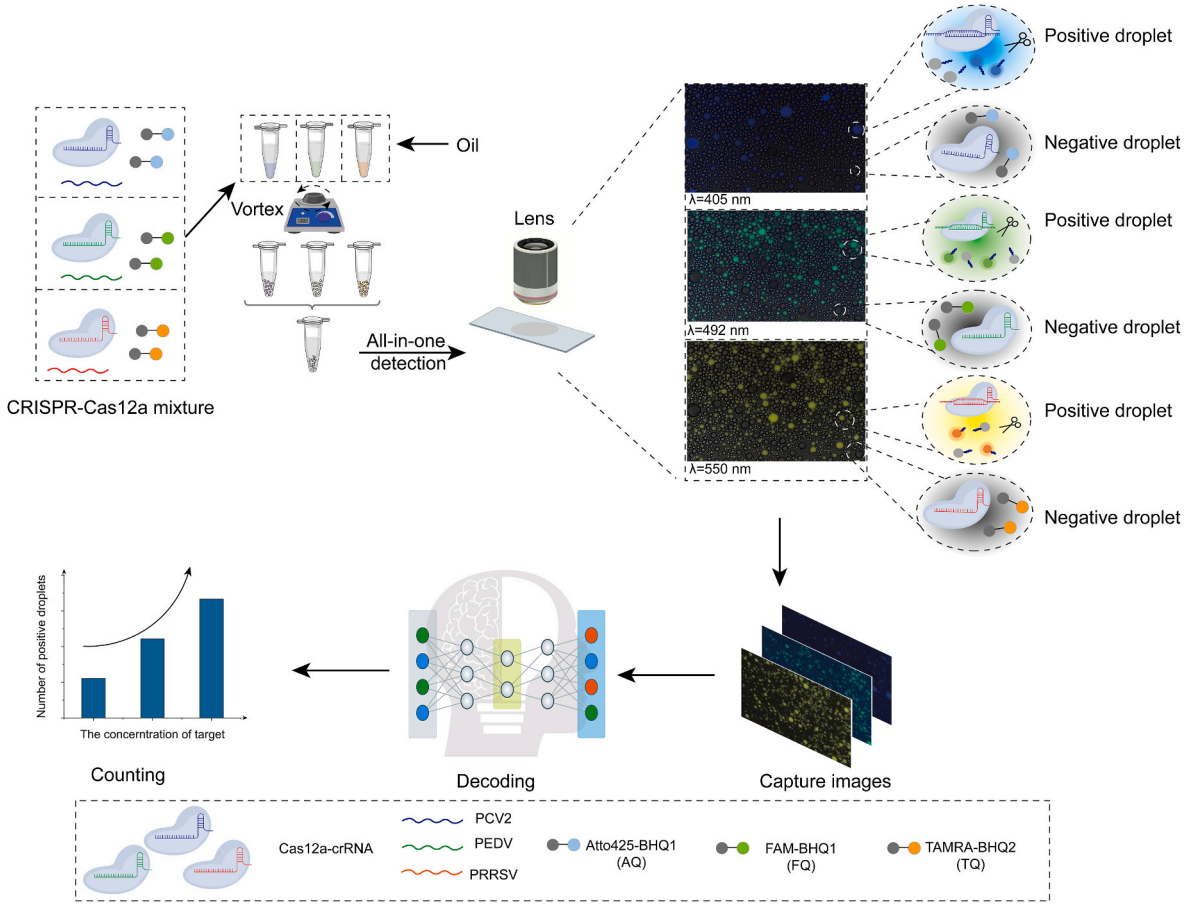
### 2.5. Image processing and automated microdroplet quantification

A custom-developed MATLAB algorithm was employed to achieve precise identification, segmentation, and quantification of individual microdroplets from raw fluorescence microscopy images. The workflow comprises four stages: (1) image pre-processing using adaptive contrast adjustment to correct illumination inhomogeneity and enhance signal-to-background ratio. (2) initial segmentation via fast k-means clustering, optimized with a parameter value of 0.5, to generate a preliminary binary mask distinguishing microdroplets from background. (3) morphological refinement through opening and closing operations with a disk-shaped structuring element (radius = 2 pixels), followed by hole-filling to produce a clean binary mask. (4) instance segmentation of overlapping microdroplets using a watershed transform based on the Euclidean distance map, yielding precise centroid coordinates. Validation was performed by superimposing centroids onto raw images, with automated enumeration for downstream analysis. The analytical threshold was defined as the average fluorescence intensity of the no-template control (NTC) plus 3 times their standard deviation to distinguish positive droplets from negative ones. More details are shown in the Supporting information.

## 3. Results and discussions

### 3.1. Principle of the DD-Cas12a assay

A key limitation of conventional single-tube CRISPR-Cas12a assays is their inability to achieve multiplexed target detection. This limitation arises from the inherent, nonspecific trans-cleavage activity of activated Cas12a, which generates fluorescent signals uncoupled from the specific recognition of multiple targets within the same reaction tube. To overcome this challenge, the DD-Cas12a platform was developed to partition large mixed reaction volumes into high-density droplet emulsions, thereby enabling effective isolation of individual detection events. As shown in Scheme 1, our system incorporates target-specific crRNAs and unique reporter probes. Upon introduction of target, the corresponding Cas12a-crRNA complex is activated, triggering trans-cleavage activity and generating a distinct fluorescent signal readout. In the absence of the target, the Cas12a-crRNA complex remains in an inactive state. To achieve high-throughput multiplex detection, target-specific reaction components are individually encapsulated into distinct droplet populations and subsequently pooled into a single tube. This strategy generates a versatile droplet library that operates as a compartmentalized one-pot system, in which the identity of each droplet is uniquely encoded by its corresponding fluorescent probe. Target identification was achieved using ADC to decode the color features of each droplet in the acquired images. Thus, the DD-Cas12a system integrates the high-order multiplexing sensitivity of droplet-based barcoding with digital detection, facilitating simultaneous, ultra-sensitive identification of multiple targets in a single assay.




**Scheme 1.** The schematic workflow of DD-Cas12a for the one-pot simultaneous detection of PCV2, PEDV, and PRRSV.

### 3.2. Feasibility validation and optimization of CRISPR/Cas12a assay

The fundamental functionality of the CRISPR/Cas12a assay was first validated through fluorescence spectroscopy and kinetic analysis (Fig. S1). Intense fluorescent emission was observed when the target, Cas12a-crRNA, and fluorescent reporters were co-incubated. In contrast, negligible fluorescence was detected without target, when only Cas12a-crRNA and fluorescent reporters were co-incubated. This result indicates that Cas12a can only be activated upon hybridization between the crRNA and its complementary DNA. Furthermore, the fluorescence spectra verified that the absence of signal crosstalk between the reporters. Collectively, these results validate the specificity of the system and confirm the successful establishment of a single-reaction, triple-target detection assay.

To systematically characterize and optimize the trans-cleavage activity of the Cas12a system, PEDV was first selected as the model target for performance evaluation. The performance of the optimized Cas12a system was investigated by comparing four kinds of widely used Cas12a buffers (Table S2). These buffers included Buffer 1, as reported for the DETECTOR (Chen et al., 2018); Buffer 2, employed in the SHERLOCK (Gootenberg et al., 2017); Buffer 3 (NEBuffer 2.1), a commercial buffer from Cas12a kit (Nguyen et al., 2020) and Buffer 4 (Enhanced buffer), previously used for the droplet-based platforms (Yue et al., 2021). As shown in Fig. S2A, NEBuffer 2.1 was determined to be optimal based on fluorescence ratio comparisons. Next, the optimal temperature for the Cas12a assay was evaluated. While Cas12a is typically operated at 37 °C for detection, reaction temperature was found to significantly affect assay efficiency in this study, with the highest signal-to-background ratio achieved at 45 °C (Fig. S2B). The impact of the Cas12a to crRNA ratio on trans-cleavage activity was also investigated, as a higher ratio was hypothesized to generate a larger signal response. A molar ratio of 2:1 was identified as optimal, achieving the highest catalytic efficiency (Fig. S2C). Based on this finding, the optimal reaction time for the entire system was determined to be 30 min (Fig. S2D). Finally, the influence of different reporter probe concentrations on the Cas12a system was evaluated. The fluorescence ratio reached a maximum value when the final concentration of AQ, FQ, and TQ was 500 nM. This result indicates that 500 nM as the optimal amount to balance the assay cost and the reaction performance, thus being selected as the optimal reporter probe concentration (Fig. 1A–B).

Following the establishment of the optimal reaction parameters, the analytical performance of the multiplexed assay was evaluated using synthetic target sequences of PCV2, PEDV, and PRRSV. A strong linear relationship was observed between the fluorescence intensity of each reporter probe and the corresponding target nucleic acid concentration. Based on the 3σ/slope criterion (Cho et al., 2006; Zhao et al., 2014), the limits of detection (LOD) were determined to be 0.02 nM for PCV2 and 0.01 nM for both PEDV and PRRSV (Fig. 1D–F). To assess the specificity of the assay, the system was challenged with non-target nucleic acids, including interfering pseudoviruses present at a ten-fold molar excess relative to the target. As depicted in Fig. 1G–I, the addition of these high-concentration interferents did not induce a significant change in the fluorescent signal, demonstrating exceptional specificity of the assay. Collectively, these findings validate that the capability of this multiplexed platform to simultaneously detect multiple targets with high sensitivity and robust selectivity.

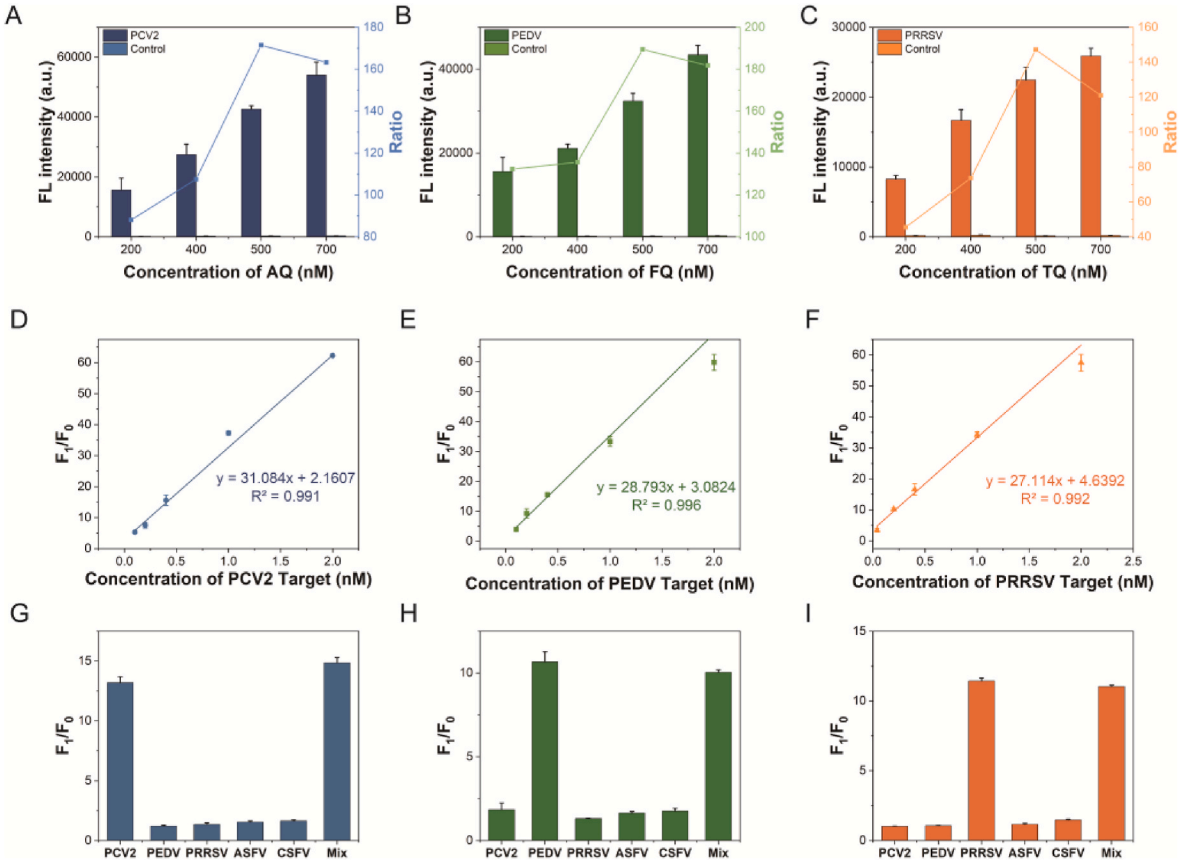



**Fig. 1. Optimization and performance evaluation of CRISPR/Cas12a assay on PCV2, PEDV, and PRRSV.** Effects of different reporter probes (AQ, FQ, and TQ) on Cas12a-based detection (A-C). Analytical performance of bulk fluorescent CRISPR/Cas12a assay for the detection of PCV2 (D), PEDV (E), and PRRSV (F). The linear relationship between target concentrations and the $F_1/F_0$ ($F_1$: the fluorescence intensity in the presence of targets; $F_0$: the fluorescence intensity of the corresponding control). The selectivity of the proposed method for PCV2 (G), PEDV (H), and PRRSV (I) detection (Target concentration: 1 nM; non-target nucleic acids concentration:10 nM).

### 3.3. Performance evaluation of multiplex DD-Cas12a detection assay

To avoid the non-specific trans-cleavage of reporter probes in a bulk mixture, which would eliminate the ability to distinguish different targets, we employed a droplet-based compartmentalization strategy. The multiplexing performance of the DD-Cas12a system was validated using three representative viral targets: PCV2, PEDV, and PRRSV (Fig. S3). In this setup, each droplet functions as an independent reaction unit encapsulated with the specific reporter-crRNA pair, where the reporter serves simultaneously as a signal transducer and identification code. Upon specific target recognition and activation of Cas12a collateral cleavage, fluorescent signals were observed exclusively in their designated Atto 425, FAM, or TAMRA channels. Both visual fluorescence inspection (Fig. S3) and quantitative heatmap analysis (Fig. S4) confirmed the absence of signal crosstalk after droplet mixing and incubation. This robust compartmentalized strategy enables high-specific, one-pot simultaneous detection of multiple pathogens by pooling individually encoded droplets into a single tube. To further enhance assay performance, the sample volume and reporter probe concentration were systematically optimized. A sample volume of 5 μL and a final reporter probe concentration of 1 μM were identified as optimal, as these parameters achieved maximum detection sensitivity and efficiency (Fig. S5 and S6). As shown in Fig. S7, distinct fluorescence signals from positive droplets were clearly observed at 30 min. Furthermore, the droplets maintained an intact morphology without obvious coalescence even after incubation for up to 60 min. Such excellent stability prevents droplet fusion and signal crosstalk, ensuring reliable multiplexed detection in this one-pot system.

To assess the analytical performance of the DD-Cas12a assay, the LOD was first determined using serial dilutions of the target nucleic acid. In the bulk Cas12a assay, the optimized protocol achieved detection of target concentrations as low as 0.01 nM. For digital quantification, fluorescence microscopy was employed to image all droplets within the generated emulsion, revealing a clear concentration-dependent increase in the number of fluorescent (positive) droplets. The target concentration was then quantified by enumerating these positive droplets using ImageJ software. This digital approach enabled the reliable discrimination of a target concentration of 0.15 fM from the NTC (Fig. 2), representing a $1.5 \times 10^5$-fold improvement in sensitivity relative to the bulk assay.

To further evaluate the performance of this system, we compared the DD-Cas12a platform with several recently reported CRISPR-based multiplexed detection methods (Table S3). Among all amplification-free methods included in the comparison, our platform achieved the shortest detection time and the lowest LOD. Additionally, the reliability of the assay was confirmed by reproducibility tests. The intra-assay and inter-assay coefficients of variation (CVs) for all three targets were below 3.97% and 4.42%, respectively (Table S4), which are in the acceptable range for diagnostic applications. Collectively, these results highlight the superior efficiency, sensitivity, and stability of the DD-Cas12a platform for rapid multiplexed detection.

To evaluate the specificity of the DD-Cas12a platform, the assay was challenged with a panel of non-target nucleic acids, including PCV2, PEDV, PRRSV, african swine fever virus (ASFV), and classical swine fever virus (CSFV). As shown in Fig. 3, the assay yielded negative results for all non-target samples, confirming that positive signals are generated exclusively in the presence of the specific target sequence. This high degree of specificity, conferred by the precise design of the crRNA and fluorescent reporter probe, enables the accurate discrimination of intended targets in unknow samples, ensuring that each fluorescent signal faithfully reports the presence of its matched pathogen.

Additionally, we evaluated the ability of the DD-Cas12a platform to detect cell culture-derived viral samples. Viral cDNA samples contain a large amount of non-specific DNA, especially from cell culture components, making them suitable for further evaluating the assay stability of the platform. Nucleic acids extracted from cultured PCV2, PEDV, and PRRSV viral supernatants were used for the assay. We evaluated the sensitivity of the DD-Cas12a platform using serial dilutions of viral cDNA from PCV2, PEDV, and PRRSV. The results showed a clear relationship between target concentration and the number of positive droplets, allowing for the detection of PCV2, PEDV, and PRRSV at minimum concentrations of 126.0, 673.5, and 548.4 fg/μL, respectively. For comparison, we performed qPCR on the same serial dilutions, which yielded minimum concentrations of 0.126, 6.735, and 5.484 pg/μL for PCV2, PEDV, and PRRSV, respectively (Fig. 4). Based on these comparative results, the novelties and distinct advantages of the current DD-Cas12a platform over the conventional qPCR method can be explicitly highlighted. Unlike traditional qPCR, which requires target pre-amplification and thermal cycling, our platform is completely isothermal and amplification-free. This design provides significant practical benefits by eliminating the requirement for complex PCR instruments and reducing the risk of aerosol contamination. Most importantly, our platform achieves a sensitivity up to a 10-fold higher than that of qPCR without the need for an amplification step. This high sensitivity is mainly due to the spatial confinement effect of the microdroplets. The microdroplets greatly reduce the reaction volume, which highly concentrates the target molecules and speeds up the Cas12a-mediated cleavage. This capability to achieve highly sensitive and absolute quantification in a simple, amplification-free manner is the core novelty of our study. In summary, our sensing platform provides an efficient, reliable, and highly sensitive method for the direct detection of multiple nucleic acid targets. It eliminates the requirement for pre-amplification, enables rapid image processing, and demonstrates significant potential for application in clinical diagnostics and field-deployable detection.

### 3.4. Development of automated microdroplet transcoding and portable device

First, the polydisperse microdroplet images were analyzed using the classic image processing software ImageJ. The images need to be filtered and denoised, and then binarized to determine the number of droplet

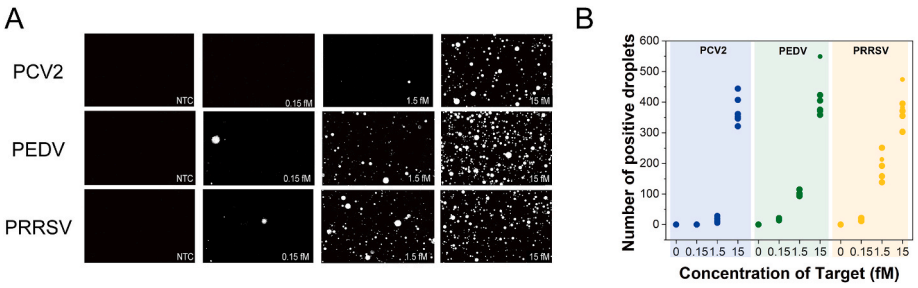



**Fig. 2. Performance evaluation of DD-Cas12a assay for PCV2, PEDV and PRRSV.** (A) Representative droplet images of the digital Cas12a assay with serial dilutions of PCV2, PEDV, and PRRSV. (B) Number of positive droplets at different target Concentrations. NTC: no-template control; Cas12a-crRNA = 50 nM, AQ = 500 nM, PCV2 Target = 0.15, 1.5, and 15 fM; FQ = 500 nM, PEDV Target = 0.15, 1.5, and 15 fM; TQ = 500 nM, PRRSV Target = 0.15, 1.5, and 15 fM.

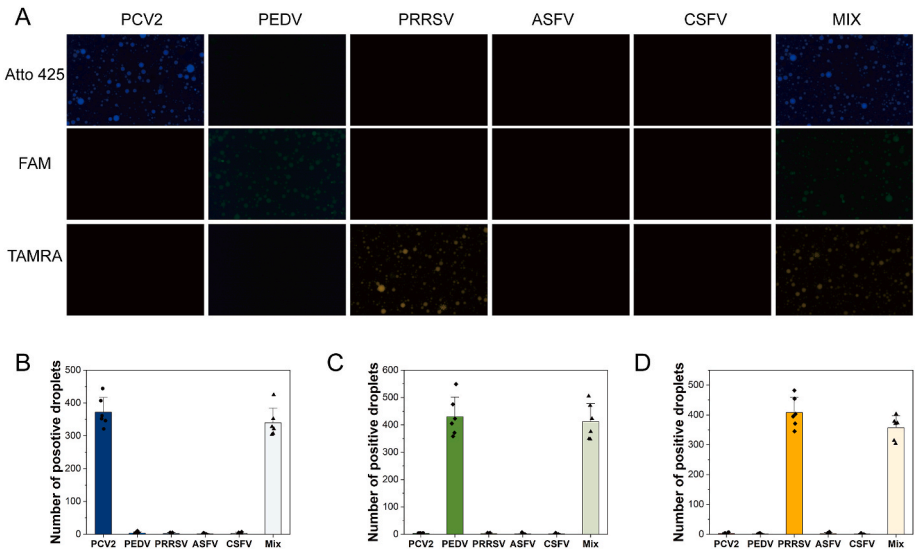



**Fig. 3. Specificity of DD-Cas12a for the detection of PCV2, PEDV, and PRRSV.** (A) Fluorescence images of different targets. The fluorescence signal was recorded in Atto 425, FAM, and TAMRA channels in the presence of targets. PCV2, PEDV, PRRSV target concentration = 15 fM; ASFV, CSFV target concentration = 150 fM. (B) Number of positive droplets of different targets.

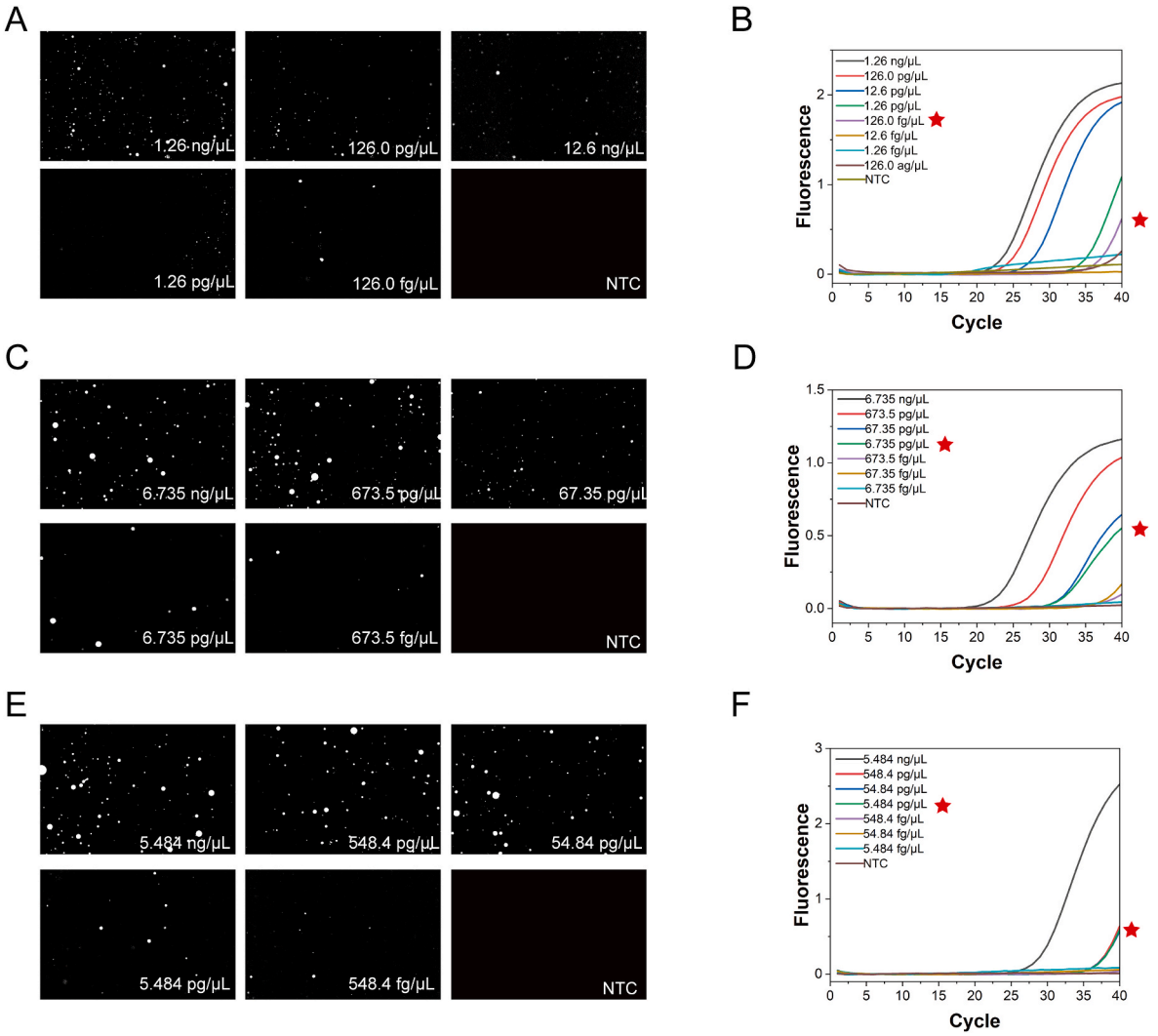



**Fig. 4. Sensitivity of DD-Cas12a assay for PCV2, PEDV, and PRRSV in transfected cells.** Representative droplet images of DD-Cas12a assay with serial dilution of PCV2 (A), PEDV (C), and PRRSV (E). Results of qPCR for PCV2 (B), PEDV (D), and PRRSV (F) detection. NTC: Water was used as the blank sample.

points. However, for the polydisperse microdroplet reactor system, conventional software exhibited insufficient processing accuracy, accompanied by poor correlation among analytical results. Therefore, a more appropriate method is required for the characterization of polydisperse microdroplet reactor systems. To address the demand for high-throughput automatic image recognition and accurate processing, an automatic integrated image processing system was developed. The ADC utilizes droplets encoded with distinct fluorescent reporter probes, which are excited at varying wavelengths to generate unique spectral signatures (Fig. 5A). The analytical workflow initiates with the classification of captured images based on the emission wavelength of the fluorescent reporter (blue, green, and yellow). These images are then sorted into designated channels corresponding to each target: the blue channel for PCV2, the green channel for PEDV, and the yellow channel for PRRSV. Subsequently, the sorted images are converted to greyscale and binarized using a defined threshold to eliminate background fluorescence. Droplets exhibiting a fluorescence intensity above this threshold are registered as a positive event ("1") and quantified, while those with a intensity below the threshold are designated as negative ("0") and excluded from the final count. As shown in Fig. 5B, the ADC quantified 267, 566, and 566 positive droplets for PCV2, PEDV, and PRRSV, respectively, showing a high degree of concordance with manual counting. In contrast, ImageJ exhibited significantly lower consistency, likely due to its lack of channel-specific processing and its sensitivity to global thresholding errors. To further substantiate the reliability of the ADC algorithm, we performed a quantitative evaluation against a manually curated ground-truth dataset (Table S5). The results show that the ADC algorithm significantly outperforms ImageJ across all evaluation metrics, with Precision, Recall, and F1-score reaching 96.42%, 98.43%, and 97.41%, respectively. Compared to ImageJ, the ADC system provides superior diagnostic accuracy equivalent to manual curation while offering much higher throughput. Collectively, these results demonstrate that the ADC platform enables rapid, multiplexed, and objective analysis of complex droplet images.

To explore the potential of DD-Cas12a for point-of-care testing (POCT) application, we developed a miniaturized portable device based on our previous work (Li et al., 2021). As shown in Fig. S8, this mobile imaging module employs a standard smartphone with a 3D-printed light-source frame, optical filters, and external lenses. To evaluate the imaging performance of our custom smartphone-based device, we first captured reference images using a standard laboratory fluorescence microscope to establish a ground truth for droplet quantitation. We then imaged the identical samples using the smartphone setup (Fig. 5C). While the smartphone images exhibited the lower overall resolution compared to the microscope, distinct fluorescence signals from positive droplets remained clearly visible across all three detection channels. Furthermore, as highlighted by the magnified insets, individual positive droplets could still be distinctly resolved under these conditions, fully demonstrating the feasibility and reliability of the smartphone device for multiplexed droplet counting. Consistent with its simplified optical design, the portable smartphone device captured a smaller portion of the overall droplets, resulting in fewer counted positive droplets compared to the fluorescence microscope. Consequently, the portable setup achieved the LOD of 1.5 fM (Fig. S9). While this sensitivity is lower than that of precision laboratory instruments, it still enabled reliable discrimination of positive samples. Given that this DD-Cas12a platform completely obviates the need for complex equipment, features user-friendly operation, and delivers results in under 40 min, this level of performance is entirely sufficient and highly suitable for POCT applications.

## 4. Conclusions

In this work, we report a DD-Cas12a platform for preamplification-free multiplex detection by encoding polydisperse droplets with distinct fluorescent reporters, enabling simultaneous and specific detection of PCV2, PEDV, and PRRSV. Our principal results demonstrate

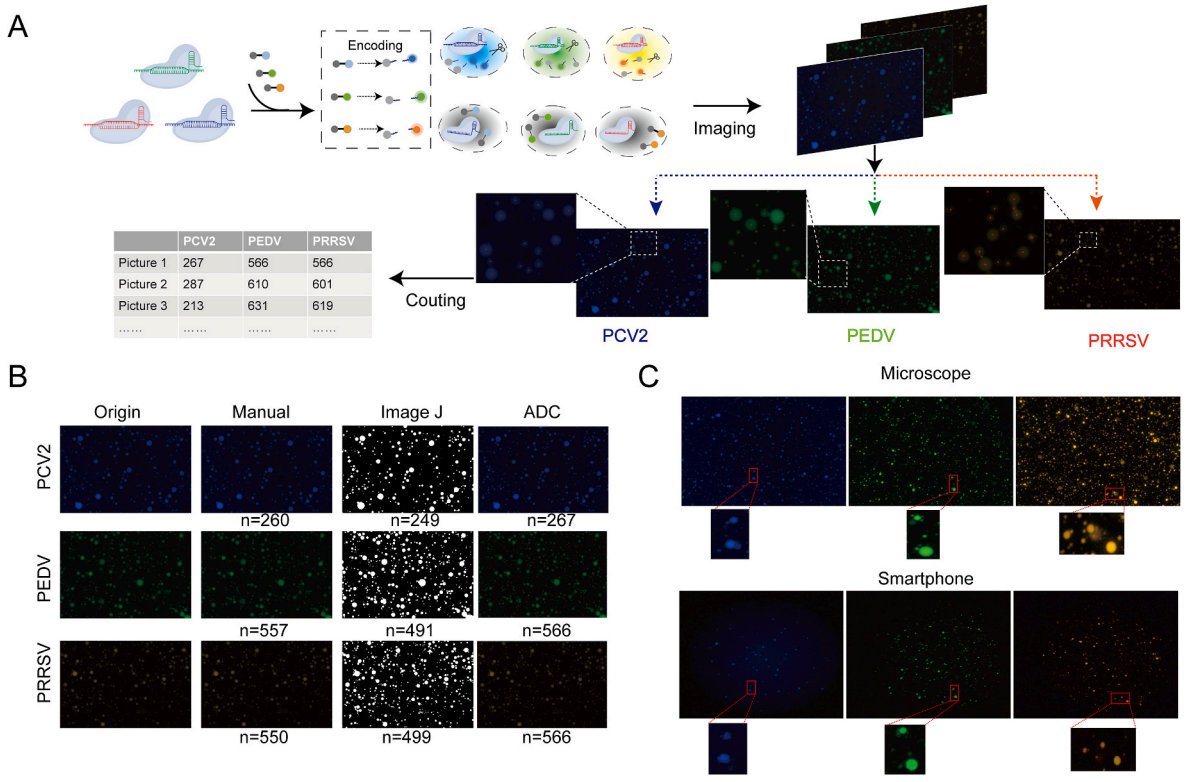



**Fig. 5. The development of ADC for the one-pot detection of PCV2, PEDV, and PRRSV.** (A) The fluorescence droplet encoding process and ADC for intelligently decoding acquired images. (B) Statistical comparison of fluorescence droplet counts in images across different software programs. (C) Laboratory microscope and smartphone imaging of the same DD-Cas12a samples.

that the platform achieved a LOD as low as 0.15 fM within 40 min, representing a 1.5 × $10^5$-fold sensitivity enhancement compared to traditional bulk assays. In complex cell-culture viral samples, it reached detection limits of 126.0, 673.5, and 548.4 fg/μL, outperforming qPCR. To improve analytical throughput and objectivity, we developed ADC system for automated droplet image processing and result decoding. To facilitate the application of DD-Cas12a in POCT, we developed a smartphone-integrated triple-channel portable detection platform. This work can be further optimized, including improving the droplet generation strategy to enhance droplet uniformity and quantitative consistency. Additionally, advances in integrated sample pretreatment and further device integration will facilitate broader deployment across different field settings. Despite these challenges, the DD-Cas12a platform enables rapid on-site screening for multiple co-infecting pathogens in pig farms and large-scale breeding facilities where specialized laboratory infrastructure is lacking. Due to its high sensitivity and modular design, this platform can be easily adapted to monitor other critical threats such as emerging zoonotic diseases. These developments provide a reliable and practical tool for improving biosecurity and early-warning systems in both animal health and environmental monitoring.

## CRediT authorship contribution statement

**Zhirun Ji:** Formal analysis, Methodology, Writing – original draft. **Yiran Zhao:** Conceptualization, Investigation. **Juncan Huang:** Software. **Yanke Shan:** Funding acquisition. **Jiahao Li:** Methodology. **Qinghua Huang:** Software. **Minghui Wu:** Writing – review & editing. **Fei Liu:** Funding acquisition, Supervision.

## 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.2026.118668.

## Data availability

Data will be made available on request.

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