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CRISPR/Cas12a-assisted Visual and On-Site Detection of Porcine Circovirus Type 2

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Abstract

Background: Porcine circovirus type 2 (PCV2) is a globally prevalent viral pathogen that causes substantial economic losses in the swine industry. Rapid and accurate on-site diagnosis is critical for controlling the spread of PCV2. In recent years, RNA-guided CRISPR/Cas12a nucleases combined with recombinase polymerase amplification (RPA) have emerged as a promising approach for nucleic acid detection. This study aimed to develop a novel RPA-CRISPR-based method for the rapid and sensitive detection of PCV2 in field settings.

Results: We designed and optimized CRISPR RNAs (crRNAs) targeting conserved regions of the PCV2 *Cap* and *Rep* genes. Upon recognition of the target sequence, the Cas12a nuclease was activated to cleave a single-stranded DNA-fluorophore quencher (ssDNA-FQ) reporter, generating a fluorescent signal detectable either by a fluorescence detector or via visual readout. The entire procedure was performed at 37 °C and completed within one hour. The assay achieved a detection limit as low as 10 copies/μL and showed no cross-reactivity with other major porcine viruses. Furthermore, a rapid-release reagent was used to replace conventional DNA extraction from serum samples, facilitating efficient on-site detection. The assay was validated using clinical samples, and the results showed strong concordance with those obtained by PCR.

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Conclusions: The RPA-CRISPR-based assay developed in this study is highly sensitive and specific, enabling detection of PCV2 within one hour. Its simplicity, rapidity, and ease of use in the field offer significant practical advantages, making it a valuable tool for the on-site diagnosis of PCV2. This method represents a promising alternative for the early and rapid detection of PCV2 infections and holds potential for contributing to the prevention and control of the disease in the swine industry.

Keywords: Porcine circovirus type 2 (PCV2), CRISPR/Cas12a, RPA, rapid-release reagent

Background

Porcine circovirus 2-systemic disease (PCV2-SD), formerly known as postweaning multisystemic wasting syndrome (PMWS), represents a major threat to global pig production, causing severe economic losses due to impaired growth performance and high mortality rates. The clinical manifestations of PCV2-SD include wasting or reduced weight gain, anemia, diarrhea, and/or respiratory distress [1]. Porcine circovirus type 2 (PCV2) is the primary etiological agent of PCV2-SD. Although most PCV2-infected pigs remain subclinical without overt symptoms [2], PCV2 infection induces immunosuppression, thereby increasing susceptibility to other pathogens and reducing vaccine responsiveness, ultimately leading to increased morbidity and mortality [3, 4].

PCV2 is a small, non-enveloped, single-stranded circular DNA virus belonging to the genus *Circovirus* within the family *Circoviridae* [5]. Its genome is approximately 1.7 kilobases in length and contains about 10 open reading frames (ORFs) [6, 7]. Among these, ORF1 and ORF2, both exceeding 600 base pairs, are two major functional ORFs. ORF1 is the largest, encoding the 37 kDa viral replication protein (Rep), which is responsible for viral replication and transcription. ORF2 encodes the sole structural capsid protein (*Cap*), which harbors the major antigen-neutralizing epitope [8, 9]. PCV2 is classified into five main genotypes, called PCV2a, PCV2b, PCV2c, PCV2d and PCV2e, among which PCV2d has been identified as the current predominant strain in Chinese pig herds [10].

The diagnosis of PCV2 primarily relies on detection of the *Cap* gene or its protein by immunological and molecular biological techniques [11]. For example, the ORF2 viral protein can be detected by the immunological methods such as ELISA [12]. Although these assays are widely used for epidemiological studies of PCV2 infections, they suffer from potential cross-reactivity and limited sensitivity. Molecular biology-based methods, including SYBR Green- and TaqMan-based real-time quantitative PCR [13] and DNA microarrays [14], offer high sensitivity and specificity; however, they require expensive instruments and trained personnel, which limits their applicability for on-site detection. To enhance accessibility and reduce costs, isothermal amplification techniques such as loop-mediated

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isothermal amplification (LAMP) PCR [15] and isothermal recombinase polymerase amplification (RPA) [16] have been introduced for PCV2 detection. Despite their high sensitivity, a major limitation of LAMP and RPA is their susceptibility to amplicon contamination.

In recent years, the clustered regular interspaced short palindromic repeats (CRISPR)-associated endonucleases (CRISPR/Cas) system has emerged as a promising diagnostic platform for nucleic acid detection [17, 18] . CRISPR/Cas nucleases, including RNA-guided RNases (Cas13a-d) and RNA-guided DNases (Cas12 and Cas14), exhibit collateral cleavage activity: upon recognition of their specific RNA or DNA targets, they cleave nearby single-stranded non-targeted nucleic acids in a non-specific manner. Given that most clinical samples require detection at picomolar levels [19], CRISPR-based assays often depend on pre-amplification of the targets. RPA and LAMP are two most commonly used isothermal amplification techniques due to their minimal instrument requirements. The RPA-CRISPR/Cas12a combination has been successfully applied to detect bacteria [20, 21] and plant pathogens [22]. Given that CRISPR/Cas12a has been used to identify viruses such as human papillomavirus type 16 (HPV16) [23] and SARS-CoV-2 [24], the RPA-CRISPR/Cas12a approach holds promise for detecting PCV2 with high sensitivity and specificity. Moreover, because the amount of PCV2 DNA in blood correlates strongly with disease severity [25], an RPA-CRISPR/Cas12a assay could facilitate rapid detection and control of PCV2 prevalence through blood sample testing. Traditional nucleic acid extraction is laboratory-based, increasing both testing time and cost. Therefore, developing a rapid-release reagent compatible with the RPA-CRISPR/Cas12a system for serum samples is of great importance for on-site PCV2 detection.

In this study, we successfully developed an RPA-CRISPR/Cas12a assay for the rapid and visual detection of PCV2. To streamline the testing process, a rapid-release reagent was incorporated for quick serum sample processing. Our assay demonstrated high sensitivity, specificity, portability, and ease of visualization, making it a promising tool for on-site PCV2 testing and potentially contributing to timely monitoring and control of PCV2 spread.

Results

Schematic diagram of PCV2 detection using the RPA-CRISPR/Cas12a assay

Serum samples are first processed with a rapid-release reagent to extract total nucleic acids. Subsequently, an RPA kit is used to exponentially pre-amplify the target gene fragment within 30 minutes. Following amplification, the Cas12a-crRNA complex is introduced into the reaction system, where the crRNA guides Cas12a to the PCV2 genome through sequence-specific base pairing. In the presence of the PAM sequence TTTV (where V represents A, C, or G), Cas12a endonuclease is activated and cleaves the target DNA. This activation also triggers trans-cleavage of the single-stranded DNA-fluorophore quencher (ssDNA-FQ)

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reporters in the system, removing the quencher group and generating a strong fluorescent signal. The fluorescence can be detected either by a fluorescence detector or visualized directly by the naked eye (Fig. 1).

Expression and purification of LbCas12a

LbCas12a was expressed in *E. coli* BL21 (DE3) following induction and cultured for 16 hours at 18°C. The protein was predominantly detected in the supernatant fraction (Fig. 2A). The His-tagged LbCas12a protein was first isolated using Ni^{2+}-immobilized metal affinity chromatography, and the His-tags were subsequently removed by TEV protease. The protein was then further purified by affinity chromatography on a HiTrap heparin HP column using an ÄKTA FPLC system. The pooled and concentrated fractions were subsequently applied to a Superdex 200 Increase 10/300 GLsize exclusion column. The elution profile revealed a single symmetrical peak at approximately 16 mL (Fig. 2B). Fractions eluted from the Superdex 200 Increase 10/300 GL column were analyzed by SDS-PAGE followed by Coomassie blue staining. The results demonstrated that LbCas12a was successfully purified, with an apparent molecular weight slightly larger than 130 kDa (Fig. 2C). From one liter of *E. coli* culture, 5 mg of purified LbCas12a was obtained for subsequent CRISPR/Cas12a assays.

Establishment of RPA-CRISPR/Cas12a assay for PCV2 detection

PCV2d is the most prevalent genotype in pigs in China. Among the PCV2d strains, PCV2-JH has been extensively characterized; therefore, it was used as the reference strain to establish the RPA-CRISPR/Cas12a assay for PCV2 detection. We observed that the Cas12a nuclease was activated only when Cas12a, crRNA and the RPA product were all present in the reaction system (Fig. 3A, lane 2). Three different crRNAs targeting the *Cap* and *Rep* genes were evaluated for PCV2 detection, and no significant differences were observed among them (Fig. 3B), indicating that all three crRNAs are suitable for detecting PCV2. To determine the optimal time for signal collection, fluorescence intensity was measured every 5 minutes through the reaction. The results shown in Fig. 3C demonstrate that the fluorescence signal became detectable as early as 5 minutes and persisted for more than 30 minutes. Finally, positive samples were visually inspected under LED blue light or UV light at 30 minutes (Fig. 3D).

Sensitivity evaluation of the RPA-CRISPR/Cas12a assay

To test the specificity of the RPA-CRISPR/Cas12a assay, DNA was extracted from PCV2-infected PK-15 cells. PK-15 cells were infected with PCV2 virus at multiplicities of infection (MOI) of 10, 5, and 1. After 12 hours, DNA was extracted and detected using the RPA-CRISPR/Cas12a assay. The fluorescence results showed that all PCV2-infected cells, regardless of the MOI, were clearly identified as positive samples (Fig. 4A), indicating that even low-level PCV2 infection is detectable by this CRISPR/Cas12a-based assay.

To further determine the detection limit of this method, the genome of PCV2-JH was cloned into the pMD18-T vector, yielding a plasmid of 4,696

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base pairs, which was used as a standard. The copy number in the 60 ng/μL of the pMD18-T-PCV2 plasmid was calculated using the formula: copies/μL = (6.02 × 10^{23} × 60 × 10^{-9}) / (DNA length × 660) = 1.165 × 10^{10}. Subsequently, a 10-fold serial dilution series was prepared, resulting in template concentrations of 1.165 × 10^{7}, 1.165 × 10^{6}, 1.165 × 10^{5}, 1.165 × 10^{4}, 1.165 × 10^{3}, 1.165 × 10^{2}, 1.165 × 10^{1} and 1.165 × 10^{0} copies/μL. Real-time qPCR results demonstrated that as few as 10 copies could be reliably detected, whereas concentrations below 10 copies fell outside of the linear range (Fig. 4B). All three crRNAs targeting different positions of the PCV2 genome successfully detected 10 copies of nucleic acids using our method (Fig. 4C, D), demonstrating the high sensitivity of this assay for PCV2 detection. Notably, crRNA-*Rep*2 (targeting nucleotides 796-815nt of the *Rep* gene) exhibited higher sensitivity compared to crRNA-*Rep*1 and crRNA-*Cap* (Fig. 4C).

Specificity test of the RPA-CRISPR/Cas12a assay

To evaluate the specificity of the RPA-CRISPR/Cas12a assay for PCV2 detection, we tested other major swine pathogens, including PRV, PRRSV, CSFV, PDCoV and PEDV. The results clearly showed that only PCV2 sample tested positive, whereas all other tested swine pathogens tested negative (Fig. 5A, B), demonstrating that our method is highly specific for PCV2 detection.

PCV2 detection in clinical samples using the RPA-CRISPR/Cas12a assay

To evaluate the feasibility of our RPA-CRISPR/Cas12a assay for detecting PCV2 in clinical samples, DNA was extracted using commercial kits from various organs (including lung, intestine, submandibular lymph node, and kidney) of a PCV2-positive pig, and the RPA-CRISPR/Cas12a assay was subsequently performed. The results showed that our assay successfully detected PCV2 in all tested tissues from the PCV2-positive pig (Fig. 6A, B).

To further assess the feasibility of the assay for clinical serum samples, 24 serum samples suspected to be PCV2-positive were collected. DNA was extracted using commercial kits, and eight of these samples were confirmed to be positive for the PCV2 *Cap* gene by both PCR and real-time qPCR. Moreover, Sanger sequencing confirmed that the PCR products originated from the *Cap* gene of PCV2-JH (Fig. 6C). To facilitate on-site application, a rapid-release reagent was adopted to replace the DNA extraction step, offering advantages of low-cost and ease of use. Serum samples were treated with the rapid-release reagent at 37 °C for 15 minutes, followed by the RPA-CRISPR/Cas12a assay. The results showed that eight samples tested positive and 16 tested negative, yielding 100% concordance with the PCR results (Fig. 6D). To exclude the possibility that the observed fluorescence signal was caused by DNase present in the serum, the assay was performed at 37 °C and 65°C (to inactivate DNase), respectively. The results showed no differences in fluorescence signal

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between two temperatures (Fig. 6E), further confirming that the fluorescence signal was specifically generated by Cas12a rather than serum DNases. Additionally, the ratio of serum sample to the rapid-release reagent was optimized. The sensitivity remained consistent at ratios of 1:5 and 1:10 but significantly decreased at a ratio of 1:20 (Fig. 6E). Taken together, the combination of the rapid-release reagent with the RPA-CRISPR/Cas12a assay is suitable for on-site PCV2 detection.

Discussion

PCV2 is one of the most serious pathogens in the global swine industry. It attacks the immune system by causing lymphoid cell depletion [26], which significantly reduces vaccine efficacy and increases the risk of secondary infections. In addition, the genetic diversity of porcine circoviruses 2 and 3 and their co-infection patterns have been widely reported in both domestic pigs and wild boar populations across different regions [27, 28], further emphasizing the importance of reliable and specific detection methods for circovirus surveillance. In response, several immunological and molecular biological detection methods have been developed and implemented. Immunological reaction-based techniques are low in cost and require minimal equipment, but suffer from low sensitivity [29]. Conversely, PCR and real-time qPCR, while highly sensitive, are too costly and inconvenient for field-based diagnostics [30]. In this study, we established a rapid, highly sensitive, and visualizable RPA-CRISPR/Cas12a assay for PCV2 detection, providing a valuable tool for on-site monitoring of this pathogen.

RPA is a simple and cost-effective technique that offers high sensitivity and great potential for clinical and field-based rapid testing. It operates at near-ambient temperature (37-42°C), whereas other isothermal techniques require heating devices (e.g., nucleic acid sequence-based amplification at 40-55°C, LAMP at 60-65 °C, and strand-displacement amplification at 60 °C) [31-33]. RPA and LAMP are the most commonly used isothermal amplification methods for viral detection, and the design of RPA primers is less complex than that of LAMP primers [34]. Although false-positive results remain a challenge for conventional RPA, combining it with downstream crRNA-based target detection improves specificity. Previously, RPA coupled with CRISPR/Cas12a system has been extensively used for virus detection, including HPV [17], SARS-CoV-2 [24], and tobacco curly shoot virus (TCSV) [35]. Based on comprehensive considerations of temperature, simplicity, and specificity, we developed an RPA-CRISPR/Cas12a assay for PCV2 detection.

The key factors for successful construction of a CRISPR/Cas12a-based nucleic acid detection system are the purity of the Cas12a protein, the specificity of the crRNA sequence, and the efficiency of the RPA primers. In this study, we expressed and purified a sufficient amount of highly pure Cas12a protein. The combination of three crRNAs targeting conserved regions of the *Cap* or *Rep* genes, along with their corresponding RPA primers, exhibited high specificity and sensitivity, achieving a detection limit of 10 copies/μL. These findings suggest that other PCV2 subtypes can

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be detected by selecting the appropriate combination, provided that no mutations occur in the crRNA-targeted regions. Given that the crRNA-*Rep*2 target region in PCV2d contains fewer base mutations, this crRNA may be applicable for detecting a broader range of PCV2d strains. In summary, the Cas12a protein, crRNAs, and RPA primers used in this study can be efficiently applied to the RPA-CRISPR/Cas12a assay for PCV2 detection.

Current nucleic acid extraction methods include guanidinium thiocyanate-phenol-chloroform extraction, column-based extraction, magnetic bead-based extraction [36]. However, these assays are costly and require professional operators and equipment. Therefore, we adopted a rapid-release reagent for processing serum samples. The components of this release reagent are simple, inexpensive, and readily available, including 25 mM NaOH, 10 mM Tris-HCl, 1% Triton X-100, and 2 mM EDTA. Notably, endogenous DNases and RNases present in clinical samples can degrade target DNA templates, potentially leading to false-negative results. The rapid-release reagent contains NaOH, which effectively inactivates these nucleases. By inactivating nucleases and stabilizing the target template, this reagent substantially reduces the risk of false negatives, thereby ensuring the accuracy and reliability of the detection results. Our results demonstrated that the combination of this rapid-release reagent with RPA-CRISPR/Cas12a assay was successfully applied to detect PCV2 in clinical serum samples. To further confirm that the fluorescence signal was specifically generated by Cas12a rather than other DNases, serum samples were processed at 37℃ or 65℃ (DNases are inactivated at 65 ℃), and the results showed no significant difference in signal, further confirming the specificity of the assay. The rapid-release reagent showed satisfactory performance for detecting serum samples but exhibited reduced sensitivity for cell and tissue samples. This may be due to the limited lysis capacity of the rapid-release reagent or the complex composition of the samples. Further testing using other types of liquid samples (e.g., saliva, urine, nasal swabs, and anal swabs) is necessary to evaluate the stability of the rapid-release reagent. In the future, more suitable and optimized extraction procedures should be developed to improve the sensitivity of this method for on-site sample detection. Our study provides insight into the development of more suitable tools for on-site viral detection with minimal resources, such as the combination of rapid-release reagents and immunochromatographic strips for the detection of other viruses.

Conclusions

The combination of a rapid-release reagent with RPA-CRISPR/Cas12a assay holds great promise for on-site PCV2 detection. This method provides rapid, low-cost, sensitive, specific, and visualizable results, thereby facilitating timely diagnosis and effective management of PCV2 spread.

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Materials

Viruses, plasmids, cell lines and clinical samples

The PCV2 strain PCV2-JH (NCBI accession number: MG245867), belonging to PCV2d, was isolated and preserved by Zhejiang Provincial Key Laboratory of Preventive Veterinary Medicine. Additionally, cDNA of pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), porcine delta coronavirus (PDCoV) and porcine epidemic diarrhea virus (PEDV) were also presented by this laboratory. Its whole genome sequence was cloned into the pMD18-T vector. The plasmid pMBP-LbCas12a was purchased from Addgene (Cat. No. 113431) and transformed into *E. coli* BL21 (DE3, TIANGEN, Cat. No. CB106) to express the LbCas12a protein with His and MBP tags. PK-15 cells were used for PCV2 infection. Clinical tissue samples including lung, intestine, submandibular lymph node and kidney were obtained from a pathological and laboratory diagnosis of a PCV2-positive pig. Twenty-four clinical serum samples of suspected PCV2 infection were collected from a pig farm in Hangzhou and their sequences of *Cap* gene were confirmed by Sanger sequencing.

Nucleic acid preparation

TIANamp viral DNA/RNA kit (TIANGEN, Cat. No. 4992285) was used to extract viral nucleic acid from cellular and clinical tissue/serum samples. cDNAs were generated from total RNAs by PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Cat. No. RR047A). The DNA of serum samples was extracted by rapid-release buffer (25 mM NaOH, 10 mM Tris-HCl, 2 mM EDTA, and 1% Triton X-100). Briefly, 2 µL samples were mixed with 10-fold volumes of rapid-release buffer and incubated at 37℃ for 15-30 minutes, then the solution was directly subjected to RPA.

Methods

RPA primer design and crRNA preparation

RPA primers were designed strictly in accordance with the primer design requirements specified in the instruction manual of the RPA kit. Targeting the highly conserved regions of PCV2 *Cap* and *Rep* genes among different prevalent strains. Alignment of the genomes of 35 PCV2d strains using SnapGene (GSL Biotech LLC) was conducted to identify the conserved regions for crRNA designing (Fig. S1). 21-nt crRNA sequences were designed by Benchling(https://www.benchling.com/), an online CRISPR guide RNA design tool, to target *Cap* gene (1484-1503nt) and *Rep* gene (499-518nt, 796-815nt) using variable PAM sequence 5′-TTTV (i.e. TTTA, TTTC, or TTTG). Sequence alignment of all crRNAs to the pig genome (sus scrofa) revealed no matches through BLAT Search Genome on UCSC (http://genome.ucsc.edu/cgi-bin/hgBlat). crRNAs and primers were synthesized by GENEWIZ (Suzhou, Jiangsu, China) (Table 1).

TABLE 1 Sequence of primers, crRNAs, and ssDNA-FQ reporter

Name	Sequence
RPA-Primers:	

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RPA-*Cap*-F	AATTCAACCTTAACCTTTCTTATTCTGTAG
RPA-*Cap*-R	ATGATGAGATTTAATATTAATGATTTTCTTC
RPA-*Rep*1-F	GCACCCTGTAACGTTTGTCAGAAATTTCCG
RPA-*Rep*1-R	TACATTCGTCTTCCAATCACGCTTCTGCAT
RPA-*Rep*2-F	GACTAAAGGTGGAACTGTACCTTTTTTGGC
RPA-*Rep*2-R	GAAGTAATCCTCCGATAGAGAGCTTCTACA
crRNAs:	
CrRNA-*Cap*	UAAUUUCUACUAAGUGUAGAUUUCCCCCAGGAGGGGG CUCA
CrRNA-*Rep*1	UAAUUUCUACUAAGUGUAGAUAAAAGUUCAGCCAGCC CGCG
CrRNA-*Rep*2	UAAUUUCUACUAAGUGUAGAUUUGGCCCGCAGUAUUC UGAU
Reporter:	
ssDNA-FQ reporter	5'- (FAM) TTATT (BHQ1) -3') [37]
qPCR:	
qPCR-F	ATAACCCAGCCCTTCTCCTACC
qPCR-R	GGCCTACATGGTCTACATTTCC
β-actin-F	AGTTATTCCAGTCTCCCTGAATG
β-actin-R	GACCTGACCGACTACCTCATG

Real-Time quantitative PCR

To detect the PCV2 *Cap* gene, the real-time quantitative PCRs were performed with TB Green® Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa, Cat. No. RR820A). *β*-actin was used as endogenous control, and the fold change was calculated via the 2-ΔΔCt method. The primers are shown in Table 1. Briefly, 12.5 μL of TB Green Premix Ex Taq (2×), 0.5 μL of forward primer (10 μM), 0.5 μL of reverse primer (10 μM), 9.5 μL of ddH_2O and 2 μL of DNA template were included. The thermal cycling protocol was set as 40 cycles of 95°C for 30 seconds for denaturation, 60°C for 30 seconds for annealing and extension, followed by the melt-curve analysis (from 60°C to 95 °C with 0.05°C/second increment) on the Applied Biosystems™ QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific).

Expression and purification of LbCas12a

To express LbCas12a protein, *E. coli* BL21 (DE3) strain harboring pMBP-LbCas12a plasmid was cultured at 37 °C in a shaking incubator at 160 rpm until reaching an OD of 0.5 to 0.6 at 600 nm, then IPTG was added to the final concentration of 200 μM to induce the expression of LbCas12a at 18°C. Sixteen hours later, cells were harvested and resuspended in the lysis buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 0.5 mM PMSF), and then lysed by a high-pressure cell crusher. After centrifugation at 15,000×*g* for 45 minutes, the supernatants were incubated with Ni-NTA resin for 1 hour. After washing three times with the washing buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20 mM Imidazole), LbCas12a was eluted using the elution buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 250 mM Imidazole). Soluble LbCas12a was then exposed to Tobacco Etch

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Virus (TEV, Beyotime, Cat. No. P2306) protease to remove the N-terminal tags. The combined sample was dialyzed overnight at 4°C with slow stirring against 2 L of the dialysis buffer (250 mM KCl, 20 mM HEPES, 1 mM DTT). The protein sample from the dialysis membrane was collected and diluted with an equal volume of 10 mM HEPES (Seebio, Cat. No. ACC0013A) and transferred to the HiTrap heparin HP column (Cytiva, Cat. No. 17040701) using a peristaltic pump. The protein was eluted using a linear gradient from 0 to 50% heparin HP elution buffer (2 M KCl, 20 mM HEPES) over 60 mL at a flow rate of 2 mL/min. Eluted fractions were pooled and concentrated with a 100 MWCO Amicon Ultra-15 mL Centrifugal Filter Unit (Sigma, Cat. No. c0005303), then loaded onto size exclusion chromatography (SEC) in SEC buffer (500 mM KCl, 20 mM HEPES, 1 mM DTT) on a GE ÄKTA FPLC (Cytiva, AKTA PURE) with the Superdex 200 Increase 10/300 GL column (Cytiva, Cat. No. 28990944) at a flow rate of 1 mL/min. Concentrated protein was diluted to 10 μM in the buffer containing 20 mM Tris-HCl, 1 mM DTT, 10 mM HEPES and 20% glycerol and stored as 5-μL aliquots at −80°C until use.

The RPA-CRISPR/Cas12a assay

For RPA, 1 μL of DNA template was added in an 8.5 μL master mix (0.5 μL of 10 μM forward and reverse primer, 6 μL of TwistAmp Rehydration Buffer and 1.5 μL of UltraPure water), and then 0.5 μL of 280 mM TwistAmp magnesium acetate was added into the reaction mixture and incubated for 15 mins at 37 °C according to the instruction of the TwistAmp® Basic kit (TwistDx, Cat. No. TABAS03KIT). The CRISPR/Cas12a-based assay was performed using the master mix containing 2 μL cleavage buffer (NEB buffer 2.1), 1 μL of Cas12a (1 μM), 1 μL of crRNA (5 μM), 0.5 μL of ssDNA-FQ reporter (10 μM), 0.2 μL of RNase inhibitor and 14.3 μL of UltraPure water. Then 1 μL of RPA product was added into the reaction, yielding a final total reaction volume of 20 μL, followed by incubation at a constant temperature of 37 °C for 15-30 minutes. The fluorescence intensity was measured by the fluorescence plate reader (EnVision® Multi-mode Plate Reader, PerkinElmer) under 485 nm excitation and 520 nm emission wavelength. With a UV transilluminator (Bio-Rad, Gel Doc XR+, Cat. No. 170-8195), fluorescence can be observed by the naked eye. The data are represented as means with SD of three independent experiments [37].

Abbreviations

ASFV	African swine fever virus
Cap	Capsid protein
CSFV	Classical swine fever virus
CRISPR	Clustered regular interspaced short palindromic repeats
crRNA	CRISPR RNA
HPV16	Human papillomavirus type 16
LAMP	Loop-mediated isothermal amplification

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NASBA	Nucleic acid sequence-based amplification
ORFs	Open reading frames
PCV2	Porcine circovirus type 2
PMWS	Postweaning multisystemic wasting syndrome
PDCoV	Porcine delta coronavirus
PRRSV	Porcine reproductive and respiratory syndrome virus
PRV	Pseudorabies virus
RPA	Recombinase polymerase amplification
Rep	Replication protein
ssDNA-FQ	Single-stranded DNA-fluorophore quencher
SEC	Size exclusion chromatography
SDA	Strand-displacement amplification
TCSV	Tobacco curly shoot virus
TEV	Tobacco etch virus

Figure Legends

Fig. 1 Schematic diagram of PCV2 detection by RPA-CRISPR/Cas12a assay**.** Specific CRISPR RNAs (crRNAs) targeting the PCV2 *Cap* gene were designed for PCV2 genome detection. The strong, non-specific trans-cleavage of single-stranded DNA (ssDNA) is triggered when Cas12a proteins cut double-stranded DNA (dsDNA) with the appropriate crRNA guide. When the quenched fluorescent ssDNA is cut, the ssDNA-FQ reporter (sequence TTATT) fluoresces. In the PAM-Target sequence, the PAM region is indicated in blue and the target sequence is indicated in orange.

Fig. 2 Expression and purification of His-tagged LbCas12a protein. The supernatant of lysed IPTG-induced cells was analyzed by SDS-PAGE and stained with Coomassie blue. 1: Un-induced sample; 2: Induced sample; The target band is marked by the red box (A). SEC elution profile of Cas12a purified by affinity chromatography using the HiTrap heparin HP column. A total of 20 tubes of 0.5 mL/tube fractions were collected (B). Coomassie blue stained SDS-PAGE of fractions from size exclusion chromatogram (C). Lane 1-5 showed the elution from peak fraction 12-16, respectively.

Fig. 3 Validation of the CRISPR/Cas12a-based fluorescence detection. Characterization of main components (RPA product, crRNA, Cas12a) (A) and different crRNA (B) of RPA-CRISPR/Cas12a assay. Detection of recombinant plasmids carrying the PCV2 genome by fluorescence detection every 5 minutes (C). Directly visualizing the results of PCV2 RPA-CRISPR/Cas12a assay by the naked eye under LED blue light or UV light (D). NC, negative control.

Fig. 4 Sensitivity of the CRISPR/Cas12a-based fluorescence detection. The detection of PCV2 in PK-15 cells infected with different

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MOI PCV2 (A). The comparison of the sensitivity of real-time qPCR and RPA-CRISPR/Cas12a assay. A series of 10-fold dilutions of pMD-18T-PCV2 plasmid was used as the template. The CT value indicated the concentration of template measured by real-time qPCR (B). The fluorescent signals produced by different dilutions of pMD18T-PCV2 plasmid were collected by the Microplate reader (C) and visualized by gel imaging system using UV light (D) after RPA-CRISPR/Cas12a assay. 6: 1.165×10^{6} copies/μL; 5: 1.165×10^{5} copies/μL; 4: 1.165×10^{4} copies/μL; 3: 1.165×10^{3} copies/μL; 2: 1.165×10^{2} copies/μL; 1: 1.165×10^{1} copies/μL; 0: 1.165×10^{0} copies/μL.

Fig. 5 Specificity of RPA-CRISPR/Cas12a assay for PCV2 detection. The fluorescent signals were collected by PE microplate reader (A) and visualized by gel imaging system (B) after performing RPA-CRISPR/Cas12a assay with DNA viruses PCV2 and PRV, RNA viruses PRRSV, CSFV, PDCoV and PEDV.

Fig. 6 RPA-CRISPR/Cas12a assay of PCV2 detection in clinical samples. Fluorescence measurement (A) and visual detection (B) of PCV2 by RPA-CRISPR/Cas12a assay from the lung, intestine, lymph node and kidney of a PCV2-positive pig. Agarose gel electrophoresis of PCR products from serum samples, with DNAs extracted by commercial kits (C). Serum samples were processed by rapid-release reagent and subsequent RPA-CRISPR/Cas12a assay, the detection was visualized by LED blue light (D). Comparison of different volume ratios of serum sample to the rapid-release reagent at 65°C and 37 °C (E).

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Declarations

Ethics approval and consent to participate

All procedures followed the guidelines of Administration of Affairs Concerning Experimental Animals of China and are in adherence with ARRIVE guidelines. In this paper, all virus isolation and genome extraction were done at Li lab. The study was approved by the Laboratory Animal Welfare and Ethics Committee of Zhejiang University (ethical approval number: ZJU20230399). The consent was obtained from all owners for the collection of samples and subsequent analysis.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing interests.

Fundings

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Authors' Contributions

XL performed the experiments, analyzed the data and drafted the manuscript. YS and JXZ assisted in performing the experiment. YP and XL conceived and designed the study, and helped in writing the manuscript. All authors read and approved the final manuscript.

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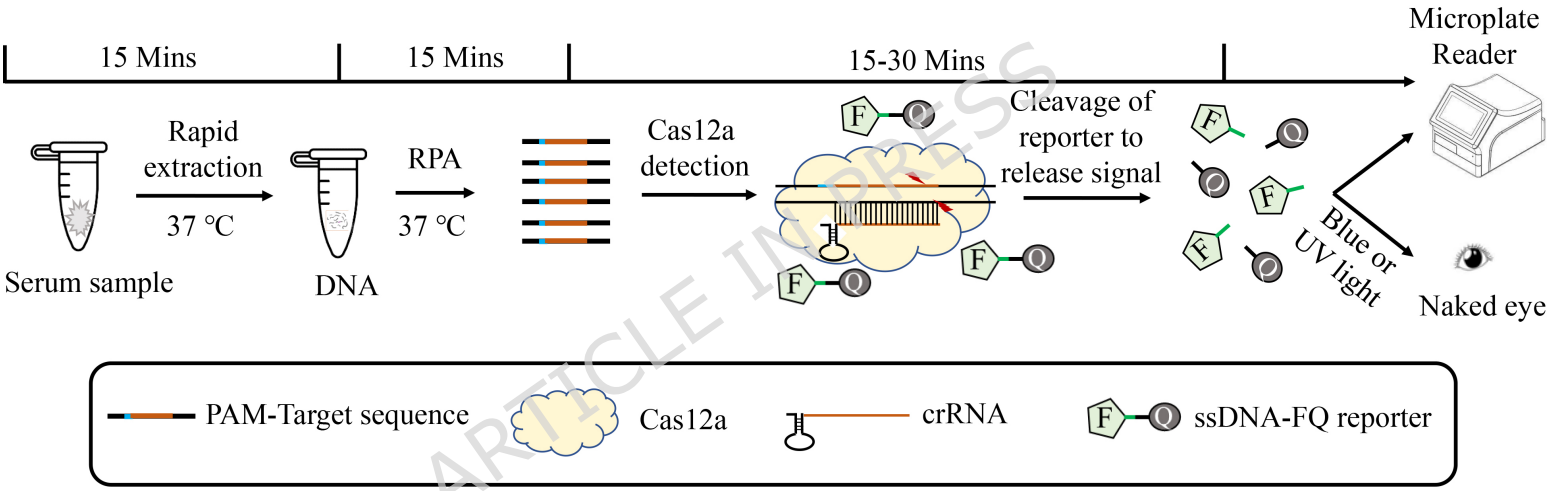
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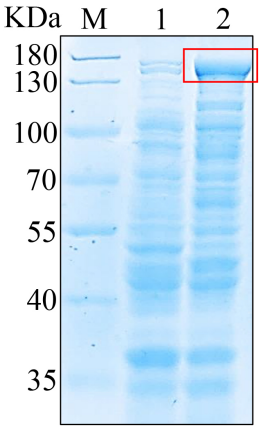
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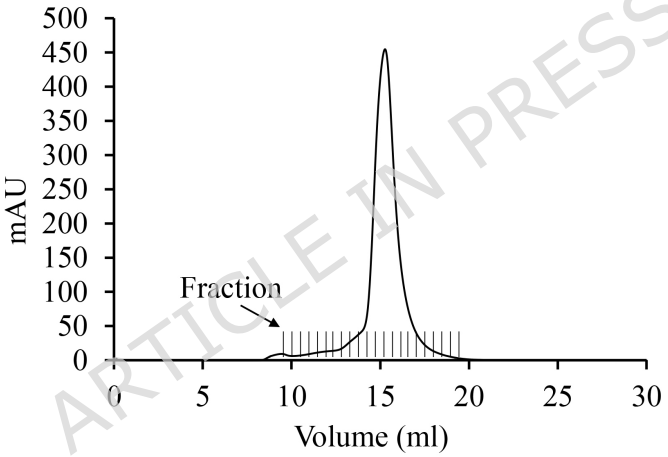
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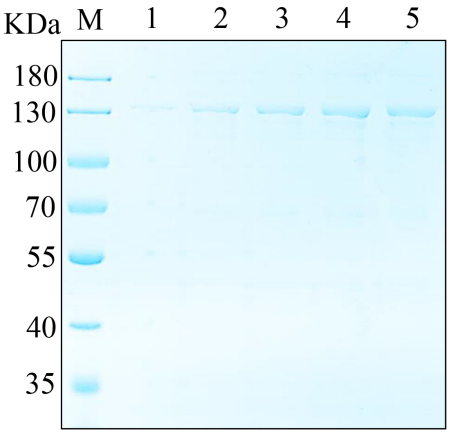
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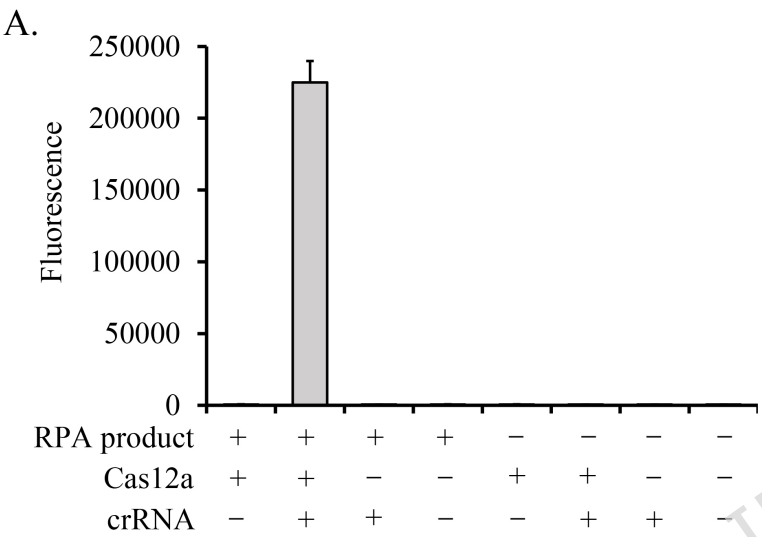


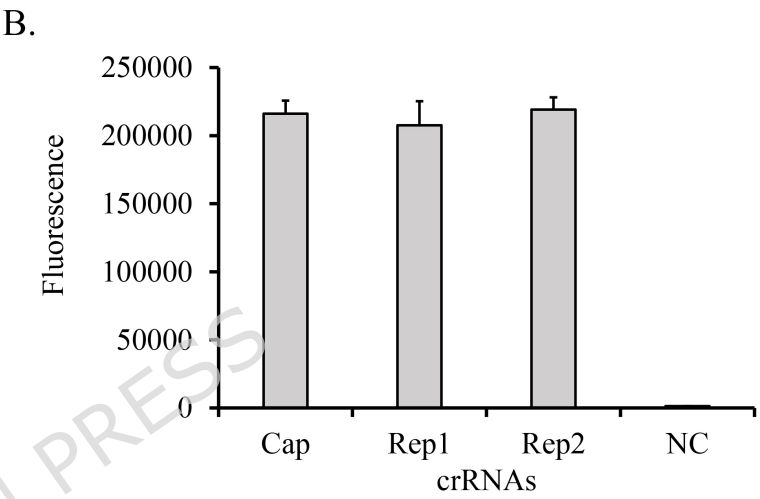
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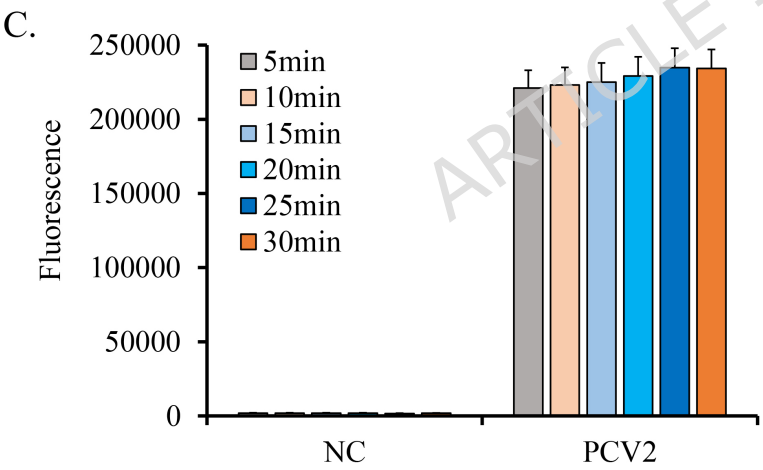


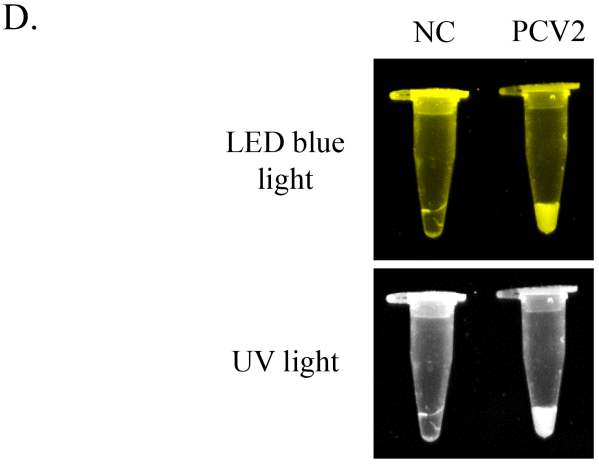
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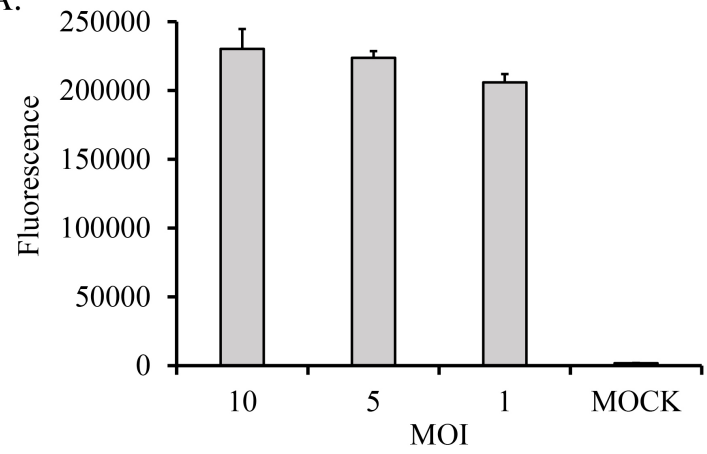




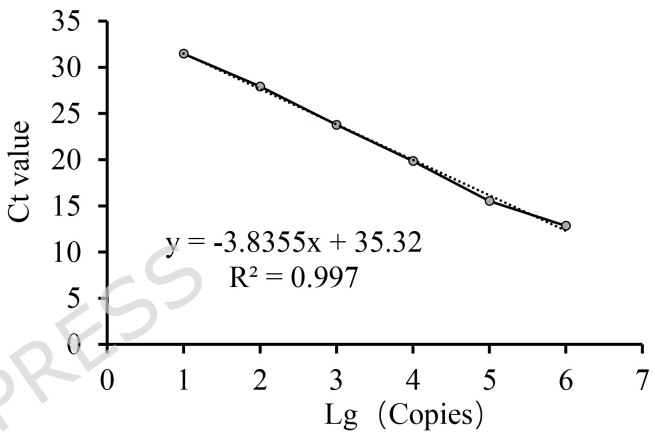
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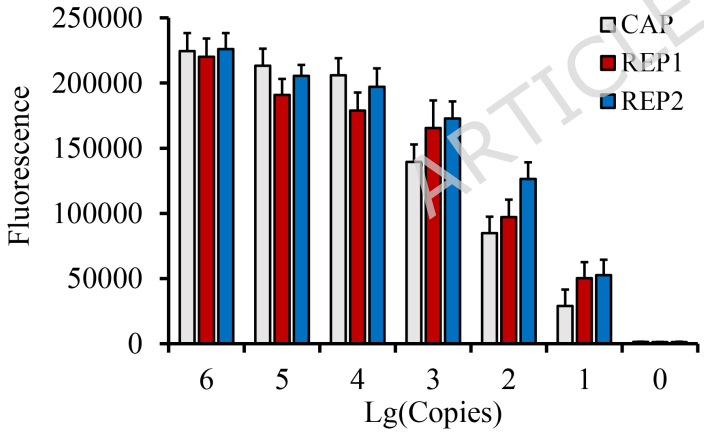
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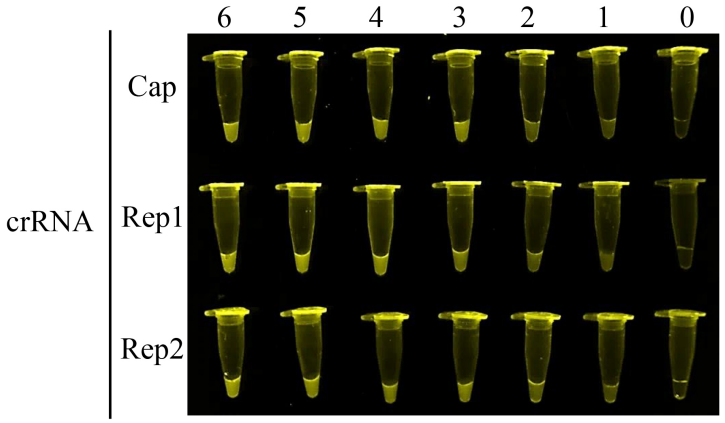
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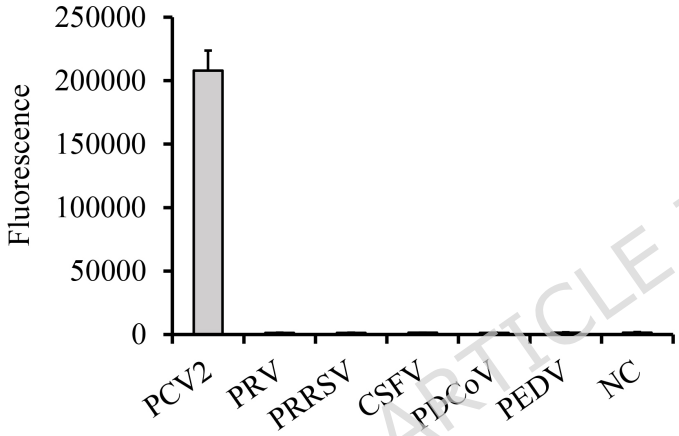
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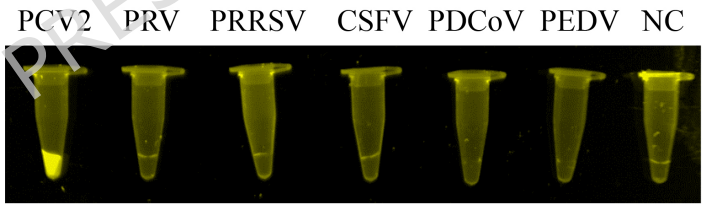
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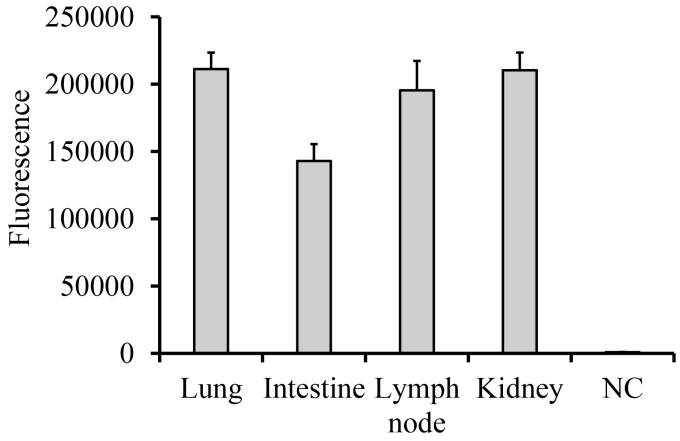
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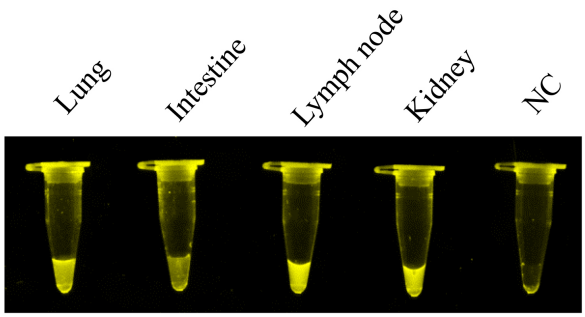
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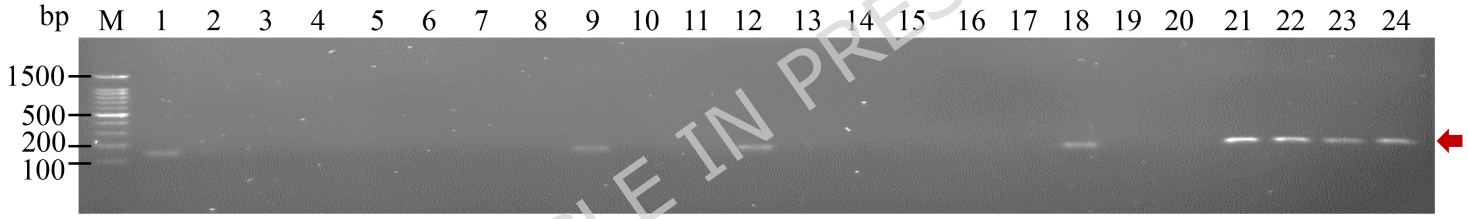
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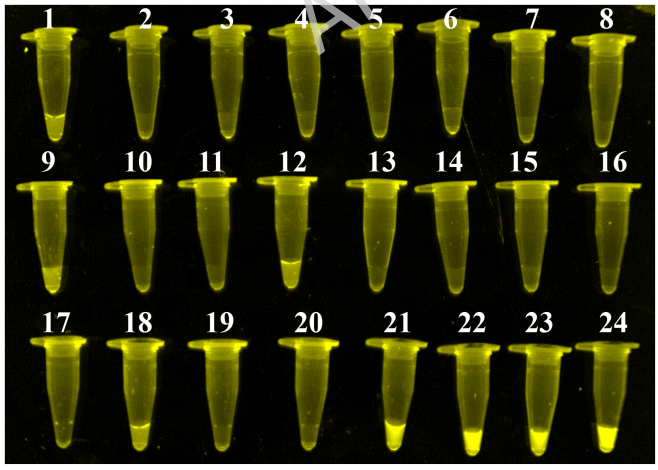
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