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Research Article

Structure-Guided design of Cas12a variants improves detection of nucleic acids

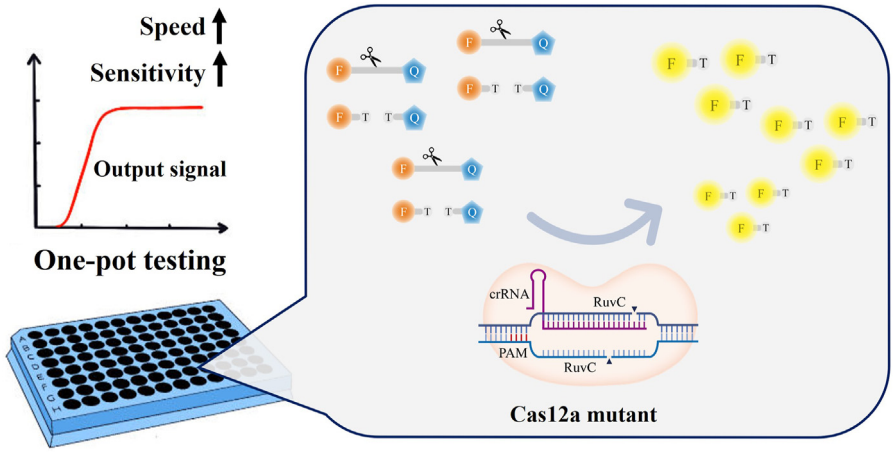
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HIGHLIGHTS

- We developed LbCas12a variants to enhance performance in CRISPR-based one-pot reactions by targeting its canonical PAM.
- This mutation is conserved across various Cas12a family members.
- The conserved mutation suggests that this approach could universally enhance one-pot reactions involving Cas12a.

GRAPHICAL ABSTRACT



ABSTRACT

CRISPR-Cas12a holds promising potential for pathogen detection. However, its performance is not optimal when combined with isothermal amplification. Hence, we engineered a mutant of LbCas12a (K595A) with reduced *cis*-cleavage activity, to minimize interference with isothermal amplification. Compared to wild-type Cas12a, the K595A mutant exhibited a 2–3 times faster reaction speed and a 1,000–10,000 times increase in sensitivity in a one-pot reaction. We applied this mutant for detection of African Swine Fever Virus (ASFV). This K595A mutant successfully detected all 30 ASFV samples within 20 minutes. Our study suggests a universal approach to improve the performance of Cas12a for pathogen detection.

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1. Introduction

The COVID-19 pandemic has underscored the urgent need for rapid, sensitive, and specific diagnostic methods (Chang et al., 1993; Dong et al., 2024; Kuiken et al., 2003; Li et al., 2023, 2024; Piot et al., 2014; Zaki et al., 2012). While reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is widely regarded as the gold standard for pathogen detection due to its exceptional sensitivity and specificity, it is limited by long processing times and the requirement for expensive laboratory equipment, making it impractical for on-site or at-home testing (Blairon et al., 2021; Osório & Correia-Neves, 2021; Safiabadi Tali et al., 2021). In contrast, antigen-based detection, though user-friendly and capable of delivering faster results, suffers from a significantly lower sensitivity—up to 1,000 times less than qPCR—thereby increasing the likelihood of false negatives (Albert et al., 2021; Corman et al., 2021; Mak et al., 2020).

Isothermal amplification techniques, such as recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP), exhibit rapid reaction kinetics and high sensitivity comparable to that of qPCR (Baek et al., 2020; Park et al., 2020; Piepenburg et al., 2006). However, their limited specificity and relatively high false-positive rates restrict broader application (Baek et al., 2020; Park et al., 2020; Piepenburg et al., 2006). This limitation can be addressed by integrating CRISPR enzymes, which offer high specificity in target recognition (Li et al., 2018). Guided by crispr RNA (crRNA), Cas12a cleaves target sequences (*cis*-cleavage) and activates nonspecific cleavage activity (*trans*-cleavage) (Chen et al., 2018; Li et al., 2018; Qiu et al., 2022; Wu et al., 2023). This rapid process, capable of cleaving thousands of ssDNA molecules per second, triggers the swift release of fluorescent signals, resulting in the cascade amplification of specific signals (Chen et al., 2018; Li et al., 2018).

While CRISPR-Cas12a alone has low sensitivity, it is commonly coupled with isothermal amplification to enhance detection (Broughton et al., 2020; Chen et al., 2018; Li et al., 2018; Swarts & Jinek, 2019; Zhang et al., 2023). For example, DETECTR (DNA Endonuclease-Targeted CRISPR Trans Reporter), a clinically validated method for SARS-CoV-2 detection, first amplifies the target substrate via LAMP, followed by CRISPR-mediated cleavage (Broughton et al., 2020). However, the two-step nature of this process increases complexity and raises the risk of cross-contamination (Arizti-Sanz et al., 2020; Joung et al., 2020). Optimizing a one-step integrated approach (one-pot reaction) could simplify operations and reduce contamination risk, but challenges remain, such as reduced speed and sensitivity (Arizti-Sanz et al., 2020; Joung et al., 2020). This is primarily due to competition between Cas proteins and the isothermal amplification reaction for the target substrate, as the high *cis*-cleavage activity of Cas proteins consumes a large portion of the amplicons, lowering amplification efficiency (Lu et al., 2022).

We demonstrated that using suboptimal PAM sequences for Cas12a can accelerate amplicon accumulation and enhance the efficiency of one-pot reactions (Lu et al., 2022). However, this strategy may not be effective for Cas12b, due to its simple PAM requirements, or for Cas13a, which lacks PAM sequences altogether (East-Seletsky et al., 2016; Teng et al., 2019; Tong et al., 2024). To address this, we have engineered Cas12b to reduce its *cis*-cleavage activity, thereby improving LAMP-mediated one-pot reactions (Tong et al., 2024). However, it remains unclear whether this strategy would be equally effective for Cas12a.

In this study, we developed LbCas12a variants to enhance performance in CRISPR-based one-pot reactions. By engineering these variants based on the ternary complex structure of LbCas12a-crRNA-double stranded DNA, we identified a single mutant, Cas12a-K595A, which exhibited faster reaction kinetics, superior sensitivity, and greater reliability compared to wild-type Cas12a targeting its canonical PAM. Notably, Lys595 is conserved across various Cas12a family members, suggesting that this approach could universally enhance one-pot reactions involving Cas12a. Our findings establish a rapid and sensitive on-site detection method leveraging Cas12a.

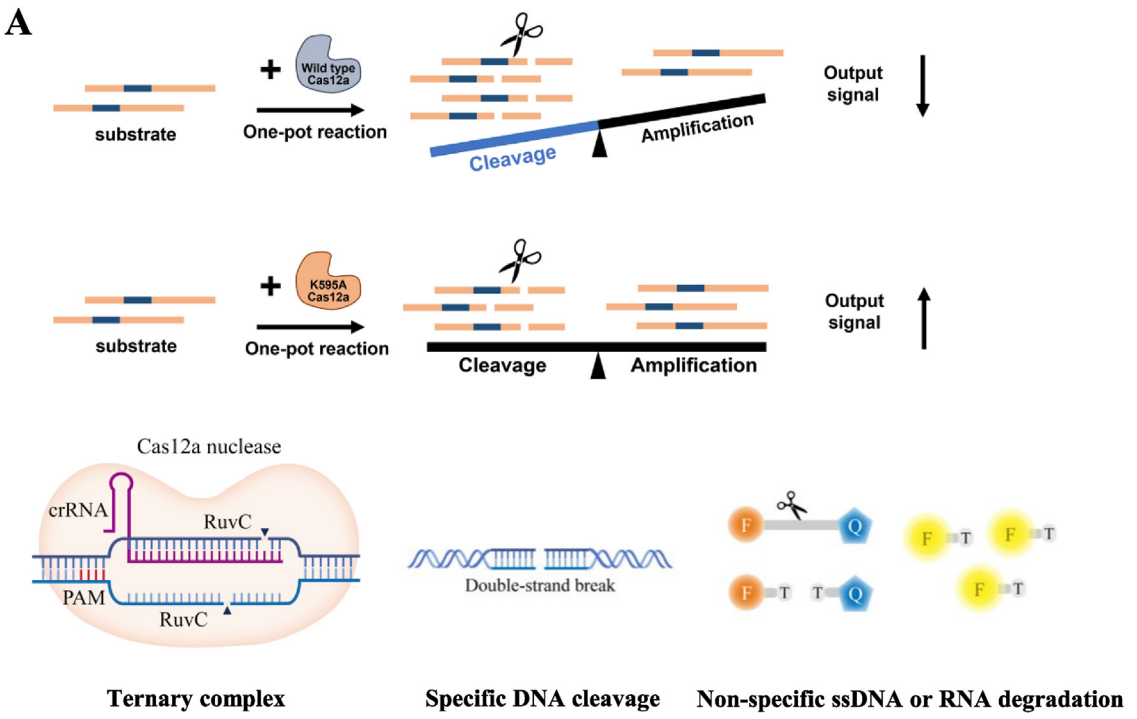
2. Results

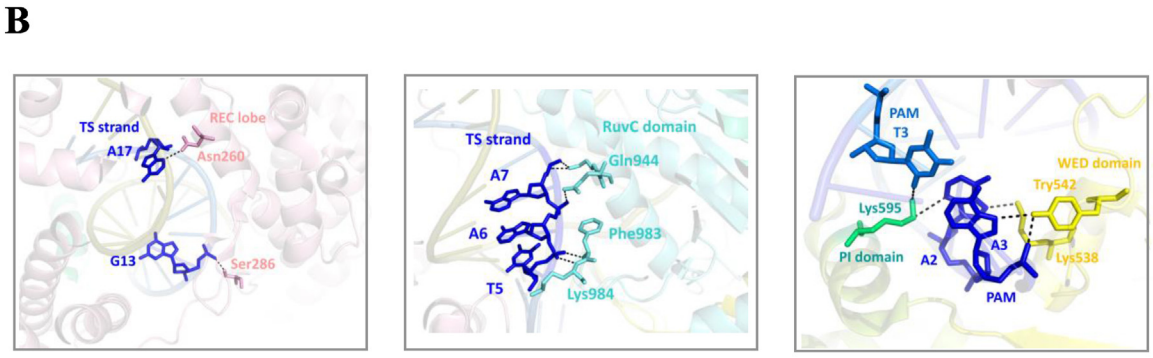
We engineered Cas12a to improve its performance in one-pot reaction (Fig. 1(A)–(D)). To generate Cas12a variants, we mutated residues interacting with the target strand of DNA (Asn260, Phe983, Gln944, Lys945, Lys984, and Ser286) and the PAM sequence (Tyr542, Lys438, and Lys595), by substituting them with alanine (Fig. 1(B)). Subsequently, these mutants were expressed and purified (Fig. S1) (Yamano et al., 2017). We evaluated their performance in RPA-mediated one-pot reaction. Compared to the wild-type Cas12a, mutants Y542A, K538A, K595A, and Y542A/K595A all exhibited enhanced fluorescent signals (Fig. 1(C)). Lys595 interacts with T3 and A2 of the PAM sequence, and the K595A mutant outperformed others in one-pot reaction (Fig. 1(B)). Therefore, we selected K595A mutant for further studies. Cas12a-K595A targeting canonical PAM demonstrated equivalent performance to wild-type Cas12a (Cas12a-WT) targeting suboptimal PAM, and both displayed much stronger fluorescent signals than the Cas12a-WT targeting canonical PAM in one-pot reaction (Fig. 1(D)).

To further evaluate the performance of the one-pot reaction mediated by Cas12a-K595A, we compared the reaction speed, sensitivity, and reproducibility of Cas12a-K595A with Cas12a-WT using spacers targeting canonical PAM of the SARS-CoV-2 *E* and *Orf1ab* genes (Fig. 2(A)–(F)). Cas12a-K595A reached the signal plateau in less than 20 min, whereas it took Cas12a-WT about 50–60 min for both spacers (Fig. 2(A) and (B)). The limit of detection (LOD) for Cas12a-K595A was about 0.2 fg per reaction for the *E* gene and the *Orf1ab* gene, making it 1,000 to 10,000 times more sensitive than Cas12a-WT (Fig. 2(C) and (D)). To assess the reliability of Cas12a-K595A, we conducted ten replicates of one-pot reaction for both Cas12a-K595A and Cas12a-WT with sufficient substrates and incubating time. The fluorescence signals of Cas12a-K595A mutant replicates were substantially more consistent than those of Cas12a-WT (Fig. 2(E) and (F)). Collectively, K595A boosted the performance of one-pot reactions, manifesting in shorter turnover time, increased sensitivity and enhanced reliability.

We hypothesized that Cas12a-K595A facilitated one-pot reaction by modulating *cis*-cleavage activity to minimize interference with isothermal amplification (Lu et al., 2022; Tong et al., 2024). Consequently, we compared the *cis*-cleavage activity and amplicon accumulation in one-pot reaction using Cas12a-WT or the K595A mutant. Cas12a-WT cleaved most of the substrates within 30 s, whereas the K595A mutant required 10 min to cleave similar amounts of substrates (Fig. 3(A)–(D)). The *cis*-cleavage rate constants of the K595A mutant were lower than those of Cas12a-WT (Fig. 3(A)–(D)). The lower *cis*--activity of the K595A mutant led to noticeable accumulation of amplicons within 2–4 min, resembling the positive control of RPA alone (Fig. 3(E) and (F)). In contrast, the WT group, with robust cutting activity, showed minimal accumulation until 6–10 min of incubation, maintaining low levels throughout the 20 min period (Fig. 3(E) and (F)).

African Swine Fever Virus (ASFV), characterized by high mortality and pathogenicity, poses a significant threat to the swine industry, requiring fast and easy diagnostic assays (Galindo & Alonso, 2017; Lu et al., 2020). We employed the K595A-mediated one-pot assay for ASFV detection, targeting its DNA polymerase sequence (Lu et al., 2020). K595A enabled rapid detection of DNA polymerase dsDNA within 15–20 min, boasting a sensitivity of 0.2 fg per reaction (Fig. 4A). Subsequently, we extracted genomic DNA from 30 ASFV viral samples, with a wide range of Ct values (from 18.1 to 35.8), and applied Cas12a-K595A to detect these samples. Remarkably, K595A-mediated one-pot assay displayed significantly enhanced fluorescence signals and accurately identified all 30 samples within 20 min, achieving a sensitivity of 100%. In contrast, LbCas12a-WT demonstrated only 70% sensitivity and lower fluorescence signals (Fig. 4B). These findings underscore the potential of Cas12a-K595A as a robust nucleic acid detection tool.





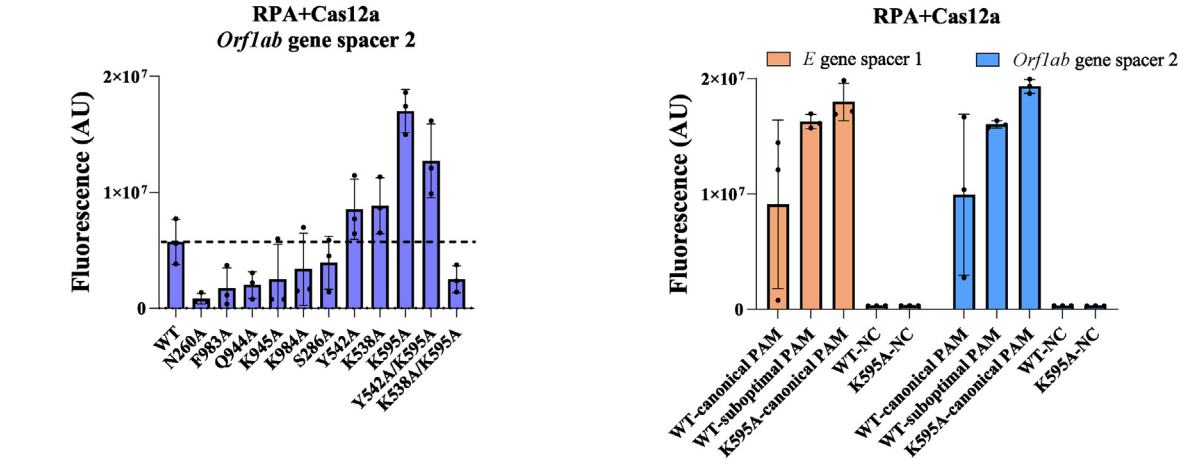


Fig. 1. One-pot detection using LbCas12a mutants. (A) Up: the schematic diagram of the Cas12a K595A promoting the one-pot reaction. Bottom: *cis*-cleavage (specific cleavage) and *trans*-cleavage (non-specific cleavage) of LbCas12a. 'F' and 'Q' represent fluorophore and quencher, respectively. 'T' indicates the single-stranded DNA fluorophore-quencher reporter sequence of TTATT. (B) The key amino acids labeled in the ternary complex structure of LbCas12a-crRNA-dsDNA (PDB number is 5XUS). (C) The fluorescence signal generated by the Cas12a mutants and WT in one-pot reactions. (D) Comparison of one-pot methods mediated by Cas12a-WT protein, K595A mutant, and suboptimal PAM-mediated approaches targeting the SARS-CoV-2 *Orf1ab* gene and *E* gene.

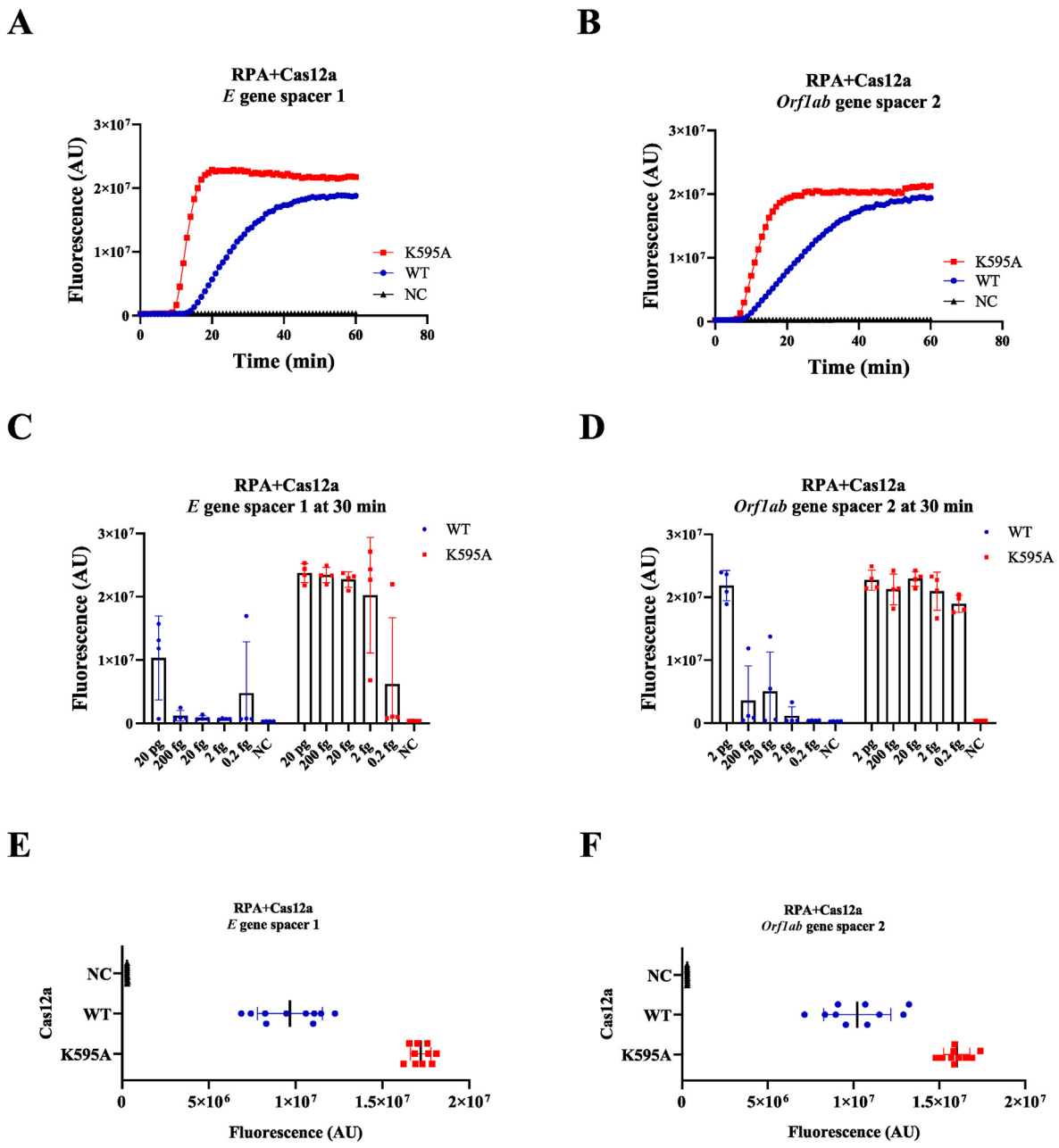


Fig. 2. The performance of LbCas12a WT and K595A mutant for detection. (A)-**(F)** The fluorescence curves ((A)–(B)), sensitivities ((C)–(D)) and reproducibilities ((E)–(F)) of one-pot reactions mediated by LbCas12a WT or K595A mutant. Mean ± s.d. of $n = 4$ technical replicates for (C) and (D), and $n = 10$ for (E) and (F). The SARS-CoV-2 *E* gene and *Orf1ab* gene were used as substrates.

Lys595 is conserved among commonly utilized Cas12a homologs, and it plays a crucial role in PAM sequence recognition in these cases (Fig. 5(A)). Similarly, in AsCas12a, Lys607 forms hydrogen bonds with A2 of the PAM, and in FnCas12a, Lys671 interacts with T2 and A3 of the PAM via hydrogen bonding (Fig. 5(B) and (C)). The conservation of this lysine residue sequence and its hydrogen bonding interactions with the PAM sequence suggest that mutations at this site could enhance the one-pot reaction for various Cas12a homologs.

3. Discussion

One-pot detection by combining CRISPR and isothermal amplification holds great promise in pathogen detection (Arizti-Sanz et al., 2020; Joung et al., 2020; Lu et al., 2022; Tong et al., 2024). We previously reported a method that utilized suboptimal PAMs to attenuate the *cis*-cleavage activity of LbCas12a, thereby reducing the interference of RPA amplification and improving the overall performance of one-step reaction (Lu et al., 2022). However, the suboptimal PAM approach is not applicable to Cas13a, which is targeting RNA without the requirement of PAM sequence (East-Seletsky et al., 2016; Lu et al., 2022; Tong et al., 2024). It is also not suitable for Cas12b, which usually have a simpler PAM sequence than Cas12a (Teng et al., 2019; Tong et al., 2024). A structurally modified Cas12b K396A/G478A mutant appropriately attenuates Cas12b cleavage activity to facilitate the one-pot reaction, suggesting that the Cas mutants may offer a universal solution (Tong et al., 2024). The Cas12b one-pot method operates at 60 °C, while Cas12a functions at 37 °C, requiring less power and simpler thermal management with comparable detection performance. Therefore, we identified Cas12a residues interacting with the PAM and protospacer sequences by analyzing the structure of the LbCas12a-crRNA-dsDNA complex. We

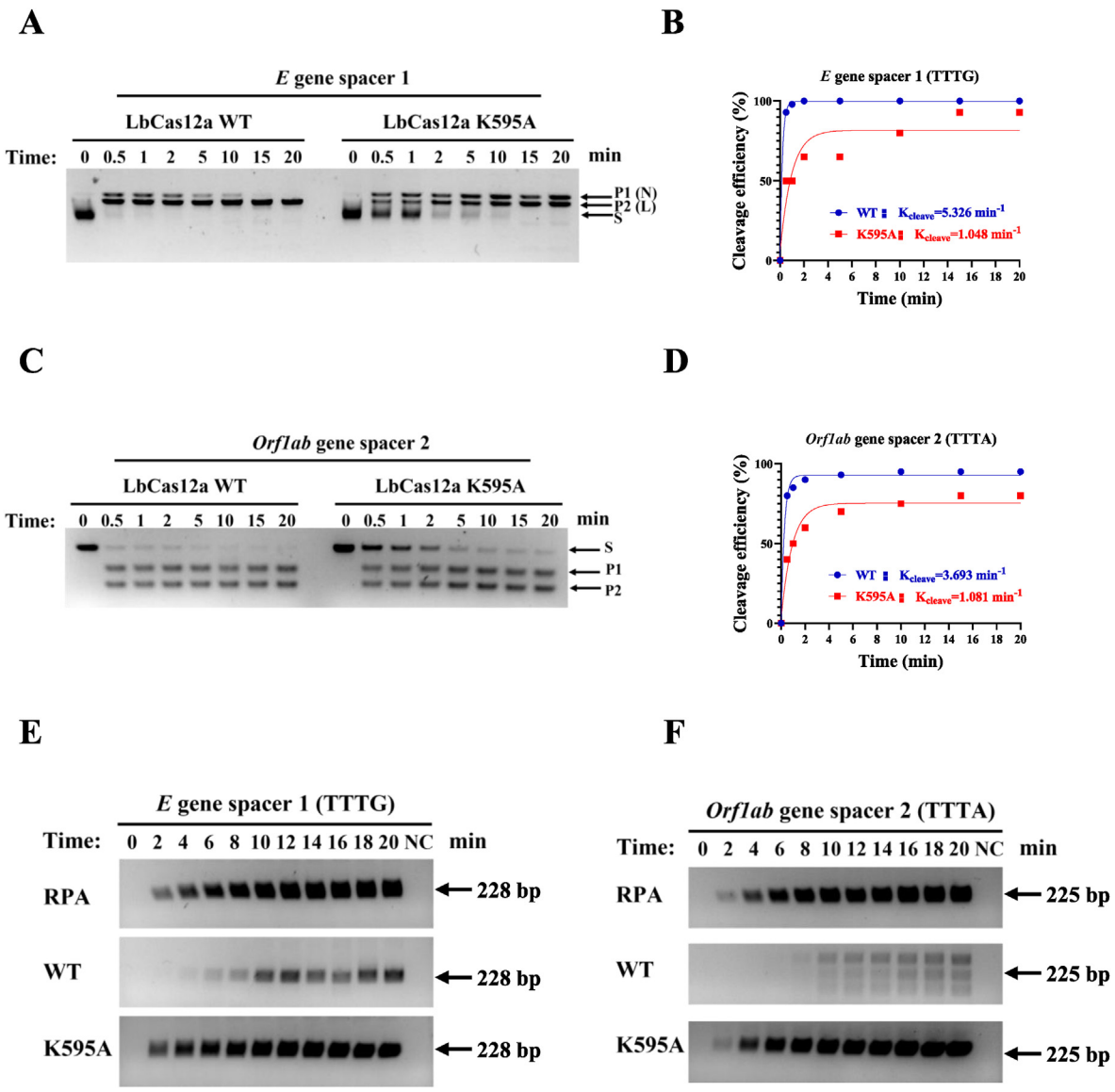


Fig. 3. Kinetics of LbCas12a WT and K595A mutant. (A)-**(D)** *Cis*-cleavage activity of LbCas12a WT and K595A mutant on *E* gene plasmid of 3314 bp and *Orf1ab* gene dsDNA of 461 bp. 'S' indicates plasmid substrates ((A), (B)) or dsDNA substrates ((C), (D)); 'P' indicates cleaved products; 'N' indicates nick, 'L' indicates linear DNA. The products of *Orf1ab* gene dsDNA are 242 bp and 219 bp. (E)-**(F)** Accumulation of RPA amplicons in one-pot reaction mediated by LbCas12a WT or K595A mutant at 37 °C for 0–20 min.

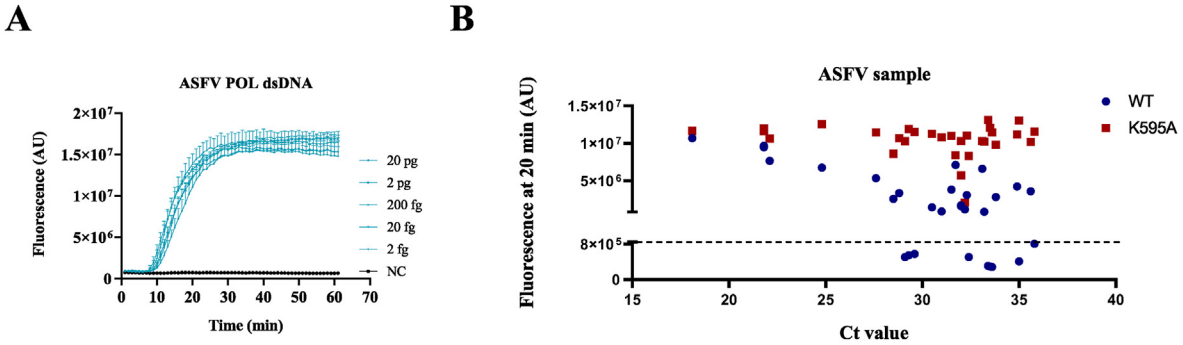


Fig. 4. Detection of ASFV using K595A mutant. (A) Limits of detection using LbCas12a K595A-mediated one-pot reaction. Mean ± s.d. of $n = 4$ technical replicates for ASFV POL dsDNA, and $n = 2$ for NC. (B) Fluorescence values of LbCas12a K595A-mediated one-pot reaction for detecting ASFV samples.

mutated these residues to alanine (Fig. 1 and Fig. S1). We found that mutants associated with PAM interactions, not those interaction with the targeting strand, exhibited stronger fluorescence signals compared to the wild-type in one-pot reaction (Fig. 1). Particularly, the K595A mutant,

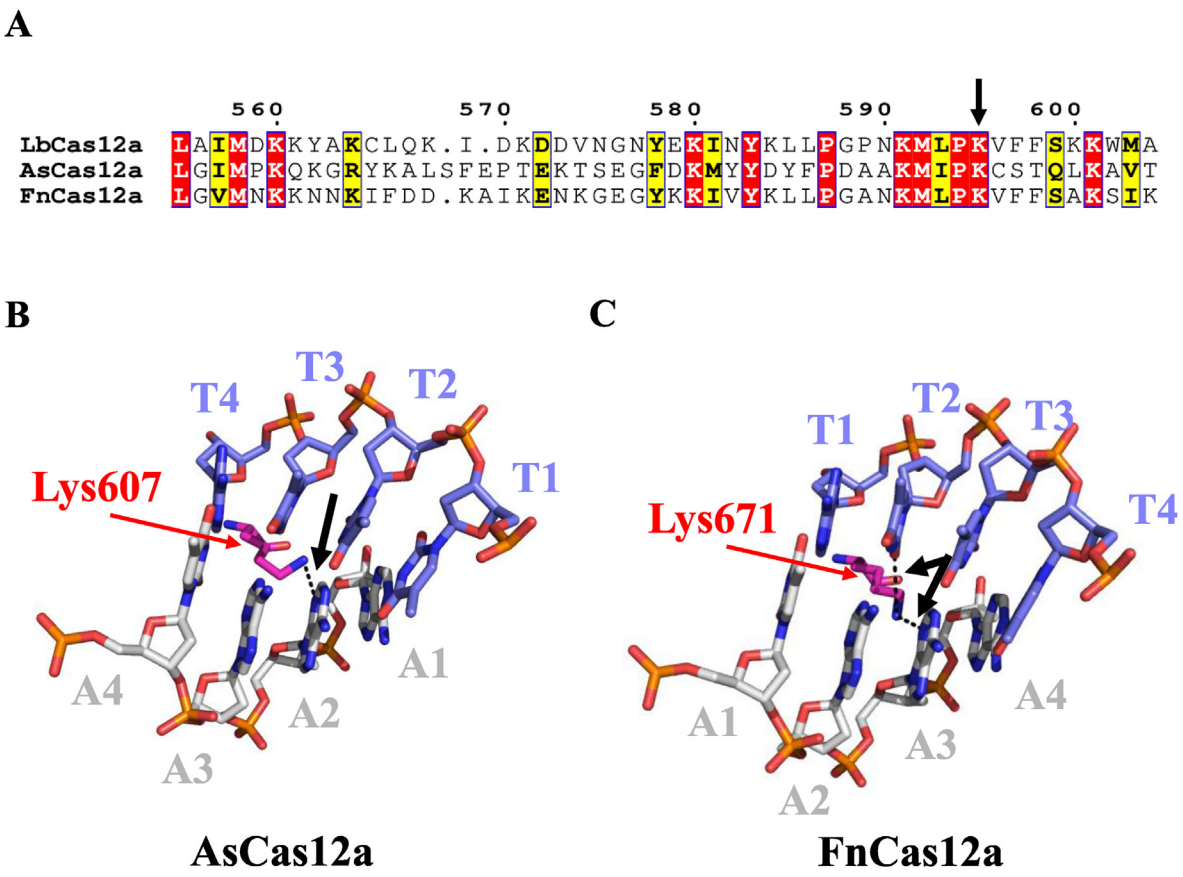


Fig. 5. Sequence and structure of Cas12a orthologs. (A) Sequence alignment of LbCas12a, AsCas12a and FnCas12a. (B)**-(C)** Hydrogen-bond interactions between PAM and the lysine residues of AsCas12a (B) and FnCas12a (C). The structures of AsCas12a (PDB number: 5B43) and FnCas12a (PDB number: 6I1K) were generated by PyMOL software.

which reduces interactions with T3 and A2 of PAM, mediated the most robust one-pot reaction (Figs. 1–2).

The LbCas12a WT exhibited excessive *cis*-cleavage activity in one-pot reaction, consuming over 80% of the substrate within 30 s, leading to slow and minimal accumulation of amplicons for amplification. Conversely, the *cis*-cleavage reduced Cas12a-K595A mutant enhanced the efficiency of amplicon accumulation, thus boosting the one-pot reaction (Fig. 3). Furthermore, the K595A mutant-mediated one-pot reaction successfully detected ASFV samples with high sensitivity, demonstrating its potential application in pathogen detection (Fig. 4).

The conservation of Lys595 among Cas12a homologs suggests that mutation of this residue may also be applicable to other Cas12a effectors (Fig. 5). For Cas12a with a simple PAM, such as FnCas12a (PAM: TTN), residue substitution may be a more suitable path than using a suboptimal PAM for one-pot reactions (Swarts & Jinek, 2019). The K595A mutant-mediated one-pot reaction using the canonical PAM further expands the range of targetable sites and enhances the flexibility of the one-pot reaction of nucleic acids detection.

4. Materials and methods

4.1. Substrate preparation

The *E* gene fragment was synthesized and cloned into pUC57 vector (GenScript). Primary porcine alveolar macrophages were infected with ASFV at an MOI of 0.1, using virus isolated from field samples. Supernatants were collected on day 3 post-infection. Seven-week-old SPF Large White and Landrace-cross pigs from the HVRI Laboratory Animal Center were intramuscularly inoculated with ASFV at 102.5 HAD50. Blood, oral, and anal swabs were collected daily for viral detection. Viral DNA was extracted from these samples using GenElute™ Mammalian Genomic DNA Miniprep Kits (Sigma Aldrich, USA) and subsequently quantified using ChamQ Universal SYBR qPCR Master Mix (Vazyme) (Lu et al., 2020). ASFV polymerase dsDNA substrates were obtained via PCR from genomic DNA of ASFV (Fig. S2). All ASFV-related experiments were approved by the Animal Ethics Committee of Harbin Veterinary Research Institute and the Animal Ethics Committee of Heilongjiang Province, and strictly adhere to the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People' s Republic of China.

4.2. Preparation of crRNA

The crRNA targeting *E* gene, *Orf1ab* gene and *ASFV* pol were synthesized via *in vitro* transcription. The dsDNA templates were prepared using overlapping PCR with two oligos. The dsDNA products, containing both T7 promoter sequence and the complete crRNA sequence, were incubated with T7 polymerase at 37 °C for a minimum of 2 h to synthesize the crRNA (Fig. S2). The transcription reaction was then treated with DNaseI to degrade the dsDNA templates, and the resulting RNA was purified using Monarch RNA cleanup kit (New England Biolabs) (Ji et al., 2024).

4.3. Protein expression and purification

The pET-based Cas12a recombinant plasmid, containing the codon sequence and a C-terminal 6 × His-tag was transformed into *Escherichia coli* BL21 (DE3) cells (WeiDibio). Colonies were cultured in LB medium at 37 °C until reaching an OD600 of 0.6–0.8. Isopropyl β-D-1-thio-galactopyranoside (IPTG) (Sangon Biotech) was added to a final

concentration of 0.5 mM, followed by incubation at 21 °C for 14 h. The cells were harvested in lysis buffer (20 mM Tris-HCl, 500 mM NaCl, and 500 mM imidazole, pH 7.4). The supernatant was purified using Ni-NTA resin in elution buffer (20 mM Tris-HCl, 500 mM NaCl, and 500 mM imidazole, pH 7.4). The purified protein was confirmed by SDS-PAGE (Fig. S1) and further purified by size-exclusion chromatography in elution buffer (20 mM Tris-HCl and 200 mM NaCl, pH 7.4) (Han et al., 2023; Lei et al., 2024; Shi et al., 2022).

4.4. Cis-cleavage

A 20 μL reaction mixture containing either 50 nM or 100 nM LbCas12a-crRNA ribonucleoprotein (RNP), 1 × NEBuffer 2.1 (New England Biolabs), and either 2.4 nM *E* gene plasmid substrate or 8.7 nM *Orf1ab* gene dsDNA substrate were incubated at 37 °C for various time points. The reaction was terminated by adding proteinase K (Thermo Fisher) and analyzed using a 1% Tris-acetate-EDTA (TAE) gel. Cleavage efficiency data were calculated using Prism 8 software to generate the K_{cleave} curve and determine values. The cleavage efficiency at each time point was calculated by analyzing the gray values of both the remaining substrate and the cleavage products.

4.5. One-pot reaction

The RPA-mediated one-pot reactions were conducted following previously established protocols (Lu et al., 2022; Tong et al., 2024), with a reaction volume of 30 μL containing 100 nM LbCas12a-crRNA RNP and 400 nM FQ ssDNA reporter (Takara Bio). The reaction was carried out at either 37 °C or 42 °C, and fluorescence values were monitored using a SpectraMax i3x instrument. To quantify amplicons, the one-pot reaction volume without FQ ssDNA reporter was terminated by proteinase K at various time points and assessed using a 2% Tris-acetate-EDTA (TAE) gel (Fig. S3). The substrate dosage of the *E* gene plasmid and *Orf1ab* gene dsDNA was 325 fM and 2,340 fM, respectively, unless otherwise specified.

CRediT authorship contribution statement

Xiaohan Tong: Writing – original draft, Investigation. **Tianle Li:** Investigation. **Kun Zhang:** Investigation. **Dongming Zhao:** Investigation. **Ying Zhang:** Investigation. **Hao Yin:** Writing – review & editing, Supervision, Conceptualization.

Data availability

The data that support the findings of this study are available within this manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hao Yin reports that financial support was provided by National Natural Science Foundation of China. H.Y.,Y.Z.,X.T. and K.Z. have filed patent applications through Wuhan University. Given her role as the Associate Editor, Ying Zhang had no involvement in the peer-review of this article and has no access to information regarding its peer-review.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellin.2025.100228.

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