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Rapid and sensitive Cas12a-based one-step nucleic acid detection with ssDNA-modified crRNA

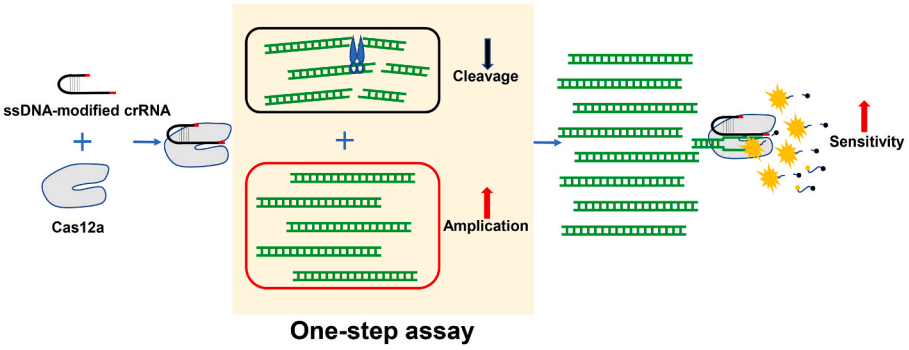
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HIGHLIGHTS

- The first time both ends ssDNA modified-crRNA utilize in one-step assay.
- The modified crRNA showed 100–1000-fold increased sensitivity in one-step assay.
- The modified crRNA was more resistant to RNase R treatment than wild-type crRNA.
- The turnaround time with CasDOS from sampling to results was within 30 min.

GRAPHICAL ABSTRACT



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ABSTRACT

CRISPR-Cas12a RNA-guided complexes have been developed to facilitate the rapid and sensitive detection of nucleic acids. However, they are limited by the complexity of the operation, risk of carry-over contamination, and degradation of CRISPR RNA (crRNA). In this study, a Cas12a-based single-stranded DNA (ssDNA)-modified crRNA (mD-crRNA)-mediated one-step diagnostic method (CasDOS) was established to overcome these drawbacks. mD-crRNA consisted of wild-type crRNA (Wt-crRNA) with ssDNA extensions at the 3′ and 5′ ends. Compared to Wt-crRNA, mD-crRNA exhibited a 100–1000-fold increase in sensitivity in the one-step assay, reducing the *cis*-cleavage activity of Cas12a to avoid excessive cleavage of the target DNA in the early stages of the reaction, leading to increased amplification and accumulation of the target amplicons, and improved the speed and intensity of the generated fluorescence signal. The detectability of CasDOS was 16.6 aM for the constructed plasmids of *Streptococcus agalactiae* (GBS), human papillomavirus type 16 (HPV16), and type 18 (HPV18). In clinical trials, CasDOS achieved 100% accuracy in identifying the known genotypes of the five HPV DNA samples. Moreover, CasDOS showed complete concordance with the qPCR results for GBS detection in ten vaginal or cervical swab samples, with a turnaround time from sampling to results within 30 min. In addition, mD-crRNA remained stable after Ribonuclease R treatment, suggesting that it might be more suitable as a raw material for the CRISPR detection kit. In conclusion, we have developed a universal, rapid, and highly sensitive one-step CRISPR detection assay.

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1. Introduction

Polymerase chain reaction (PCR)-based nucleic acid detection techniques, including quantitative PCR (qPCR), reverse transcriptase qPCR, and digital droplet PCR, are crucial for detecting infectious diseases [1–3]. However, their requirement for bulky and expensive equipment and well-trained personnel limits their application, particularly in resource-poor or developing countries. Several isothermal amplification techniques, such as recombinase polymerase amplification (RPA) [4] and loop-mediated isothermal amplification (LAMP) [5], have been developed as alternative methods for PCR due to their simplicity, portability, and speed. Nonetheless, they often generate false-positive results due to nonspecific amplification products [6,7].

A promising alternative to PCR and isothermal amplification techniques is the nucleic acid detection method using clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated nucleases (CRISPR/Cas), including Cas12, Cas13, and Cas14 [8,9]. The key principle underlying the CRISPR nucleic acid detection assay is *trans*-cleavage, also known as collateral cleavage activity [10–13]. CRISPR RNA (crRNA) can base pair with a target sequence when the Cas protein binds to it, triggering enzymatic *cis*-cleavage that specifically cleaves the target sequence [14,15] and *trans*-cleavage that nonspecifically degrades ambient single-stranded DNA (ssDNA) or RNA [10–13]. Combined with isothermal amplification, CRISPR-based nucleic acid detection platforms, such as SHELOCK [10], DETECTR [12], and CDetection [16], show high sensitivity and specificity, facilitating the development of point-of-care (POC) tests.

However, the enzymatic reactions of isothermal amplification and CRISPR detection are incompatible [17,18], requiring a two-step process of pre-amplification and detection of the amplified product using CRISPR-based techniques [10,12,16,19–21]. Given that the complexity of the operation and the risk of carry-over contamination limit this two-step process, ongoing research aims to streamline and simplify the process by developing one-step assays that combine the amplification and detection steps into a single reaction. Researchers have investigated physical separation [17,22], concentration optimization of various amplification and detection components [18,23,24], and unconventional CRISPR detection [25–27]. The implementation of these one-step methods has substantially advanced the clinical applications of CRISPR detection.

In this study, we aimed to establish a one-step diagnosis method, named CasDOS, using a Cas12a-based ssDNA-modified crRNA (mD-crRNA) to streamline the process. The wild-type crRNA (Wt-crRNA) was modified with ssDNA at the 3′ and 5′ ends. The sensitivity of CasDOS was demonstrated by detecting the constructed plasmids of Streptococcus agalactiae (GBS), human papillomavirus type 16 (HPV16), and type 18 (HPV18). Furthermore, its clinical testing performance was validated using DNA samples and vaginal or cervical swab samples. The stability of the mD-crRNA was verified by Ribonuclease R (RNase R) digestion, and the principle of the one-step assay mediated by mD-crRNA was elucidated. This study provided the first evidence that mD-crRNA can be used for a one-step assay, which offers insight into the potential use of other types of modified or engineered crRNAs for one-step assay and promotes the further development of Cas12a-based detection assay.

2. Materials and methods

2.1. Source of samples and ethics approval

Clinical samples were collected from Changsha Hospital for Maternal and Child Health Care and Hunan Jiahui Genetics Hospital, and informed consent was acquired from all sample donors. The study was conducted in accordance with the Declaration of Helsinki guidelines and approved by the Ethics Committee of School of Life Sciences, Central South University, China (No. 2019-1-16 and No. 2023-1-31).

2.2. Materials

All oligonucleotides, including FAM-BHQ1-labeled ssDNA, FITC-biotin-labeled ssDNA, and RNA were synthesized by Sangon Biotech (Shanghai, China), and their detailed sequences are in Tables S1 and S2. The plasmids, including sequence A, GBS *cfb*, HPV16 *L1*, and HPV18 *L1* genes, were synthesized by Tsingke Biotech (Beijing, China), and their detailed sequences are in Table S3. AsCas12a was acquired from Ezassay Biotech (Guangdong, China). RPA kit was purchased from Amplification Future Biotech (Jiangsu, China). RNase R was obtained from Haigene Biotech (Heilongjiang, China). A HybriDetect kit was purchased from Milenia Biotech (Gießen, Germany). ChamQ Universal SYBR qPCR Master Mix was obtained from Vazyme Biotech (Jiangsu, China). One-Stop miRNA Urea-PAGE packages were purchased from Zeye Biotech (Shanghai, China).

2.3. Target double-stranded DNA (dsDNA) preparation

The target dsDNA was prepared by hybridizing an equivalent molar amount of the two complementary oligo DNAs, the target strands (TS) and non-target strands (NTS) for GBS, HPV16, and HPV18, at 20 μM in 1 × AsCas12a reaction buffer at 95 °C for 5 min, followed by a slow cooling process to 25 °C at a rate of 0.1 °C s^{-1}.

2.4. RPA assay

For the RPA assay, a 10 μL reaction mixture was prepared consisting of 4 μL of C buffer, 0.3 μL of dNTPs (10 mM), 0.4 μL of RPA forward primer (10 μM), 0.4 μL of RPA reverse primer (10 μM), 1 μL of L buffer, 2.4 μL of P-core, 0.5 μL of B buffer, and 1 μL of DNA template. The C buffer, dNTPs, RPA primer pairs, L buffer, and P-core were first mixed and added to the reaction tube. B buffer and the DNA template were then added to the same tube. The reaction was performed at 39 °C for 30 min.

2.5. Cis-cleavage assay

For the *cis*-cleavage assay, a 20 μL reaction mixture was prepared containing 13 μL of RNase-free water, 2 μL of 10 × AsCas12a reaction buffer, 2 μL of AsCas12a (2 μM), 2 μL of crRNA (2 μM), and 1 μL of RPA amplicons. The AsCas12a and crRNA ribonucleoprotein (RNP) were incubated at room temperature for 10 min before being mixed with RPA products. The mixture was then incubated at 39 °C for 0, 5, 10, 15, 20, 25, and 30 min. The reaction was stopped at different time points by heating the mixture at 80 °C for 10 min, and the products were visualized on agarose gels.

2.6. Collateral cleavage assay

To perform the collateral cleavage assay, a 20 μL reaction mixture was prepared containing 15.5 μL of RNase-free water, 2 μL of 10 × AsCas12a reaction buffer, 0.5 μL of AsCas12a (2 μM), 0.5 μL of crRNA (2 μM), 0.5 μL of FAM-BHQ1-labeled ssDNA (10 μM), and 1 μL of target dsDNA (5-100 nM). The mixture was incubated at 39 °C for 40 min, and the fluorescence signal was recorded every minute using a CFX96 Real-Time PCR Detection System (Bio-Rad, USA).

2.7. One-step assay

To perform the one-step assay, a 10 μL reaction mixture was prepared containing 1.3 μL of RNase-free water, 0.2 μL of 10 × AsCas12a reaction buffer, 0.25 μL of AsCas12a (1-2 μM), 0.25 μL of crRNA (1-2 μM), 0.5 μL of FAM-BHQ1-labeled ssDNA (10 μM), 6.5 μL of RPA mixture, and 1 μL of DNA template. The RNP were first incubated at room temperature for 10 min in 1 × AsCas12a reaction buffer, followed by mixing with the RPA mixture and FAM-BHQ1-labeled ssDNA in a reaction tube. Finally, B buffer and the DNA template were added to the

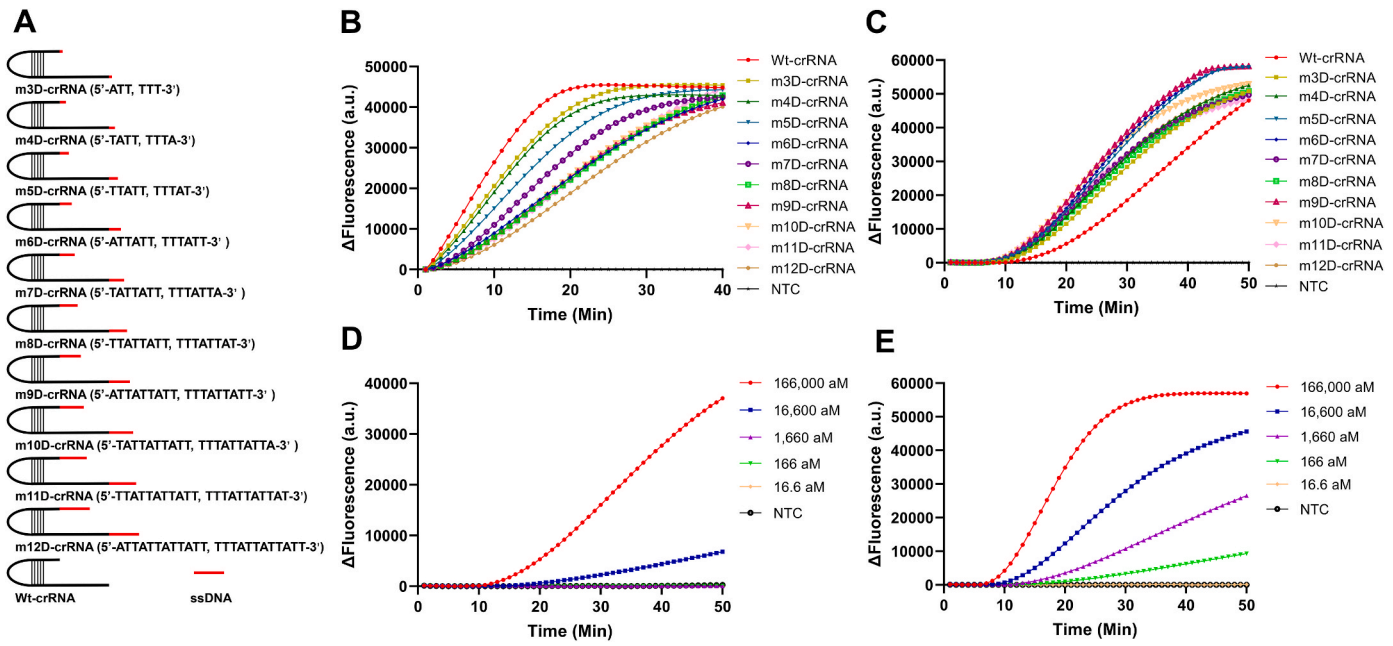


Fig. 1. Comparison of Wt-crRNA and corresponding mD-crRNAs in one-step assays. (A) Schematic illustration of the designed mD-crRNAs. m3D-to m12D-crRNAs are Wt-crRNA with 3–12 DNA bases added in both ends, respectively. The fluorescence signals of Wt- and mD-crRNAs targeting sequence A in collateral cleavage assay (B) and one-step assay (C). Sensitivity of one-step assays using Wt-crRNA (D) and m9D-crRNA (E) targeting sequence A. NTC represents no template control. Δ, represents subtracted the background fluorescence. a.u. represents arbitrary units.

same tube. The mixture was incubated at 39 °C for 50 min, and the signal was measured every minute using the CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The sensitivity of the one-step assay was performed using 10-fold gradient dilution of the plasmids. The limit of detection (LoD) is defined as the lowest concentration at which the generated fluorescent signal values show statistically significant differences compared to the fluorescent signal values generated by the no template control in three repeated experiments.

2.8. Accumulation of RPA products in one-step assay

The assay was performed according to section 2.6, except that the reaction did not include FAM-BHQ1-labeled ssDNA and the DNA template concentration was 3,320 fM. The reaction mixture was incubated at 39 °C for 0, 4, 8, 12, 16, and 20 min, and the reaction was terminated by heating at 80 °C for 10 min at various time points. The resulting products were analyzed using agarose gels.

2.9. Lateral flow strip-based one-step assay

The lateral flow strip assay was conducted following the protocol described in 2.6, with the only difference being the use of FITC-biotin-labeled ssDNA instead of FAM-BHQ1-labeled ssDNA at a concentration of 1 μM. After the reaction, 10 μL of the product was mixed with 40 μL of HybriDetect assay buffer in a 1.5 mL tube. The lateral flow strip was then inserted into the tube and incubated for 3–5 min before the result was observed by the naked eye.

2.10. qPCR assay

For the qPCR assay, a 20 μL reaction mixture was prepared consisting of 7 μL of RNase-free water, 10 μL of 2 × ChamQ Universal SYBR qPCR Master Mix, 1 μL of qPCR forward primer (10 μM), 1 μL of qPCR reverse primer (10 μM), and 1 μL of DNA template. The reaction was carried out by incubating at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. Fluorescence signals were recorded during annealing, and a melt curve analysis was conducted by cooling the reaction to 60 °C and heating to 95 °C at a rate of 0.5 °C s^{-1} with data collection every second.

2.11. RNase R digestion assay

For RNase R digestion assay, a 20 μL reaction containing 7 μL of RNase-free water, 2 μL of 10 × RNase R buffer, 0.5 μL of Rnase inhibitor (40 U μL^{-1}), 0.5 μL of RNase R (40 U μL^{-1}), and 10 μL of crRNA (10 μM) was incubated at 37 °C for 0, 2, 5, 10, 20, 30, and 40 min. The reaction was stopped by heating at 80 °C for 10 min, and the products were analyzed by collateral cleavage and polyacrylamide gel electrophoresis (PAGE) assays.

2.12. PAGE assay

The RNase R digestion products were separated on a 15% denaturing polyacrylamide gel containing 7 M urea in 1 × TBE buffer by electrophoresis at 150 V for 30–40 min. GelRed staining was used for visualization.

2.13. Statistical analysis

The statistical analysis was based on three experimental replicates and results are expressed as means ± S.D. GraphPad Prism 9 was used for statistical analysis using the unpaired, two-tailed *t*-test for two-group comparisons and Dunnett's multiple comparisons test for multi-group comparisons. Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Effects of using mD-crRNA in one-step assay

To determine whether mD-crRNA activated the collateral cleavage activity of Cas12a, we designed a Wt-crRNA and modified crRNAs targeting sequence A, in which the modification involved adding 3-12 AT-rich DNA bases at both ends of the Wt-crRNA (Fig. 1A). All mD-crRNAs activated Cas12a but were less effective than Wt-crRNA when treated

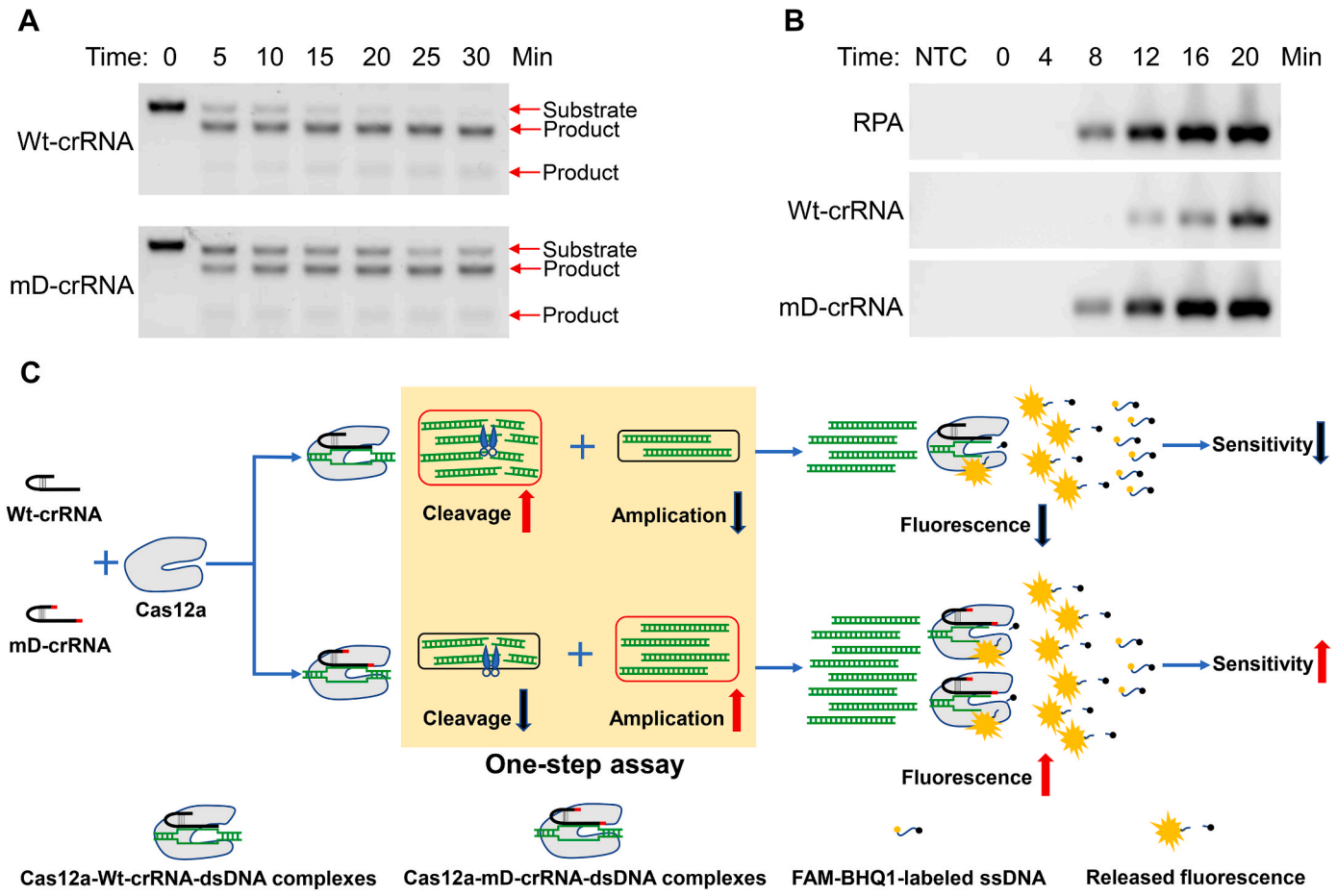


Fig. 2. The principle of the one-step assay mediated by mD-crRNA. (A) *Cis*-cleavage activities of Cas12a using Wt-crRNA and the corresponding m9D-crRNA targeting sequence A. (B) Accumulation of RPA products in one-step assays using Wt-crRNA and the corresponding m9D-crRNA targeting sequence A. The fragment size of the RPA amplicons is 192 bp. (C) Schematic illustration of the principle of one-step assays using Wt-crRNA and mD-crRNA. For the one-step assay using Wt-crRNA, the high *cis*-cleavage activity of Cas12a resulted in excessive consumption of target DNA in the early stages of the reaction, thereby reducing the amount of target DNA for nucleic acid amplification. Conversely, for the one-step assay using mD-crRNA, the weakened *cis*-cleavage activity of Cas12a allowed for more target DNA to be amplified and accumulated, which increases the fast and intense of the fluorescence signals. The up arrows in one-step assays show the dominating process.

with dsDNA containing the targeted sequence A (Fig. 1B). However, unexpectedly, the mD-crRNAs exhibited faster fluorescence signal kinetics than the Wt-crRNA in the one-step assay (Fig. 1C), in which RPA and CRISPR system were detected simultaneously in the same tube. The fluorescence signal kinetics did not show a regular change with an increased number of DNA bases added by Wt-crRNA. m5D-crRNA, m6D-crRNA, and m9D-crRNA showed faster fluorescence signal kinetics than other mD-crRNAs, and m9D had a low background fluorescence signal (Fig. S1). Therefore, m9D-crRNA was used in the subsequent experiments.

The LoD for m9D-crRNA was evaluated using serial dilutions of plasmids containing sequence A. m9D-crRNA could detect as little as 166 aM of the target plasmid (Fig. 1E), which was 100-fold lower than the LoD of Wt-crRNA (Fig. 1D). Sensitivity analysis showed that the one-step assay using mD-crRNA had better detection performance than the one-step assay using Wt-crRNA.

3.2. *The principle of one-step assay mediated by mD-crRNA*

Since RPA and CRISPR detection compete for the target DNA in the one-step assay, the detectability depends on the balance of these two reactions [17,22,26,27]. Specifically, in the one-step assay, high *cis*-cleavage activity of Cas12a consumes a significant amount of target DNA in the early stages of the reaction, resulting in insufficient target DNA amplification for RPA and subsequently reducing detectability. Conversely, Cas12a with low *cis*-cleavage activity reduces cleavage of the target DNA in the reaction, allowing RPA to amplify and accumulate a larger amount of target DNA and, thereby enhancing detectability. Hence, we hypothesized that the improved performance of the mD-crRNA-mediated one-step assay was due to the reduced activity of Cas12a in cleaving the target DNA, which led to increased amplification and accumulation of the target amplicons, resulting in the excitation of a fast and intense fluorescent signal production. To verify this possibility, we performed a *cis*-cleavage experiment using Wt-crRNA and m9D-crRNA targeting sequence A. Agarose gel analyses showed that the Cas12a-m9D-crRNA complexes cleaved the target amplicons significantly less than the Cas12a-Wt-crRNA complexes (Fig. 2A and Fig. S2A). We further validated the accumulation of the amplification products in the one-step assay. For the one-step assay using m9D-crRNA or RPA alone, amplification products were observed at the 8th minute, while for the one-step reaction using Wt-crRNA, amplification products were only observed at the 12th minute (Fig. 2B and Fig. S2B). Moreover, the amount of amplification products in the one-step reaction using m9D-crRNA or RPA alone was significantly higher than that produced in the one-step reaction using Wt-crRNA at the 12th, 16th, and 20th time points (Fig. 2B and Fig. S2B). These experiments demonstrated that the superior performance of mD-crRNA compared to that of Wt-crRNA in the one-step reaction could be attributed to its ability to reduce Cas12a cleavage activity for target DNA and bias the reaction toward RPA, leading to increased accumulation of amplicons and improved the detectability (Fig. 2C).

Regarding the mechanism underlying the reduction in Cas12a *cis*-

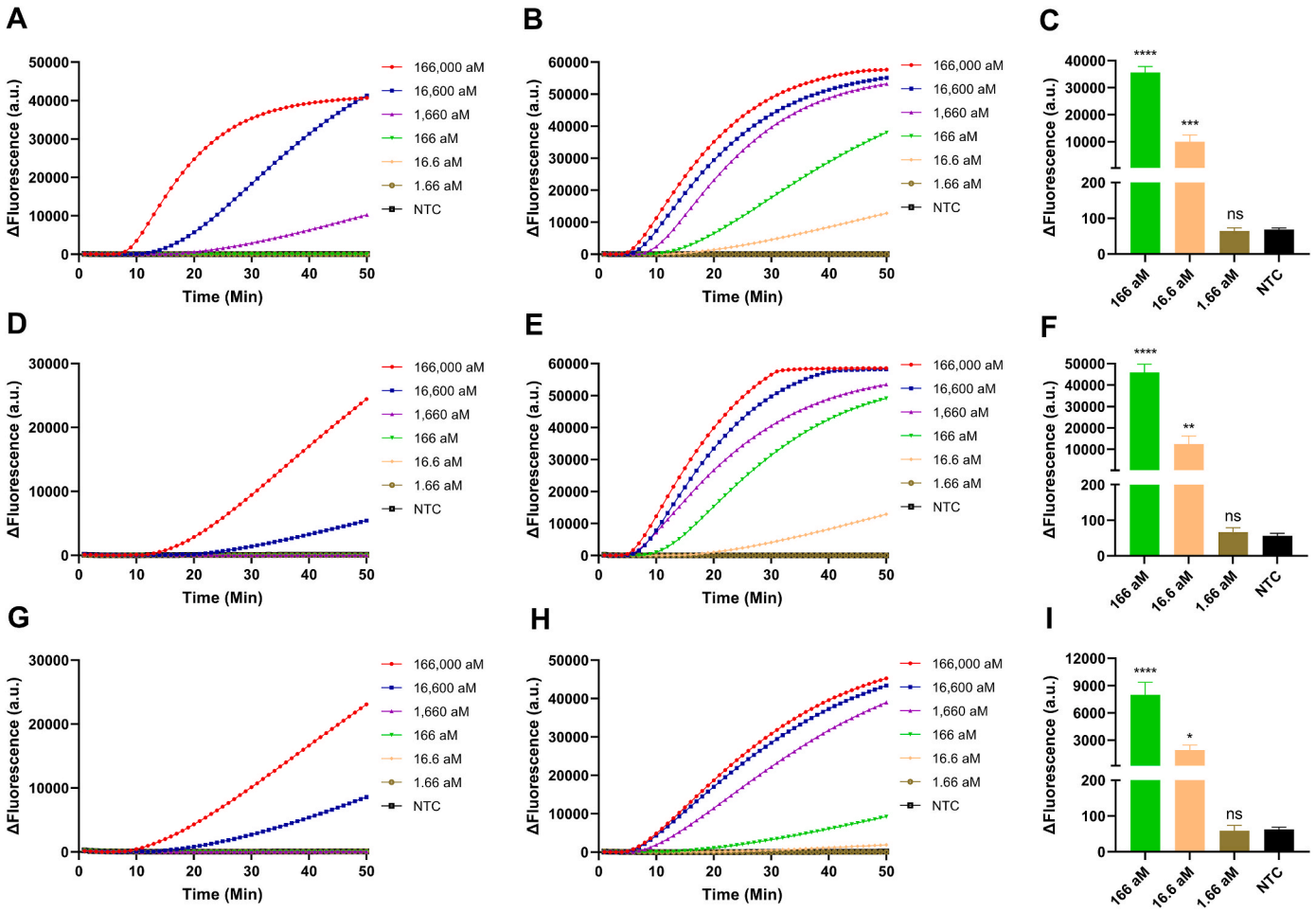


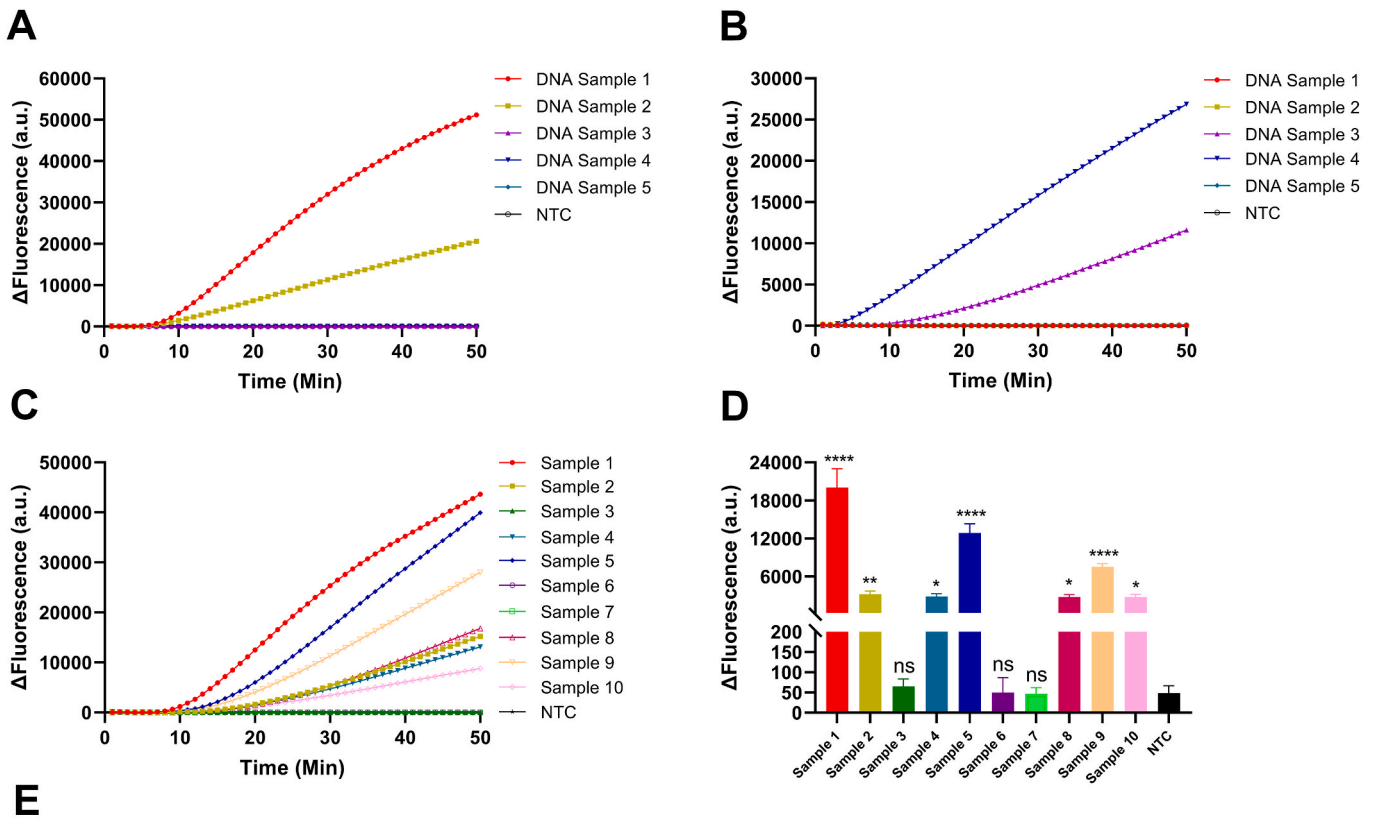
Fig. 3. Sensitivity of one-step assays using Wt-crRNAs and corresponding m9D-crRNAs for GBS, HPV16, and HPV18. Sensitivity of one-step assays using Wt-crRNA (A) and m9D-crRNA (B) targeting the GBS *cfb* gene. Sensitivity of one-step assays using Wt-crRNA (D) and m9D-crRNA (E) targeting the HPV16 *L1* gene. Sensitivity of one-step assays using Wt-crRNA (G) and m9D-crRNA (H) targeting the HPV18 *L1* gene. Fluorescence values of the CasDOS for GBS (C), HPV16 (F), and HPV18 (I) obtained at the 50th minute. Dunnett's multiple comparisons test was used to analyze the difference from the NTC. Error bars in (C), (F), and (I) represent the mean ± SD (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns: not significant), with n = 3 technical replicates. NTC represents no template control. Δ, represents subtracted the background fluorescence. a.u. represents arbitrary units.

cleavage activity caused by mD-crRNA, we hypothesized that this might be due to the conformational change induced by mD-crRNA in the Cas12a RNP complexes, which results in decreased affinity between the RNP and target DNA. Upon binding to Wt-crRNA, Cas12a undergoes a structural rearrangement to bind to the target DNA [28]. Adding DNA bases to both ends of the Wt-crRNA might further alter the RNP conformation, resulting in decreased affinity of the RNP complexes for the target DNA that could delay and reduce the cleavage of the target DNA by RNP, resulting in more target DNA being amplified and accumulated in the one-step reaction [26,27]. Thus, mD-crRNA was more suitable for the one-step assay because it balanced amplification and detection. Nguyen et al. [29] reported a study on the impact of crRNA with DNA base modifications at the 3′- or 5′-end on the activity of Cas12a. Their study found that the first uracil on the 5′-end of crRNA is cleaved by Cas12a in the absence of target DNA, resulting in a minimal effect on the enzymatic activity of Cas12a for the 5′-end DNA base-modified crRNA. The effects of the 3′-end DNA base-modified crRNA on different orthologs of Cas12a varied. The 3′-end DNA base-modified crRNA did not alter the *cis*-cleavage activity of LbCas12a but enhanced its *trans*-cleavage capability, while it did not affect the *cis*-cleavage activity of AsCas12a but reduced its *trans*-cleavage ability. However, in our study, mD-crRNA not only reduced the *cis*-cleavage activity of AsCas12a but also decreased its *trans*-cleavage activity. These findings demonstrated the complexity of the interaction mechanism between Cas12a and DNA base-modified Wt-crRNA, highlighting the scientific value of further research.

3.3. CasDOS detect GBS, HPV16, and HPV18

To assess the versatility of CasDOS, we selected GBS, HPV16, and HPV18 for further validation using RPA primers and Wt-crRNAs based on the previous studies (Tables S1 and S2) [12,30]. The corresponding m9D-crRNAs were designed. As expected, m9D-crRNAs had a lower ability to activate the collateral cleavage activity of Cas12a (Figs. S3A, C, and E) and faster kinetics of the fluorescence signals in the one-step assays compared to those of Wt-crRNAs (Figs. S3B, D, and F). Surprisingly, the LoD of the one-step assays for GBS, HPV16, and HPV18 employing m9D-crRNAs were 16.6 aM (Fig. 3B, C, E, F, H, and I), while those using Wt-crRNAs were only 1,660 aM, 16,600 aM, and 16,600 aM (Fig. 3A, D, and G), respectively. In addition, we combined CasDOS with a lateral flow strip owing to its portability. The LoD of the lateral flow strip assay for GBS, HPV16, and HPV18 was 166 aM, 166 aM, and 1,660 aM, respectively (Figs. S4A, B, and C).

The results of the validation assays for GBS, HPV16, and HPV18 were like those obtained for sequence A. These m9D-crRNAs exhibited a ~100- to 1000-fold increase in sensitivity compared to Wt-crRNAs in one-step assays. This suggests that the superiority of m9D-crRNA over Wt-crRNA in the one-step assay might be a widespread phenomenon and



E

Method	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
CasDOS	Positive	Positive	Negative	Positive	Positive	Negative	Negative	Positive	Positive	Positive
qPCR	Positive	Positive	Negative	Positive	Positive	Negative	Negative	Positive	Positive	Positive

Fig. 4. Detection of HPV16, HPV18, and GBS clinical samples by CasDOS. (A) Fluorescence signals for detecting HPV16 in CasDOS. (B) Fluorescence signals for detecting HPV18 in CasDOS. (C) Fluorescence signals for detecting GBS in CasDOS. (D) Fluorescence values of vaginal or cervical swab samples obtained at the 25th minute using CasDOS. (E) Uniformity table between CasDOS and qPCR methods for vaginal or cervical swab samples. Dunnett's multiple comparisons test was used to analyze the difference from the NTC. Error bars in (C) represent the mean ± SD, with n = 3 technical replicates (****$p < 0.0001$, **$p < 0.01$, *$p < 0.05$, ns: not significant). NTC represents no template control. Δ, represents subtracted the background fluorescence. a.u. represents arbitrary units.

that CasDOS could be utilized to detect a wide range of organisms. Notably, the LoD of these one-step assays met the requirement for clinical testing (attomolar sensitivity), comparable to that of two-step assays in which RPA and cleavage of Cas12a are performed separately [12,31]. In addition, we observed that the minimum detectable concentrations of GBS, HPV16, and HPV18 could be explicitly distinguished from the negative control at the 25th minute (Fig. S5). This indicates that the detection time of CasDOS could be reduced to 25 min. Overall, the developed CasDOS appears to be a universal, easy-to-use, rapid, and highly sensitive nucleic acid testing tool.

3.4. Clinical validation of CasDOS

To evaluate the applicability of CasDOS for molecular diagnostics, we performed a clinical study using five DNA samples with known genotypes and ten vaginal or cervical swab samples. The five DNA samples with known genotypes consisted of two HPV16-positive samples, two HPV18-positive samples, and one negative sample, which were provided by Changsha Hospital for Maternal and Child Health Care (Table S4). CasDOS was utilized to detect HPV16 and HPV18 in each of these five samples, and the results showed complete concordance between the detected genotypes and the known genotypes of these DNA samples (Fig. 4A, B and Table S4).

Ten vaginal or cervical swab samples were collected by Hunan Jiahui Genetics Hospital. They were diluted in 200 μL of water and subjected to crude DNA extraction by heating at 100 °C for 5 min. Subsequently, CasDOS was employed for GBS detection, and seven GBS-positive and three GBS-negative samples were identified. The outcomes were compared with those obtained from the qPCR method established in our laboratory (the sensitivity of qPCR was 11.1 aM, as shown in Fig. S6). The analysis revealed that CasDOS had 100% concordance with the results obtained by the qPCR method for both GBS-positive and GBS-negative samples (Fig. 4C, E and Figs. S7A–B). In addition, all vaginal or cervical swab samples were accurately identified within 25 min (Fig. 4D).

qPCR is a commonly used nucleic acid detection method known for its high sensitivity in detecting infectious diseases [32,33]. In this study, qPCR was used as a comparison to CasDOS for GBS detection. The LoD of CasDOS was comparable to that of the qPCR method, indicating that CasDOS possesses high sensitivity, The 100% accuracy of CasDOS in detecting the five DNA samples and ten vaginal or cervical swab samples demonstrated its excellent clinical detection performance. Furthermore, CasDOS completed the entire process in 30 min, including 5 min for sample processing and 25 min for nucleic acid detection, highlighting its outstanding speed advantage. Notably, compared to the two-step assays [12,31], CasDOS was easier to handle and reduced the possibility of cross-contamination by eliminating the need to open the cap. These results suggest that CasDOS could be a suitable tool for POC testing to detect infectious diseases, especially for tests that require urgent reporting to clinicians.

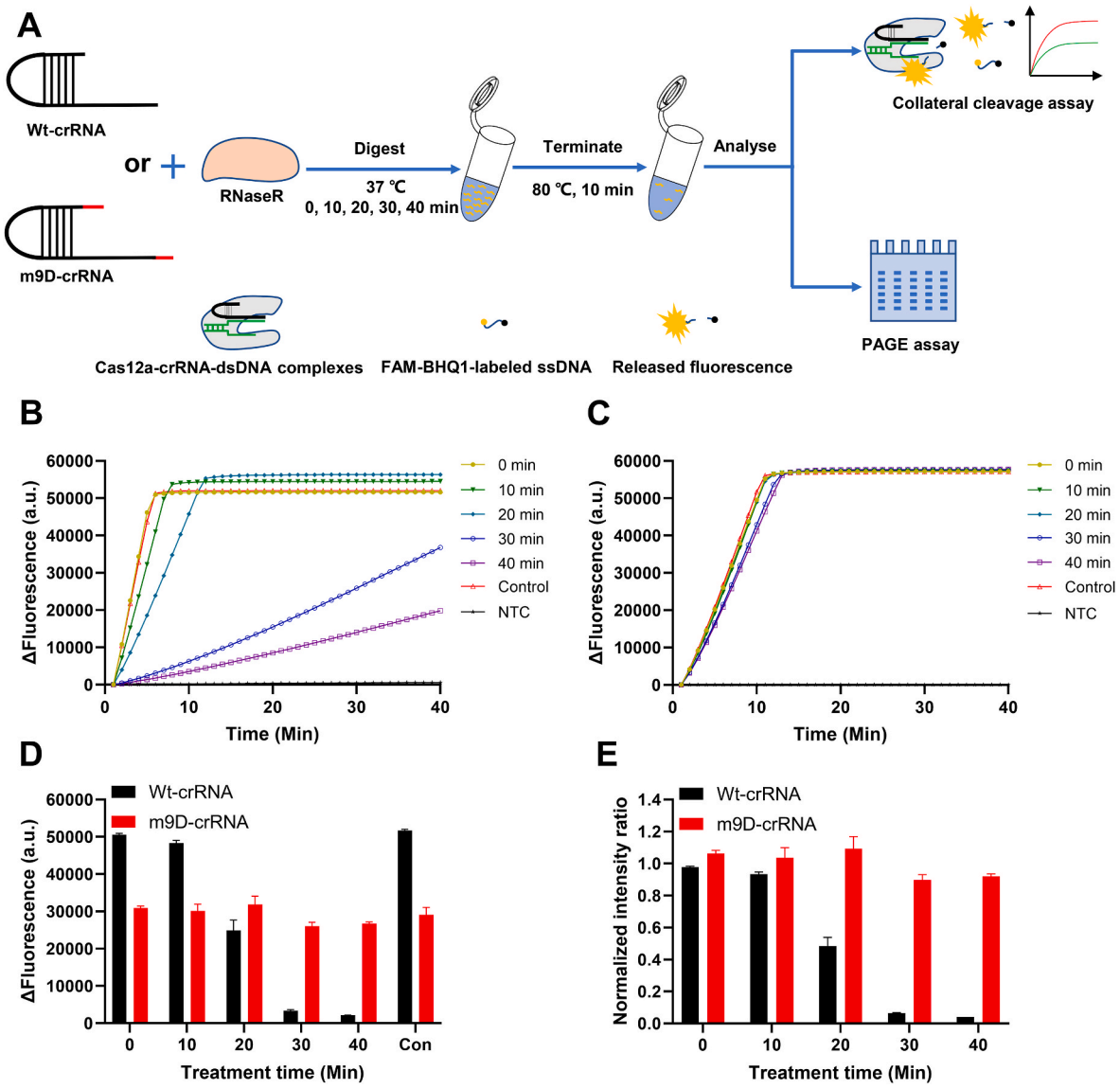


Fig. 5. Effects of Wt-crRNA and corresponding m9D-crRNA treated by RNase R. (A) Schematic workflow of Wt-crRNA and m9D-crRNA targeting the GBS *cfb* gene treated by RNase R. Fluorescence signals of Wt-crRNA (B) and m9D-crRNA (C) treated by RNase R in the collateral cleavage assay. Fluorescence values (D) and normalized intensity ratio (E) of Wt-crRNA and m9D-crRNA treated by RNase R obtained at the 5th minute in the collateral cleavage assay. Con represents the untreated Wt-crRNA or m9D-crRNA by RNase R. The normalized intensity ratio represents the ratio of fluorescence values of RNase R-treated and untreated Wt-crRNA or m9D-crRNAs, respectively. Error bars in (D) and (E) represent the mean ± SD, with n = 3 technical replicates. NTC represents no template control. Δ, represents subtracted the background fluorescence. a.u. represents arbitrary units.

3.5. *Effect of RNase R treatment on mD-crRNA*

To validate the stability of the mD-crRNA, an RNase R digestion assay was conducted using Wt-crRNA and the corresponding m9D-crRNA targeting GBS *cfb*. The process of the RNase R digestion assay is shown in Fig. 5A. The collateral cleavage experiment results revealed that the fluorescence signal kinetics of Wt-crRNA decreased significantly compared to those of m9D-crRNA after incubation with RNase R (Fig. 5B and C). To visually observe the impact of RNase R treatment, fluorescence measurements were compared at the 5th minute reaction point. Compared to the corresponding untreated group, the fluorescence value of Wt-crRNA decreased to 35% after 20 min of incubation and to approximately 3% after 40 min of incubation (Fig. 5D and E), whereas the fluorescence value of m9D-crRNA decreased by less than 10% even after 40 min of incubation (Fig. 5D and E). Based on the results of the PAGE analysis, it was observed that the Wt-crRNA band showed a significant decrease in intensity after incubation with RNase R for 20 min and was almost completely degraded after 30 min (Fig. S8A). In contrast, the m9D-RNA band remained bright even after 40 min of incubation with RNase R (Fig. S8B).

These findings demonstrate that RNase R efficiently degrades Wt-crRNA but has little effect on m9D-crRNA, which is crucial for the CRISPR detection method, as RNA is extremely unstable in the aqueous phase. Unstable RNA (crRNA) may not only affect the accuracy of the CRISPR detection assay, but also shorten the shelf life of CRISPR detection kits. However, no feasible method exists for solving this problem. Kim et al. [34] discovered that replacing the last eight RNA bases at the 3′-end of crRNA with DNA bases not only improved the specificity of Cas12a in target DNA cleavage but also made the CRISPR/Cas system more convenient for commercialized products and gene editing. Inspired by that study, we successfully used mD-crRNAs for a one-step detection. Furthermore, we demonstrated the stability of the mD-crRNA under RNase R treatment. Therefore, mD-crRNA may be a more suitable raw material for use in the CRISPR detection kits. However, maintaining crRNA stability remains a long-term challenge.

4. Conclusion

In the present study, we developed a universal, rapid, and highly sensitive one-step nucleic acid detection assay based on Cas12a. We achieved a one-step reaction compatible with RPA and CRISPR detection by adding DNA bases to both ends of Wt-crRNA. The mD-crRNA showed a sensitivity increase of 100-1000-fold compared to that of the Wt-crRNA in the one-step assay. This was due to the reduced *cis*-cleavage activity of Cas12a caused by mD-crRNA, which facilitated the equilibrium transition of the reaction towards isothermal amplification. As a result, there was more amplification and accumulation of the target amplicons, leading to faster and stronger fluorescent signal than those of Wt-crRNA. The LoD of CasDOS was 16.6 aM for plasmids GBS, HPV16, and HPV18. In clinical testing, CasDOS demonstrated 100% accuracy in detecting the known genotypes of the five HPV DNA samples. Moreover, CasDOS exhibited 100% agreement with the qPCR results for GBS detection in ten vaginal or cervical swab samples, with results obtained in 30 min. Additionally, we found that mD-crRNA was more resistant to RNase R treatment than Wt-crRNA, indicating that it might be a more suitable raw material for CRISPR detection kits. However, our study has some limitations. First, we did not investigate how mD-crRNA led to reduced *cis*- and *trans*-cleavage activities of Cas12a. Understanding this mechanism is crucial to further improve the performance of CasDOS and promote its clinical application. Second, the number of samples used in our clinical validation was small, and further validation with a larger number of clinical samples is necessary for the practical application of CasDOS. We aim to address these limitations in future studies. In conclusion, the CasDOS can potentially be used as a POC test for detecting infectious diseases.

CRediT authorship contribution statement

Qinlong Zeng: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **Miaojin Zhou:** Conceptualization, Methodology, Funding acquisition, Supervision, Writing – review & editing. **Zhiqing Hu:** Formal analysis, Data curation. **Weiheng Deng:** Validation, Formal analysis. **Zhuo Li:** Conceptualization, Funding acquisition, Project administration, Supervision. **Lingqian Wu:** Funding acquisition, Project administration, Resources. **Desheng Liang:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2023.341622.

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