analytical chemistry

pubs.acs.org/ac Article

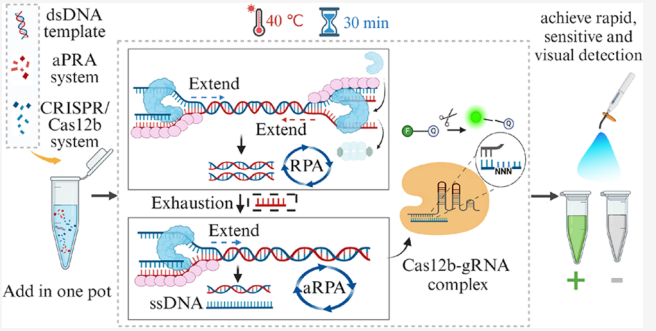
A PAM-Free One-Step Asymmetric RPA and CRISPR/Cas12b Combined Assay (OAR-CRISPR) for Rapid and Ultrasensitive DNA Detection

Lei Yang,[#] Guanwei Chen,[#] Jian Wu, Wei Wei, Cheng Peng, Lin Ding, Xiaoyun Chen, Xiaoli Xu, Xiaofu Wang,* and Junfeng Xu*

Cite This: *Anal. Chem.* 2024, 96, 5471−5477 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Current research endeavors have focused on the combination of various isothermal nucleic acid amplification methods with CRISPR/Cas systems, aiming to establish a more sensitive and reliable molecular diagnostic approach. Nevertheless, most assays adopt a two-step procedure, complicating manual operations and heightening the risk of contamination. Efforts to amalgamate both assays into a single-step procedure have faced challenges due to their inherent incompatibility. Furthermore, the presence of the protospacer adjacent motif (PAM) motif (e.g., TTN or TTTN) in the target double-strand DNA (dsDNA) is an essential prerequisite for the activation of the Cas12-based method. This requirement imposes constraints on crRNA selection. To overcome such limitations, we have developed a novel PAM-free one-step asymmetric recombinase polymerase amplification (RPA) coupled with a CRISPR/Cas12b assay (OAR-CRISPR). This method innovatively merges asymmetric RPA, generating single-stranded DNA (ssDNA) amenable to CRISPR RNA binding without the limitations of the PAM site. Importantly, the single-strand cleavage by PAM-free crRNA does not interfere with the RPA amplification process, significantly reducing the overall detection times. The OAR-CRISPR assay demonstrates sensitivity comparable to that of qPCR but achieves results in a quarter of the time required by the latter method. Additionally, our OAR-CRISPR assay allows the naked-eye detection of as few as 60 copies/μL DNA within 8 min. This innovation marks the first integration of an asymmetric RPA into one-step CRISPR-based assays. These advancements not only support the progression of one-step CRISPR/Cas12-based detection but also open new avenues for the development of detection methods capable of targeting a wide range of DNA targets.



Downloaded via SHANGHAI NORMAL UNIV on March 30, 2025 at 08:05:50 (UTC).
See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

CRISPR/Cas12, an RNA-guided DNase, exhibits collateral cleavage activity toward nontarget ssDNA when activated by a target DNA template. This indiscriminate cleavage capability holds significant promise for the application of the CRISPR system in the field of molecular diagnosis.[1,2] While CRISPR/Cas12-based detection methods offer simpler and faster alternatives, they may sometimes fall short in terms of sensitivity. To address the sensitivity challenge, isothermal amplification techniques such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) have been incorporated.[3−5] This dual recognition approach, combining isothermal amplification methods with the Cas gRNA complex, significantly enhances the sensitivity and reliability of the molecular detection.[6] The integration of RPA with the CRISPR/Cas system, as exemplified by methods like DETECER, HOLMES, or HOLMESv2, has emerged as powerful tools for augmenting detection specificity and have garnered considerable interest in pathogen detection applications.[1,7,8]

Traditional CRISPR/Cas12-assisted RPA assays involve a two-step process. Initially, the nucleic acid target undergoes preamplification via RPA, followed by the addition of Cas12-crRNA mix to induce collateral cleavage activity. However, this two-step procedure complicates manual operations, enhances instrumentation requirements, and increases the risk of carryover contamination. Earlier attempts to integrate RPA with CRISPR/Cas12 involved adding RPA reagents to the bottom of the tube and the Cas12/crRNA complex to the cover of the tube.[9,10] Innovations such as the photocleavable linker,[11,12] which need to be cleaved through an ultraviolet irradiation step to release the blocked crRNA, despite being

Received: December 5, 2023
Revised: March 14, 2024
Accepted: March 18, 2024
Published: March 29, 2024



ACS Publications © 2024 American Chemical Society

https://doi.org/10.1021/acs.analchem.3c05545
Anal. Chem. 2024, 96, 5471−5477

ingenious, did not achieve a true one-step assay. Despite numerous CRISPR/Cas-assisted assays reported in recent years, only a handful of single-step CRISPR/Cas12-assisted RPA assays have been documented to date. Much of this limitation can be attributed to the low reaction compatibility between these two assays, resulting in poor sensitivity.[13,14] One potential explanation for this is the excessive activation of Cas12 by crRNA with a canonical PAM motif, overwhelming the RPA amplification and compromising the synergy between these two reactions. Furthermore, the presence of the PAM motif (e.g., TTN or TTTN) in the target dsDNA is essential for the Cas12-based method.[1] The low frequency of the PAM motif in dsDNA sequences constrains its applicability. Asymmetric PCR or RPA is favored for its ability to generate a large amount of ssDNA from minimal dsDNA input,[15−17] and it offers a potential solution. Since Cas12 targets dsDNA substrate in a PAM-dependent manner while recognizing the target ssDNA substrate in a PAM-independent manner,[8] utilizing ssDNA to activate the *trans*-cleavage activity of Cas12 is a promising approach to establish a PAM-free RPA-CRISPR assay. Moreover, the *cis*-cleavage toward ssDNA did not affect the early stage amplification of RPA, thus enabling the development of a one-step CRISPR/Cas12-assisted RPA assay.

Considering all of the issues above, we have introduced a one-step PAM-free asymmetric RPA (aRPA) and CRISPR/Cas12b assay, referred to as the OAR-CRISPR. This approach circumvents the PAM requirement and simplifies the detection process. Our study leverages aRPA to generate ssDNA at constant temperatures, which in conjunction with Cas12b's heightened sensitivity to ssDNA[8] and significantly improves the assay's performance. To validate the effectiveness of the OAR-CRISPR, we demonstrate its application in detecting bacterial fruit blotch (BFB) and genetically modified soybean DBN9004. BFB causes significant economic losses in cucurbit fruit production,[18] and there are currently no effective means to prevent seed infection.[19] DBN9004 is a multiple herbicide-resistant transgenic soybean, which obtained a safety certificate in China in 2021.[20] Therefore, the accurate and timely detection of these agents holds broad prospects. Our developed OAR-CRISPR assay achieves rapid, sensitive, specific, and visually detectable results. This innovation not only mitigates the PAM constraint in CRISPR/Cas systems but also demonstrates the feasibility of a one-pot, one-step assay with broad applications for the rapid and sensitive detection of DNA pathogens.

MATERIALS AND METHODS

Development of the OAR-CRISPR Assay. The OAR-CRISPR assay was assembled by first creating separate premixes for the RPA and CRISPR components. RPA component A included 12 μL of rehydration A buffer, 0.8 μL of each primer with varied ratios, 4.6 μL of DEPC−water, and a recommended dose of recombinase and polymerase. RPA component B consisted of 0.8 μL of target DNA and 1 μL of B-buffer containing MgOAc. The CRISPR/Cas12b premix included a blend of crRNA, Cas12b, tracrRNA, and DNA-FQ probe, followed by the addition of 2 μL of a 10× HOLMES buffer 1, diluted with DEPC−water to a final volume. The concentrations of Cas12b and tracrRNA were added according to the recommended concentrations of the ssDNA-based CRISPR/Cas12b reaction system (see the Supporting Information for details, Figure S1). These components were combined to initiate the assay. All crRNAs, tracrRNA, primers, and probes are listed in Table S1.

Further experiments were aimed at refining the conditions for the OAR-CRISPR assay, focusing on the concentration of Cas12b and tracrRNA, reaction temperature, and primer concentration ratio. The optimal concentration of Cas12b was determined by testing four concentration gradients: 31, 62, 125, and 250 nM, using the most promising crRNA candidate. Then, the optimization of tracrRNA was evaluated by using the same four concentration gradients. The CRISPR/Cas12b reaction experiments were conducted at various temperature gradients ranging from 35 to 65 °C, with 5 °C intervals. The optimal RPA primer concentration ratio was established through the end point fluorescence signals. The ratios of forward to reverse primer in nM were set as follows: 10,000:10,000, 10,000:2000, 10,000:1000, 10,000:500, 10,000:250, 10,000:125, 10,000:62.5, 10,000:31.25. The fluorescence was recorded at 40 s intervals for a total 36 times.

Assay Sensitivity and Specificity Evaluation. The sensitivity of the OAR-CRISPR assay was assessed by testing diluted target *Acidovorax citrulli* DNA (15, 30, 6×10, 6×10^2, 6×10^3, 6×10^4, and 6×10^5 copies/μL), with DEPC−water as the negative control. The lowest detection limit was determined by conducting 20 independent tests at 60 copies/μL. Real-time monitoring captured the reactions at 4-min intervals to ascertain the detection threshold for low-copy-number targets. To assess the specificity of the OAR-CRISPR assay, we examined the potential cross-reactivity of the common watermelon-infecting pathogen strains (*Pseudomonas syringae*, *Xanthomonas cucurbita*, *Fusarium solani*, and *Fusarium oxysporum*) with the RPA primers specific to *A. citrulli*. Gel electrophoresis was conducted to visualize the RPA results. The OAR-CRISPR assay was employed to determine if there was any nonspecific cleavage activity activated.

Statistical Analysis. The end point signals of all experiments were visualized and integrated into the corresponding figures, demonstrating the viability of the OAR-CRISPR assay in terms of visualization. To ensure data accuracy, all experiments were conducted in triplicate. Significance analysis was performed using one-way analysis of variance (ANOVA) and Duncan's multiple-range test ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). The threshold was established at the 95% confidence level, computed as the mean with two standard deviations (mean + 2 SD) from 60 randomly chosen nontemplate controls (NTC).[21,22] All graphical plots presented in this study were generated and analyzed by using Origin 10.0 software.

RESULTS AND DISCUSSION

Design of the OAR-CRISPR Assay. In our investigation, we developed an innovative PAM-free one-step method that synergizes aRPA and CRISPR/Cas12b reagents (Figure 1). The RPA utilizes single-stranded DNA binding protein (SSB) alongside recombinase to facilitate primer extension, leading to the synthesis of numerous DNA duplexes (Figure 1A). As the reverse primer becomes depleted, recombinase-bound forward primers bind to the RPA-produced dsDNA and initiate the generation of ssDNA (Figure 1B). Simultaneously, the Cas12b/tracrRNA/crRNA complex recognizes and binds to the target ssDNA, with the unique ability of crRNA to detect ssDNA without the need for a PAM site, activating the cleavage of FQ reporters and generating a distinct fluorescence signal (Figure 1C). In contrast, dsDNA remains unrecognized

https://doi.org/10.1021/acs.analchem.3c05545
Anal. Chem. 2024, 96, 5471−5477

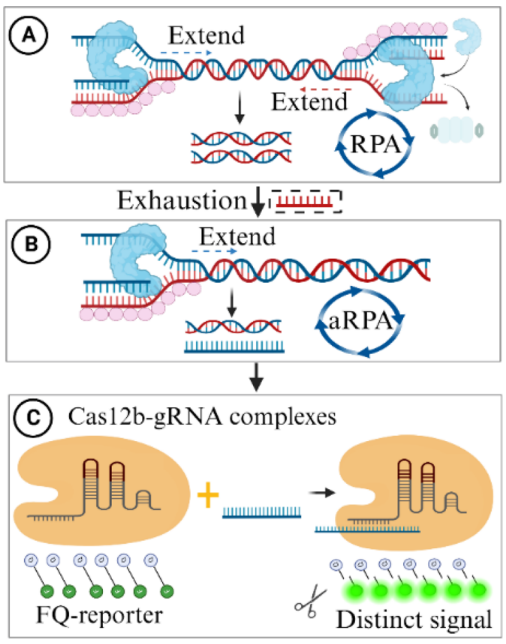


Figure 1. Schematic illustration of the principle of the OAR-CRISPR assay. (A) RPA reaction involves the binding of recombinase−primer complexes to target dsDNA, initiating both the forward and reverse primer extensions and the formation of multitude dsDNA templates. (B) Upon the deletion of a reverse primer, asymmetric RPA reaction triggers the synthesis of ssDNA based on the dsDNA templates. (C) ssDNA amplicons were recognized by Cas12b−gRNA complexes without PAM site constraints, leading to the cleavage of FQ reporter and the production of fluorescence signals.

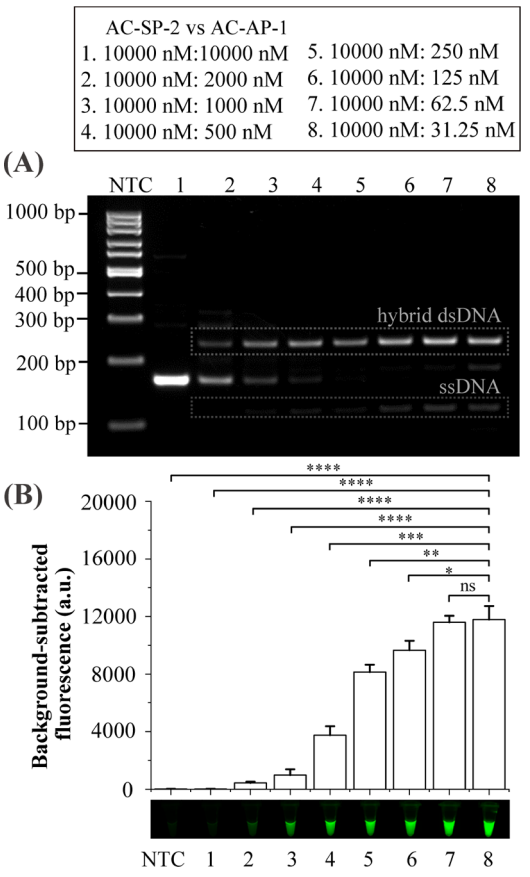


Figure 2. Optimizing primer ratios in the OAR-CRISPR assay. (A) aRPA amplification visualized on agarose gel, with reverse to forward primer ratios ranging from 10,000 nM:10,000 nM (1:1) to 10,000 nM:31.25 nM (320:1). (B) Bar charts and fluorescence images document the OAR-CRISPR end point signals across varying primer ratios. NTC, nontemplate control reaction. Error bars denote means ± standard deviation from three replicates, and asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

by PAM-free crRNA, and consequently, cleavage does not occur. This effort eliminates the impediment of RPA amplification in early stages, establishing compatibility between the RPA and Cas12b systems. Consequently, it enables the integration of RPA and CRISPR into a single reaction.

Analysis of Asymmetric RPA. We conducted an aRPA assay with varied primer concentration ratios using the optimal RPA primer pair (AC-SP-2/AC-AP-2, Figure S2). The results showed the presence of three prominent DNA bands. Multiple-sequence alignments confirmed that the lower band is an ssDNA consisting of the sense strand of the RPA-SP-2/RPA-AP-1-amplified fragment (Figures S3 and S4), whereas the upper band comprises a hybrid dsDNA structure (Figures S5−S7).

The concentration of the reverse to forward primers was varied to optimize the aRPA primer ratio. While previous studies typically employed ratios ranging from 10:1 to100:1, the actual concentration of the restricted RPA primer is the vital factor for ssDNA production, as the concentration of the unrestricted primers is often redundant.[23] Previous studies suggested a restricted RPA primer concentration range from 42 to 93 nM for maximum ssDNA production.[16,24−26] As the ratio of reverse to forward primers reduced, the intensity of the RPA amplicon band at approximately 160 bp weakened, with the lower bands being more visible compared to those observed in the standard RPA assay (Figure 2A). Gel analysis determined that setting the primer ratio from 10,000 nM:62.5 nM (160:1) to 10,000 nM:31.25 nM (320:1) resulted in the maximum production of ssDNA, as indicated by the most prominent band at 130 bp.

Establishment and Characterization of the OAR-CRISPR Assay. To validate the feasibility of the OAR-CRISPR assay, we incubated PAM-free crRNA/tracrRNA/Cas12b complexes with RPA and aRPA amplicons to clarify the mechanism of the aRPA with Cas12b. The results showed that RPA products could not be cleaved by the Cas12b complex with PAM-free crRNA (Figure S8). However, degradation of the ssDNA band in the aRPA/Cas12b assay confirmed that aRPA could catalyze Cas12b cleavage activities. This degradation, specific to aRPA and not observed with the RPA amplicon, confirmed that the amplicon from aRPA is a prerequisite for Cas12b activation using PAM-free crRNA.

We then tested varying RPA primer ratios to optimize the OAR-CRISPR assay. Initial testing with a 1:1 primer ratio did not yield any significant fluorescence signals. As the reverse to forward primer ratios decreased, both the fluorescence growth rate and the fluorescence value increased (Figure 2B). There were no differences in the end point signals between the primer concentration ratios from 10,000 nM:62.5 nM (160:1) to 10,000 nM:31.25 nM (320:1). These findings were consistent with results obtained from gel analysis, and 10,000 nM:31.25 nM was selected for the OAR-CRISPR assay. We further conducted experiments to fine-tune the conditions for

https://doi.org/10.1021/acs.analchem.3c05545
Anal. Chem. 2024, 96, 5471−5477

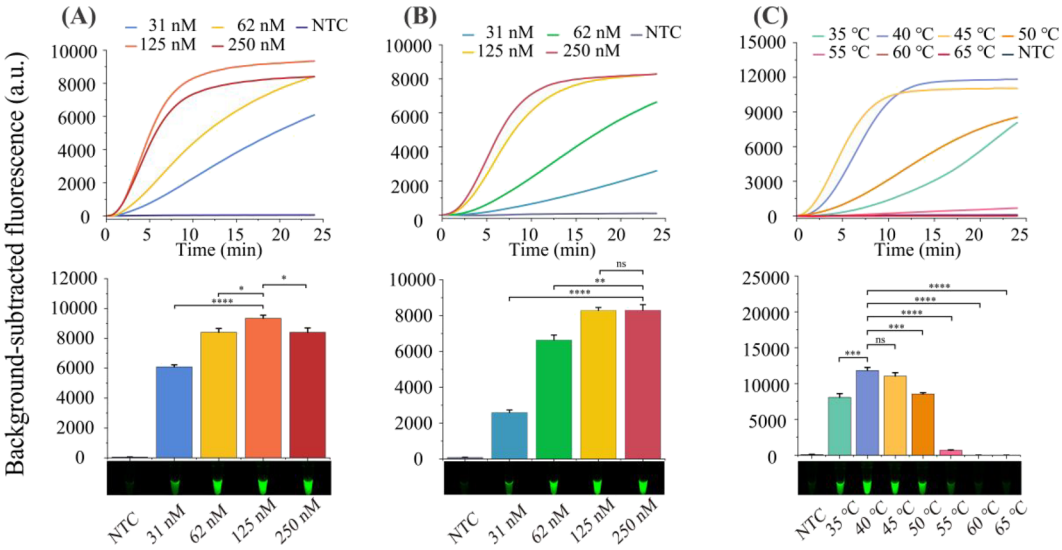


Figure 3. Optimizing OAR-CRISPR reaction conditions. (A) Selection of Cas12b concentration, (B) tracrRNA concentration, and (C) optimal working temperature. Display includes reaction real-time curves, bar charts, and fluorescence images of the OAR-CRISPR end point assay. Error bars are means ± standard deviation from triplicate experiments, with asterisks denoting statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

the OAR-CRISPR assay, including optimizing the concentration of Cas12b, tracrRNA, and the reaction temperature. Results showed that 125 nM Cas12b, 250 nM tracrRNA, and 40 °C incubation temperature were the optimal conditions for the OAR-CRISPR assay (Figure 3).

As the OAR-CRISPR assay was established, the evaluation of the detection specificity and sensitivity of the OAR-CRISPR assay is a crucial step in assessing its performance. Genomic DNAs from various prevalent watermelon pathogens were chosen as the targets for the specificity test since we focused on *A. citrulli*, which is the causative agent of BFB and mainly poses significant threat to watermelon production worldwide. The RPA reaction specificity was confirmed through agarose gel electrophoresis; only the target DNA addition yielded distinct bands, while no amplification occurred for the other pathogens (Figure S9A). The OAR-CRISPR assay substantiated these findings, as it generated significant fluorescence signals solely in response to *A. citrulli*, with no cross-reactivity observed (Figure S9B). These results affirm the assay's high specificity in detecting target DNA.

To ascertain the sensitivity of the OAR-CRISPR assay, we compared the OAR-CRISPR assay with the established qPCR method using serially diluted *A. citrulli* target DNA. The OAR-CRISPR assay demonstrated the capability to detect target DNA at concentrations as low as 30 copies/μL, showing effective sensitivity (Figure 4A). Reproducibility testing was also performed, and the results consistently showed reliable detection, further confirming the assay's effective sensitivity (Figure S10). In contrast, the qPCR assay displayed marginally higher sensitivity with detection limits down to 15 copies/μL (Figure S11A). Notwithstanding this slight variance in detection threshold, the rapidity of the OAR-CRISPR assay was its distinguishing feature, completing detection within 24 min, while qPCR required in excess of 100 min, including a protracted initial incubation and numerous amplification cycles. Both assays exhibited excellent linear relationships in quantitative analyses, with R^2 values surpassing 0.99, indicating that both methods are suitable for quantitative assessments (Figure 4B and Figure S11B). These findings suggest that the OAR-CRISPR assay, while slightly less sensitive than qPCR, is significantly faster and remains a robust and accurate method for low-level DNA detection. For real-time detection, we periodically captured images of the reaction tubes at 4-min intervals. This approach allowed us to ascertain the assay's capability to detect 600 copies/μL within the first 4 min (Figure 4C). While the fluorescent values for 30 copies/μL DNA were slightly higher than the threshold values, they did not significantly exceed the threshold values and were challenging to detect with the naked eye. Thus, we concluded that the OAR-CRISPR assay could detect 60 copies/μL within just 8 min and 30 copies/μL within just 12 min.

Field sample detection by the OAR-CRISPR assay. To verify the real sample applicability of the OAR-CRISPR assay, we tested 24 watermelon seed samples from Xinjiang, China, to screen for contamination by *A. citrulli*. Samples underwent a standardized pretreatment procedure and were detected through both the established qPCR method and our OAR-CRISPR assay with PAM-free crRNA. A comparative analysis of the results underscores the effectiveness of the latter approach. The results of our OAR-CRISPR assay revealed the presence of *A. citrulli* in six of the samples. Cross-verification with qPCR yielded a 100% concordance rate (Figure 5 and Figure S12), strengthening the case for the OAR-CRISPR assay's diagnostic accuracy in real sample conditions.

To demonstrate the generality of the OAR-CRISPR assay, we applied it to the detection of DBN9004 in real samples. To develop an effective OAR-CRISPR assay for DBN9004, optimal RPA primers and crRNA, along with the RPA primer concentration ratio was screened (Figures S13–S15). The assay's application to real soybean samples demonstrated a 100% consistency with qPCR results (Figures S16 and S17). These data further emphasize the broad applicability of the assay and its potential for reliable on-site DNA detection.

Comparison of One-Pot RPA Combined CRISPR/Cas12 Assays. The OAR-CRISPR assay was compared with other one-pot RPA combined CRISPR/Cas12 assays in the literature (Table S2). The evaluated assays were classified into five distinct classes based on their design principles. While the

https://doi.org/10.1021/acs.analchem.3c05545
Anal. Chem. 2024, 96, 5471−5477

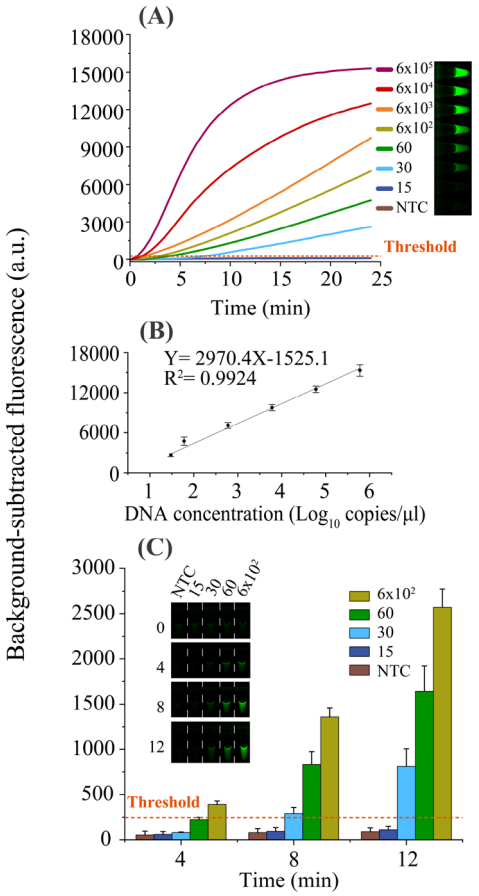


Figure 4. Sensitivity assessment of the OAR-CRISPR assay. (A) Real-time detection curves and fluorescence images at various DNA concentrations. (B) Standard curve plot generated from the results of the qPCR assay, displaying the slope, *Y*-intercept, and R^2 value. (C) Fluorescent signal quantification and time-course fluorescence images for low-DNA-concentration detection, presented in a bar chart format. Data points are means ± the standard deviation from triplicate experiments. NTC represents the nontemplate control. The horizontal dashed line indicates the threshold fluorescence intensity (average of 60× NTC plus two times of their standard deviations).

methods described in these articles demonstrated notable strengths, such as novelty and high sensitivity, they also exhibited certain limitations. For instance, some assays employed temporary blocking of crRNA using photocontrolled materials, resulting in ultra sensitivity but falling short of being true one-step methods.[11,12,27] Other approaches focused on optimizing the concentrations of reaction components to achieve a one-step RPA-CRISPR/Cas assay,[28−30] which, although relatively straightforward to operate, necessitated extensive optimization efforts. Moreover, researchers explored the utilization of suboptimal PAM-site crRNAs[14,31,32] or strategically positioning crRNAs adjacent to RPA primers to strike a balance between amplification efficiency and fluorescence signaling.[33−35] While these assays have demonstrated commendable performance, their reliance on crRNA design posed challenges and hindered the optimization of crRNA selection. In contrast, our OAR-CRISPR assay obviates the need for a PAM recognition site, substantially expanding the repertoire of crRNA options. Comparative analysis with other one-pot RPA combined CRISPR/Cas12 assays revealed that our assay excelled in terms of the detection speed. This highlights the efficiency of the OAR-CRISPR assay in providing rapid and sensitive results, which is crucial for timely diagnostics.

CONCLUSIONS

Overall, our study has introduced a pioneering OAR-CRISPR assay that demonstrates robustness and sensitivity, achieving visualized detection thresholds as low as 60 copies/μL within just 8 min. Moreover, the assay exhibits quantification capabilities, as evidenced by its excellent linear response, with R^2 values exceeding 0.99. The distinction of our assay lies in its generation of ssDNA and without the limitation of the PAM site, which allows for greater flexibility in target detection crRNA optimization and the feasibility of a one-step assay. Additionally, by bypassing the need for RPA preamplification, our OAR-CRISPR assay markedly reduces the overall detection time. Although the OAR-CRISPR is established for BFB detection, the universal applicability of the OAR-CRISPR positions it as a versatile tool suitable for all DNA diagnoses, catering to the demands of both rapid and precise detection. Therefore, our OAR-CRISPR assay represents a breakthrough in on-site DNA diagnosis and constitutes a leap forward in the realm of CRISPR/Cas12-based assays.

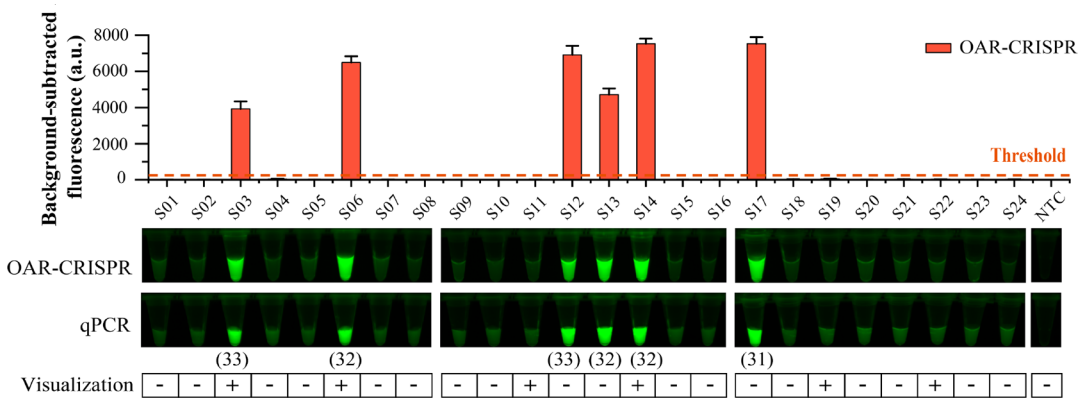


Figure 5. Field sample detection comparing the OAR-CRISPR and qPCR assays. End point OAR-CRISPR results are depicted as bar charts and fluorescence images. Error bars reflect means ± standard deviation from triplicate tests. NTC represents the nontemplate control. The horizontal dashed line indicating the threshold fluorescence intensity (average of 60× NTC plus two times of their standard deviations). The end point qPCR results are depicted as fluorescence images and the average Ct values from triplicate tests.

https://doi.org/10.1021/acs.analchem.3c05545
Anal. Chem. 2024, 96, 5471−5477

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.3c05545.

Experimental section (1.1 reagents and materials; 1.2 construction of a plasmid template and design of *Acidovorax citrulli* RPA primers and crRNAs; 1.3 RPA primer optimization; 1.4 ssDNA-based CRISPR/Cas12b AC-crRNA screening and reaction system optimization; 1.5 RPA primer concentration ratio optimization and amplified aRPA amplicons analysis; 1.6 cleavage mechanism of Cas12b toward aRPA amplicons; 1.7 sensitivity of the qPCR assay toward bacterial fruit blotch (BFB) detection; 1.8 watermelon seed sample validation; 1.9 application of OAR-CRISPR in genetically modified soybean DBN9004); results (2.1 AC-crRNA screening and ssDNA-based CRISPR/Cas12b reaction parameter optimization; 2.2 selection of the optimal RPA primer combination in BFB detection; 2.3 analysis of aRPA amplicons; 2.4 mechanism of aRPA with CRISPR/Cas12b using PAM-free crRNA; 2.5 specificity of OAR-CRISPR assay for BFB detection; 2.6 reproducibility testing of OAR-CRISPR at the lowest detection limit for BFB detection; 2.7 qPCR assay sensitivity and real-time curves for field samples in BFB detection; 2.8 establishment of OAR-CRISPR assay for DBN9004 detection); tables (3.1 Table S1 the sequence list of primers, tracrRNA, crRNAs, ssDNA target, FQ reporter used in this study; 3.2 Table S2 comparison of assay design, operation, sensitivity, speed, and experimental design of the one-pot CRISPR/Cas12-assisted assay combined with RPA process) (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Xiaofu Wang − *State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, Key Laboratory of Traceability for Agricultural Genetically Modified Organisms, Ministry of Agriculture and Rural Affairs, P.R.China, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China;* orcid.org/0000-0001-9323-4022; Email: yywxf1981@163.com

Junfeng Xu − *State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, Key Laboratory of Traceability for Agricultural Genetically Modified Organisms, Ministry of Agriculture and Rural Affairs, P.R.China, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China*; Email: njjfxu@163.com

Authors

Lei Yang − *State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, Key Laboratory of Traceability for Agricultural Genetically Modified Organisms, Ministry of Agriculture and Rural Affairs, P.R.China, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China;* orcid.org/0000-0002-5983-3061

Guanwei Chen − *State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, Key Laboratory of Traceability for Agricultural Genetically Modified Organisms, Ministry of Agriculture and Rural Affairs, P.R.China, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China; College of Life Sciences, Zhejiang Normal University, Jinhua 321004, China*

Jian Wu − *College of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou 310058, China;* orcid.org/0000-0003-4518-7027

Wei Wei − *State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, Key Laboratory of Traceability for Agricultural Genetically Modified Organisms, Ministry of Agriculture and Rural Affairs, P.R.China, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China*

Cheng Peng − *State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, Key Laboratory of Traceability for Agricultural Genetically Modified Organisms, Ministry of Agriculture and Rural Affairs, P.R.China, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China;* orcid.org/0000-0003-1782-1350

Lin Ding − *State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, Key Laboratory of Traceability for Agricultural Genetically Modified Organisms, Ministry of Agriculture and Rural Affairs, P.R.China, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China;* orcid.org/0009-0000-0865-8622

Xiaoyun Chen − *State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, Key Laboratory of Traceability for Agricultural Genetically Modified Organisms, Ministry of Agriculture and Rural Affairs, P.R.China, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China*

Xiaoli Xu − *State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, Key Laboratory of Traceability for Agricultural Genetically Modified Organisms, Ministry of Agriculture and Rural Affairs, P.R.China, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.analchem.3c05545

Author Contributions

#L.Y. and G.C. contributed equally. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The work was supported by the Major project of agricultural biological breeding (2022ZD0401908), the "San Nong Jiu Fang" Technology Collaboration Program Project of Zhejiang (2023SNJF043), Key Projects of Zhejiang Natural Science Foundation (LZ23D030001), the Basic Public Welfare Research Projects in Zhejiang Province (LGN22C140017), and the Science and Technology Innovation Leading Talent Project of Zhejiang (2021R52046).

■ REFERENCES

(1) Chen, J. S.; Ma, E. B.; Harrington, L. B.; Da Costa, M.; Tian, X. R.; Palefsky, J. M.; Doudna, J. A. *Science* **2018**, *360* (6387), 436−439.

(2) Ramachandran, A.; Huyke, D. A.; Sharma, E.; Sahoo, M. K.; Huang, C.; Banaei, N.; Pinsky, B. A.; Santiago, J. G. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 29518−29525.
(3) Notomi, T.; Okayama, H.; Masubuchi, H.; Yonekawa, T.; Watanabe, K.; Amino, N.; Hase, T. *Nucleic Acids Res.* **2000**, *28* (12), 63e DOI: 10.1093/nar/28.12.e63.
(4) Zhao, Y.; Chen, F.; Li, Q.; Wang, L.; Fan, C. *Chem. Rev.* **2015**, *115* (22), 12491−12545.
(5) Lobato, I. M.; O'Sullivan, C. K. *Trac-Trends Anal. Chem.* **2018**, *98*, 19−35.
(6) Mukama, O.; Wu, J.; Li, Z.; Liang, Q.; Yi, Z.; Lu, X.; Liu, Y.; Liu, Y.; Hussain, M.; Makafe, G. G.; Liu, J.; Xu, N.; Zeng, L. *Biosens. Bioelectron.* **2020**, *159*, No. 112143.
(7) Guo, Y.; Xia, H.; Dai, T.; Liu, T.; Shamoun, S. F.; CuiPing, W. *Front. Cell. Infect. Microbiol.* **2023**, *13*, 1218105.
(8) Li, L.; Li, S.; Wu, N.; Wu, J.; Wang, G.; Zhao, G.; Wang, J. *ACS Synth. Biol.* **2019**, *8* (10), 2228−2237.
(9) Sun, Y.; Yu, L.; Liu, C.; Ye, S.; Chen, W.; Li, D.; Huang, W. *J. Transl. Med.* **2021**, *19* (1), 74.
(10) Wang, Y. Q.; Ke, Y. Q.; Liu, W. J.; Sun, Y. Q.; Ding, X. T. *ACS Sens.* **2020**, *5* (5), 1427−1435.
(11) Hu, M.; Qiu, Z.; Bi, Z.; Tian, T.; Jiang, Y.; Zhou, X. *Proc. Natl. Acad. Sci. U. S. A.* **2022**, *119* (26), No. e2202034119.
(12) Chen, Y.; Xu, X.; Wang, J.; Zhang, Y.; Zeng, W.; Liu, Y.; Zhang, X. *Anal. Chem.* **2022**, *94*, 9724.
(13) Aman, R.; Mahas, A.; Marsic, T.; Hassan, N.; Mahfouz, M. M. *Front. Microbiol.* **2020**, *11*, No. 610872.
(14) Wang, P.; Guo, B.; Zhang, X.; Wang, Y.; Yang, G.; Shen, H.; Gao, S.; Zhang, L. *J. Agric. Food Chem.* **2023**, *71* (16), 6490−6498.
(15) Yenice, C. P.; Chahin, N.; Jauset-Rubio, M.; Hall, M.; Biggs, P.; Dimai, H.; Obermayer-Pietsch, B.; Ortiz, M.; O'Sullivan, C. K. *Anal. Chem.* **2023**, 14192 DOI: 10.1021/acs.analchem.3c01668.
(16) Kober, C.; Niessner, R.; Seidel, M. *Biosens. Bioelectron.* **2018**, *100*, 49−55.
(17) Nakagawa, T.; Tanaka, J.; Harada, K.; Shiratori, A.; Shimazaki, Y.; Yokoi, T.; Uematsu, C.; Kohara, Y. *Anal. Chem.* **2020**, *92* (17), 11705−11713.
(18) Martin, H. L.; Horlock, C. M. *Plant Dis* **2002**, *86* (12), 1406.
(19) Islam, M. R.; Hossain, M. R.; Kim, H.-T.; Jesse, D. M. I.; Abuyusuf, M.; Jung, H.-J.; Park, J.-I.; Nou, I.-S. *Int. J. Mol. Sci.* **2019**, *20* (11), 1223−1228.
(20) Xiang, D.; Luo, M.; Jiang, F.; Wen, Z.; Chen, X.; Wang, X.; Xu, X.; Wei, W.; Xu, J. *Sci. Rep.* **2023**, *13* (1), 5445.
(21) Mohammadniaei, M.; Zhang, M.; Ashley, J.; Christensen, U. B.; Friis-Hansen, L. J.; Gregersen, R.; Lisby, J. G.; Benfield, T. L.; Nielsen, F. E.; Rasmussen, J. H.; et al. *Nat. Commun.* **2021**, *12* (1), 5089.
(22) Patchsung, M.; Jantarug, K.; Pattama, A.; Aphicho, K.; Suraritdechachai, S.; Meesawat, P.; Sappakhaw, K.; Leelahakorn, N.; Ruenkam, T.; Wongsatit, T.; et al. *Nat. Biomed. Eng.* **2020**, *4* (12), 1140−1149.
(23) Wang, B.; Jiao, Y.; Li, X.; Zheng, F.; Liang, H.; Sun, Z.; Guo, G. *Biotechnol. Appl. Bioc.* **2009**, *52*, 41−44.
(24) Bemetz, J.; Kober, C.; Meyer, V. K.; Niessner, R.; Seidel, M. *Anal. Bioanal. Chem.* **2019**, *411* (10), 1943−1955.
(25) Elsäßer, D.; Ho, J.; Niessner, R.; Tiehm, A.; Seidel, M. *Anal. Biochem.* **2018**, *546*, 58−64.
(26) Thoeny, V.; Melnik, E.; Huetter, M.; Asadi, M.; Mehrabi, P.; Schalkhammer, T.; Pulverer, W.; Maier, T.; Mutinati, G. C.; Lieberzeit, P.; et al. *Anal. Chim. Acta* **2023**, *1281*, No. 341922.
(27) Hu, M. L.; Liu, R. H.; Qiu, Z. Q.; Cao, F.; Tian, T.; Lu, Y. X.; Jiang, Y. Z.; Zhou, X. M. *Angew. Chem., Int. Ed.* **2023**, *62* (23), No. e202300663.
(28) Chen, Y.; Mei, Y.; Zhao, X.; Jiang, X. *Anal. Chem.* **2020**, *92* (21), 14846−14852.
(29) Malc, K.; Walls, L. E.; Rios-Solis, L. *ACS Synth. Biol.* **2022**, *11* (4), 1555−1567.
(30) Aman, R.; Marsic, T.; Rao, G. S.; Mahas, A.; Ali, Z.; Alsanea, M.; Al-Qahtani, A.; Alhamlan, F.; Mahfouz, M. *Front. Bioeng. Biotechnol.* **2022**, *9*, No. 800104, DOI: 10.3389/fbioe.2021.800104.
(31) Lu, S.; Tong, X.; Han, Y.; Zhang, K.; Zhang, Y.; Chen, Q.; Duan, J.; Lei, X.; Huang, M.; Qiu, Y.; et al. *Nat. Biomed. Eng.* **2022**, *6* (3), 286−297.
(32) Lin, M.; Yue, H.; Tian, T.; Xiong, E.; Zhu, D.; Jiang, Y.; Zhou, X. *Anal. Chem.* **2022**, *94* (23), 8277−8284.
(33) Shao, F.; Park, J. S.; Zhao, G.; Hsieh, K.; Wang, T.-H. *Anal. Chem.* **2023**, *95* (7), 3873−3882.
(34) Ding, X.; Yin, K.; Li, Z.; Lalla, R. V.; Ballesteros, E.; Sfeir, M. M.; Liu, C. *Nat. Commun.* **2020**, *11* (1), 4711.
(35) Lee, C. Y.; Degani, I.; Cheong, J.; Lee, J.; Choi, H.; Cheon, J.; Lee, H. *Biosens. Bioelectron.* **2021**, *178*, No. 113049.

https://doi.org/10.1021/acs.analchem.3c05545
Anal. Chem. 2024, 96, 5471−5477