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CRISPR/Cas12a-based method to discriminate Plasmodium ovale wallikeri and Plasmodium ovale curtisi

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Abstract

Background Plasmodium ovale

(*P. ovale*) is one of five malaria parasites known to infect humans. Current research confirms the existence of two genetically distinct species, *P. ovale wallikeri* (*Pow*) and *P. ovale curtisi* (*Poc*), which differ in their pathogenicity and virulence in humans. Accurate discrimination between these species is crucial for appropriate patient management and epidemiological surveillance. Commonly employed methods such as qPCR and serological assays are limited by high cost, long processing times, and inadequate sensitivity or specificity.

Method

We designed five candidate crRNAs and selected two highly specific ones to construct separate detection systems: one for *Pow* and another for *Poc*. PCR-CRISPR/Cas12a detection system was then established and optimized. The limit of detection and specificity of the system were evaluated, and its detection performance was further validated using clinical samples.

Results

The *Pow* CRISPR/Cas12a system demonstrated the detection limit of 100 copies/μL, while the *Poc* system achieved the detection limit of 30 copies/μL, with no cross-reactivity observed between the two species, indicating high sensitivity and specificity. We further validated the assay using 137 clinical samples from imported malaria patients, yielding the sensitivity of 96.77% and specificity of 99.05% for *Poc* and sensitivity of 83.78% and specificity of 100% for *Pow*.

Conclusion

This method represents a significant step forward in the molecular diagnosis of *P. ovale* malaria. Its combination of specificity, ease of use, and potential for adaptation makes it a highly promising tool for enhancing epidemiological surveillance and supporting malaria elimination efforts, particularly in the context of managing malaria cases.

Background

Malaria remains a major global public health threat, which is an infectious disease accused by mosquito-borne pathogen transmission (1). According to the latest data, there were approximately 263 million malaria cases reported globally in 2023, an increase of about 11 million cases compared to 2022, with an estimated 597,000 deaths due to malaria. The disease burden is heavily concentrated, with the WHO African Region accounting for about 94% of cases and deaths, while parts of Southeast Asia also face

significant transmission (2). Among the Plasmodium species infecting humans, *Plasmodium ovale* (3) is often overlooked in malaria research and control programs compared to the more prevalent and virulent *P. falciparum* (*P. f*) and *P. vivax* (*P. v*). This is primarily due to the significantly higher incidence and mortality rates associated with the latter two species. However, *P. ovale* possesses distinct biological features, including its specificity for infecting reticulocytes and, critically, its ability to form hypnozoites, which are dormant liver stages that can reactivate months or even years after the initial infectious mosquito bite, causing clinical relapses (4). Therefore, to achieve the goals of malaria elimination, it is essential to enhance understanding of *P. ovale* in terms of epidemiological patterns, clinical diagnosis and treatment, and further research into its unique biological characteristics are essential.

Recent studies have confirmed that *Plasmodium ovale* comprises two distinct species, *Poc and Pow*, rather than being a single species with two subspecies (5). These two species differ in several clinical and pathological manifestations, including the degree of thrombocytopenia (6, 7), latency period (8, 9), and levels of LDH (10), creatinine and hemoglobin(10), which may affect treatment decisions. Therefore, accurate identification of the infecting species is critical for guiding appropriate clinical management, highlighting an urgent need for robust molecular tools to reliably discriminate between the two species. The development of such tools would significantly benefit the epidemiological surveillance, outbreak traceability and precise prevention and control strategies.

Conventional methods for distinguishing the two species include microscopy, immunological and molecular diagnosis techniques (11, 12). Microscopy demonstrates low sensitivity and fails to distinguish the two species due to their morphological similarities(13). The immunological detection, primarily using Rapid Diagnostic Test (RDT) targeting the *P. ovale* lactate dehydrogenase (PoLDH) antigen (14), often suffers from limited sensitivity. Notably, studies have reported a higher false-negative rate for *Poc* compared to *Pow* using this approach (15), which may be attributed to natural genetic variability within the species or frequently low levels of parasitemia. To improve the accuracy of species discrimination, molecular techniques such as real-time quantitative PCR (qPCR) and nested PCR (nPCR) have been developed. Oguike *et al*. (16) had succussed to use qPCR to differentiate *Poc* and *Pow* by analyzing their distinct melt curves profiles. Similarly, Tanomsing *et al*. (17) reported a semi-nested PCR assay capable of detecting the two species with 2–10 parasites/μL of blood, demonstrating high sensitivity. Despite their excellent sensitivity and specificity, these molecular methods involve complex procedures, are relatively time-consuming, and require well-equipped laboratories, limiting their utility for rapid screening in resource-limited settings. Therefore, more convenient and efficient diagnostic tool for the two species is required.

The clustered regularly interspaced short palindromic repeats associated with Cas12a (CRISPR/Cas12a) system has emerged as a powerful alternative to PCR due to its advantages in nucleic acid detection (18–20). Double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA) could serve as activator for CRISPR/Cas12a, which is specifically recognized by a guide CRISPR RNA (crRNA). Upon activation, the Cas12a nuclease exhibits collateral cleavage activity, non-specifically cleaving fluorescently-quenched ssDNA reporter and generating detectable signal(21).This system is characteristic by high sensitivity

and specificity, low equipment requirements. These features have led to its integration into various biosensing platforms, including microfluidics(22), logic gate(23) and isothermal amplification techniques (24), and with applications in detection of various organisms, such as virus (25), pathogen(26), and parasite(27). Therefore, CRISPR/Cas12a presents a promising foundation for developing a novel method to distinguish between *Poc* and *Pow*.

Inspired by the high specificity of CRISPR/Cas12a system in recognizing sequence differences (28), we proposed a strategy employing a CRISPR/Cas12a-based assay to identify the distinction between two species in *P. ovale*. In this approach, initial PCR amplification ensures specific target enrichment from low-abundance genomic DNA, reducing the non-specific amplified products compared to isothermal amplification technique. Subsequently, two species-specific crRNAs are designed to target divergent genomic regions unique to *Poc* or *Pow*. The counterpart amplification product could bind with crRNA, which then activates Cas12a nuclease activity to cleave fluorescent probe and generate positive signal (Schematic 1). We believe that this study will provide an efficient tool for the epidemiological investigation of *P. ovale*, support the process of malaria elimination, and contribute to accurate diagnosis and medical strategy.

Methods and materials

Materials

All oligonucleotides, including primers, crRNAs, reporter labeled with 6-FAM and BHQ-1, as well as the plasmid DNA standards for *Pow* and *Poc*, were synthesized and purified by Sangon Biotechnology Co., Ltd. (Shanghai, China). The sequences are provided in Supplementary Table S1. The Cas12a nuclease (LbCas12a) was purchased from New England Biolabs (Beijing, China). The 2×MasterPCR Mix was purchased from Promega (Wisconsin, USA). The QIAamp DNA Blood Mini Kits was obtained from QIAGEN (Dusseldorf, Germany). Clinical samples were sourced from the Sample Resource Library of the Henan Provincial Reference Laboratory for Malaria Diagnosis.

Target Pow and Poc Amplification by PCR

Given its high specificity and stability compared to isothermal amplification methods, conventional PCR was employed to amplify target sequences *Pow* and *Poc*. The 25 μL PCR mixture consisted of 12.5 μL of 2× MasterPCR Mix, 1 μL of forward primer (8 μM), 1 μL of reverse primer (8 μM), 5 μL of DNA template (from *Pow*, *Poc* synthetic standard sample or clinical samples), and 5.5 μL of nuclease-free water. The thermal cycling conditions were as follows: initial denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 45 seconds; and final extension at 72°C for 5 minutes. PCR amplification products were analyzed using 1% agarose gel electrophoresis. The gel was then visualized under UV light using a Bio-Rad automated gel imaging system (California, USA).

CRISPR/Cas12a Detection Assay

The CRISPR/Cas12a reaction was carried out in a 20 μL volume containing 0.4 μL of Cas12a nuclease (500 nM), 0.5 μL of crRNA (1 μM), 1 μL of ssDNA fluorescent reporter (10 μM), 2 μL of 10× NEB buffer, and 1 μL of PCR amplification product. The CRISPR/Cas12a reaction mixture was incubated at 37°C for 30 minutes with fluorescence measurements taken every 30 seconds in a QuantStudio 7 flex Real-Time PCR system (Thermo Fisher, USA). Fluorescence intensity is calculated by subtracting the initial value from the fluorescence endpoint value.

crRNA Screening

To identify the optimal crRNA for specifically detecting *Pow* and *Poc*, a panel of 12 whole blood samples (pre-characterized by commercial qPCR kits into low [20 < Ct < 25], medium [25 < Ct < 30], and high [Ct > 30] parasitemia groups for each species) was used. Genomic DNA was first extracted from these samples using the QIAamp DNA Blood Mini Kits according to the manufacturer's instructions. Then, the extracted DNA was amplified via PCR. The PCR products were subsequently used as activators in the CRISPR/Cas12a reaction. The crRNA yielding the highest fluorescence signal for its target species (*Pow* or *Poc*) with no cross-reactivity was selected for subsequent experiments.

Optimization Assay

Key reaction conditions were systematically optimized by measuring the resulting fluorescence intensity. The concentration of PCR primers was tested at 2, 4, 6, 8, 10 μM. The annealing temperature of PCR was evaluated at 46, 50, 54, 58 and 62 ℃. CRISPR/Cas12a Reaction Components: the concentrations of crRNA (100, 250, 350, 500, and 1000 nM) and Cas12a nuclease (100, 200, 300, 400, and 500 nM) were optimized. For each optimization step, the reaction products were analyzed using the CRISPR/Cas12a assay, and the condition yielding the suitable signal was selected.

Clinical Sample Testing

A total of 137 archived whole blood samples from patients with imported malaria mainly originating from countries in Africa and Southeast Asia were obtained and analyzed. Genomic DNA was extracted and subjected to both the optimized CRISPR/Cas12a-based assay and a reference qPCR method(29). The results from both methods were compared to assess the clinical performance of the CRISPR/Cas12a-based detection system.

Results

Assay Development and crRNA Screening for discrimination of Pow and Poc

To establish a CRISPR/Cas12a-based method for discriminating *Pow* and *Poc*, we selected the 18s rRNA gene as target gene due to its highly conserved sequences and the presence of species-specific polymorphic regions. A single pair of primers was designed to amplify a common fragment from both species. The sequence alignment diagram is shown in Fig. 1A. The designed primers successfully amplified the target sequence from both standard and clinical samples without non-specific amplification by gel electrophoresis assay, as shown by clear and single bands (Fig. S1).

In order to screen the optimal crRNA for detecting *Pow* and *Poc*, which could specifically identify the corresponding species. We designed candidate crRNAs for species-specific detection: three for *Pow* and two for *Poc*. The assay demonstrated that all five crRNAs could mediate Cas12a activation upon binding to their respective amplification products and produce fluorescent signal (Fig. 1B, 1C). Nevertheless, the crRNA3 for *Pow* exhibited significant cross-reactivity, producing strong fluorescent signals with both *Pow* (S-W and W) and *Poc* sample (S-C and C). In contrast, the crRNA1 and crRNA2 produced strong fluorescence signals specific to *Pow* samples, with only minimal background for *Poc* sample. Similarly, the crRNA4 and crRNA5 for *Poc* exhibited high specificity for *Poc* sample without cross-reaction to *Pow* sample. Between the two *Poc* crRNAs, crRNA4 yielded a stronger fluorescence signal than crRNA5, the crRNA4 was therefore selected for subsequent *Poc* CRISPR/Cas12a detection system.

Specificity Assessment and Selection of Optimal crRNA for Pow

Further refinement was necessary to select the optimal crRNA for *Pow* detection. Although crRNA1 generated a stronger fluorescence signal for *Pow* samples than crRNA2, it also produced considerable background signal in S-C and C samples, indicating potential for false positives (Fig. 1B). Conversely, crRNA 2 showed negligible fluorescence in negative controls, suggesting superior specificity (Fig. 1B). To evaluate their performance in a clinically relevant context, crRNA1 and crRNA2 were detected against a panel of 12 pre-characterized whole blood samples (6 *Pow* and 6 *Poc*) previously confirmed by qPCR to represent low, medium and high parasite loads.

As shown in Fig. 2A, while crRNA1 detected all 6 *Pow* samples with the good sensitivity, it also produced false-positive signals in *Poc* samples with low and medium Ct values. Conversely, crRNA2 correctly identified all 6 *Pow* samples without generating any detectable signals from any of the 6 *Poc* samples (Fig. 2B). Based on this excellent specificity, the crRNA2 was selected as the optimal guide for the *Pow* CRISPR/Cas12a detection system.

Optimization of reaction conditions

To acquire the optimal condition for the CRISPR/Cas12a-based method to discriminate *Poc* and *Pow*, key parameters of both the PCR amplification and CRISPR/Cas12a reaction were systematically optimized to maximize the sensitivity and specificity of the assay.

The concentration of primers was optimized from 2 μM to 10 μM. The fluorescent signal increased with the primer concentration up to 8 μM for both targets, indicating maximal amplicon yield, and was thus

selected as optimal (Fig. 3A, 3B) .

Among the tested annealing temperatures (49, 52, 55, 58, and 61°C), 58 ℃ yielded the strongest fluorescent signal for both *Pow* and *Poc* systems and was chosen for all subsequent PCR reaction (Fig. 3C, 3D).

In the CRISPR/Cas12a system, the concentration of crRNA was optimized from 100 nM to 600 nM. The fluorescence signal plateaued at 500 nM for both crRNA2 (*Pow*) and crRNA4 (*Poc*), which was therefore selected as the optimal concentration considering both cost and efficacy (Fig. 3E, 3F).

The concentration of Cas12a nuclease was optimized from 100 nM to 500 nM. 400 nM was found to generate the highest trans-cleavage activity and was adopted for the CRISPR/Cas12a reaction (Fig. 3G, 3H).

Analytical Sensitivity and Specificity of the Optimized Assay

Under the systematically optimized condition, the performance of the assay for *Poc* and *Pow* detection was evaluated. Other common malaria species, *P. falciparum*, *P. vivax* and *P. malariae*, were detected for the evaluation of the specificity, the *Poc* and *Pow* specific detection systems could produce significant fluorescence signals only for their respective targets, with negligible cross-reactivity (Fig. 4A, 4B).

The limit of detection (LOD) was determined using serial dilutions of target plasmids. The fluorescence intensity increased logarithmically with the target copy number for both systems (Fig. 4C, 4D). The LOD was determined to be 30 copies/μL for *Poc* and 100 copies/μL for *Pow*. Compared with previous study, the CRISPR/Cas12a-based method exhibit the comparable sensitivity, which was suited for discrimination of two *P. ovale* species in real sample (Table S2).

Clinical Validation with Patient Samples

The clinical performance of the assay was evaluated using 137 archived patient samples infected with different *Plasmodium* species, including *P. falciparum* (n = 39), *P. vivax* (n = 20), *P. malariae* (n = 9), *Poc* (n = 31), *Pow* (n = 37), 1 of mixed *Poc* and *Pow*, all of them were confirmed by qPCR. Among the 31 *Poc* samples, 30 were successfully detected with crRNA4, while only one sample remained undetected. For the 37 *Pow* samples, 31 were successfully detected using crRNA2, while 6 samples yielded no signal. Notably, the sample 28 yielded fluorescent signals with both crRNA2 and crRNA4, indicating it was a mixed infection type. Upon re-verification using qPCR, the returned Ct values of 33.26 for *Poc* and 27.09 for *Pow*, indicates that the conclusions of the two methods are consistent. In contrast, sample 56 confirmed as *Pow*-positive via qPCR exhibited weak non-specific fluorescence when detected with crRNA4, a signal clearly distinguishable from the specific fluorescent readouts generated by its target crRNA2 assay. (Fig. 5A and 5B). The assay demonstrated high specificity for its respective targets. Additionally, the CRISPR/Cas12a detection systems showed complete specificity against non-*ovale* malaria species (Fig. S2). The fluorescence signals between positive and negative groups were highly

significant for both *Poc* and *Pow* detection systems (Fig. 5C). Based on the receiver operating characteristic (ROC) curve analysis, the assay demonstrated a clinical sensitivity of 96.77% and a specificity of 99.05% for *Poc*, a sensitivity of 83.78% and a specificity of 100% for *Pow*, compared to the qPCR reference method (Tables 1 and 2). The ROC curve analysis further confirmed the robust diagnostic performance. The area under the curve (AUC) was 0.9994 for *Poc* CRISPR/Cas12a detection system and 0.9850 for *Pow* CRISPR/Cas12a detection system (Fig. 5D), validating the satisfactory clinical performance of the CRISPR/Cas12a-based method. These results validate the reliability of the CRISPR/Cas12a-based method to discriminate *Poc* and *Pow* among the complex clinical samples accurately.

The CRISPR/Cas12a detection method developed in this study exhibits specificity comparable to qPCR, while offering operational simplicity and reduced dependence on complex and expensive equipment, thereby showing greater potential for application in resource-limited settings. It also demonstrates stable and reliable diagnostic performance in clinical samples. However, further optimization is still required to improve the sensitivity of *Pow* detection and to simplify the workflow.

Table 1
Comparison between qPCR and CRISPR/Cas12a-based method to detect *Poc*.

CRISPR/Cas12a-crRNA2	**qPCR**		**Total**	**Sensitivity**	**Specificity**
	Poc	***Pow* and non-*ovale***			
Positive.	30	1	31	96.77%	---
Negative.	1	104	105	---	99.05%
Total	31	105	136		
Note: One case of mixed infection was excluded.					

Table 2
Comparison between qPCR and CRISPR/Cas12a-based method to detect *Pow*.

CRISPR/Cas12a-crRNA4	**qPCR**		**Total**	**Sensitivity**	**Specificity**
	Pow	***Poc* and non-*ovale***			
Positive.	31	0	31	83.78%	---
Negative.	6	99	105	---	100%
Total	37	99	136		
Note: One case of mixed infection was excluded.					

Discussion and conclusion

This study successfully establishes a novel CRISPR/Cas12a-based assay for the effective and precise discrimination of two species of *P. ovale*. The core of this methodology centers on the strategic design and meticulous screening of species-specific crRNAs. From a panel of five crRNA candidates, crRNA2 and crRNA4 were selected for their exceptional specificity, enabling the construction of two distinct detection systems. The optimized assay demonstrated robust analytical performance, with the *Poc* system exhibiting a superior detection limit of 30 copies/μL compared to 100 copies/μL for the *Pow* system. Crucially, both detection systems absolutely no cross-reactivity with other major human malaria parasites (*P. falciparum*, *P. vivax*, *P. malariae*), underscoring their high specificity.

The clinical validity of the assay was rigorously evaluated using 137 archived patient samples. Our results showed strong concordance with the reference qPCR method. Notably, one sample that had initially been diagnosed as a *Poc*-type infection was reclassified as a mixed infection, which was first indicated by our assay and then definitively confirmed via a follow-up qPCR test. Overall, this CRISPR/Cas12a-based approach achieved a clinical sensitivity of 96.77% and specificity of 99.05% for *Poc*, and a sensitivity of 83.78% with a specificity of 100% for *Pow*. The ROC curve analysis, yielding an AUC of 0.9994 for *Poc* and 0.9850 for *Pow*, further validates the excellent diagnostic power of this CRISPR/Cas12a-based approach.

The accurate discrimination of *Pow* and *Poc* has been a challenge due to their high genetic similarity. Our approach leverages the high specificity of the CRISPR/Cas12a system for single-nucleotide difference to discriminate them. Meanwhile, the initial screening of the five crRNAs was critical. While all candidates demonstrated CRISPR/Cas12a system activity, significant differences in specificity were observed. crRNA3 for *Pow* was discarded due to pronounced cross-reactivity, highlighting the importance of careful crRNA selection. The final selection of crRNA2 for *Pow* and crRNA4 for *Poc* was based on their ability to generate strong fluorescence signals exclusively for their intended targets, both in standard plasmids and clinically relevant sample panels, thereby ensuring reliable species identification.

The obtained detection limits (LOD of 30 copies/μL for *Poc* and 100 copies/μL for *Pow*) are comparable to those of established qPCR assays, but the LOD for *Pow* is higher than that for *Poc.* Meanwhile, Studies have shown that when distinguishing highly similar parasitic subtypes, eliminating cross-reactions which may compromise diagnostic accuracy and lead to false-positive results(30), often requires prioritizing specificity over sensitivity(31). Based on this principle, the core of this study lies in the design of specific crRNAs targeting the unique genomic regions of *Pow* and *Poc*. Among several candidate crRNAs, the selected crRNA2 exhibited excellent specificity, but the fluorescence signal intensity it generated was weaker than that of crRNA1. This screening strategy, which sacrifices some fluorescence intensity to ensure maximal specificity, may have resulted in the relatively lower sensitivity of the *Pow* detection system. The reduced sensitivity of *Pow* compared to *Poc* may be attributed to systematic bias arising from minor genomic differences between the two species.Specifically, the optimal crRNA for *Pow* may be inherently less efficient than that for *Poc* in terms of binding affinity or R-loop formation kinetics, thereby leading to reduced cleavage efficiency of the CRISPR/Cas12a system. Regarding clinical applicability, according to the WHO's evaluation criteria for RDTs, a detection rate above a certain

threshold (typically > 95%) is required at a parasite density of 200 parasites/μL. Conventional rapid diagnostic tools often show poor performance at low parasite densities (e.g., < 200 parasites/μL). In this study, the sensitivity of the *Pow* detection system at 100 copies/μL meets or even exceeds the WHO performance expectations for primary healthcare settings, indicating its suitability for clinical application.

Although our method does not surpass the highest sensitivity reported in some researches, it offers an excellent balance between sensitivity, specificity, and practical utility (Table S2). The absolute specificity against non-ovale species is a particular strength, addressing a limitation of cross-reactivity observed in some earlier studies (32). The high clinical specificity and sensitivity confirm the assay's robustness and reliability for clinical sample application, providing a valuable tool for epidemiological surveillance and clinical diagnosis of *P. ovale*.

Annealing temperatures and primer concentrations optimization plays an essential role in the efficiency, specificity, and sensitivity of PCR reaction (33, 34). Similarly, the temperature and some reaction condition of CRISPR/Cas12a could influence the trans-cleavage activity of Cas12a and the sensitivity of the method (35–37), then the annealing temperature and primer concentration in PCR and the crRNA, Cas12a concentration and reaction temperature in CRISPR/Cas12a system was optimized in this study. The detection limits were 30 copies/μL for the *P. ovale curtisi* system and 100 copies/μL for the *P. ovale wallikeri* system. It should be acknowledged that, compared with previously reported assays (32, 38), our method did not exhibit superior sensitivity. Nevertheless, both detection systems showed no cross-reactivity with other *Plasmodium* species and demonstrated excellent specificity, which represents a clear advantage over some earlier studies that involve cross-reactivity (32). Finally, We applied the established detection methods to clinical malaria samples and obtained excellent sensitivity and specificity, indicating that the CRISPR/Cas12a-based method meets the requirements for clinical application.

It is important to acknowledged it contains some limitations in this study. The primary limitation is the lower sensitivity of the *Pow* detection system, which led to false negatives in samples with high qPCR Ct values (low parasite density). Future work should focus on enhancing the sensitivity for *Pow*, potentially through exploring alternative crRNA target sites, incorporating signal amplification strategies like DNAzymes, or optimizing the pre-amplification step. Secondly, the requirement for two separate detection reactions increases operational complexity and the risk of cross-contamination. A significant future direction is the development of the one-pot reaction or multiplexed assay capable of simultaneously differentiating *Pow* and *Poc* using differentially labeled reporters. Finally, while the clinical sample size is sufficient to validate the method, broader validation with a larger, prospective cohort from diverse geographical regions would further strengthen the evidence for its widespread applicability.

In conclusion, we have developed and validated the CRISPR/Cas12a-based method that accurately differentiates between *P. ovale wallikeri* and *P. ovale curtisi*. Despite the noted limitations, this method

represents a significant step forward in the molecular diagnosis of *P. ovale* malaria. Its combination of specificity, ease of use, and potential for adaptation makes it a highly promising tool for enhancing epidemiological surveillance, guiding appropriate treatment, and supporting malaria elimination efforts, particularly in the context of managing malaria cases.

Abbreviations

Pow
Plasmodium ovale wallikeri
Poc
Plasmodium ovale curtisi
PCR
polymerase chain reaction
DNA
desoxyribonucleic acid
CRISPR/Cas12a
Clustered regularly interspaced short palindromic repeats associated with Cas12a
RDT
Rapid Diagnostic Test.

Declarations

Supporting Information

Supplementary data associated with this article can be found in the online version.

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Competing interests

The authors declare no conflicts of interest.

Ethics approval

The purpose of this study was demonstrated to participants before sampling, who agreed to participate in the survey. This study was approved by the Medical Ethics Committee of Henan Province Center for

Disease Control and Prevention (2018-KY-016-01).

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Author Contribution

SR, SY and RZ contributed to the study design. PJ and SL collected samples and carried out the laboratory work. HZ, YD and DZh conducted field activities. SR drafted the initial manuscript. SR, XZ and RZ contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information

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Scheme

Scheme 1 is available in the Supplementary Files section.

Figures

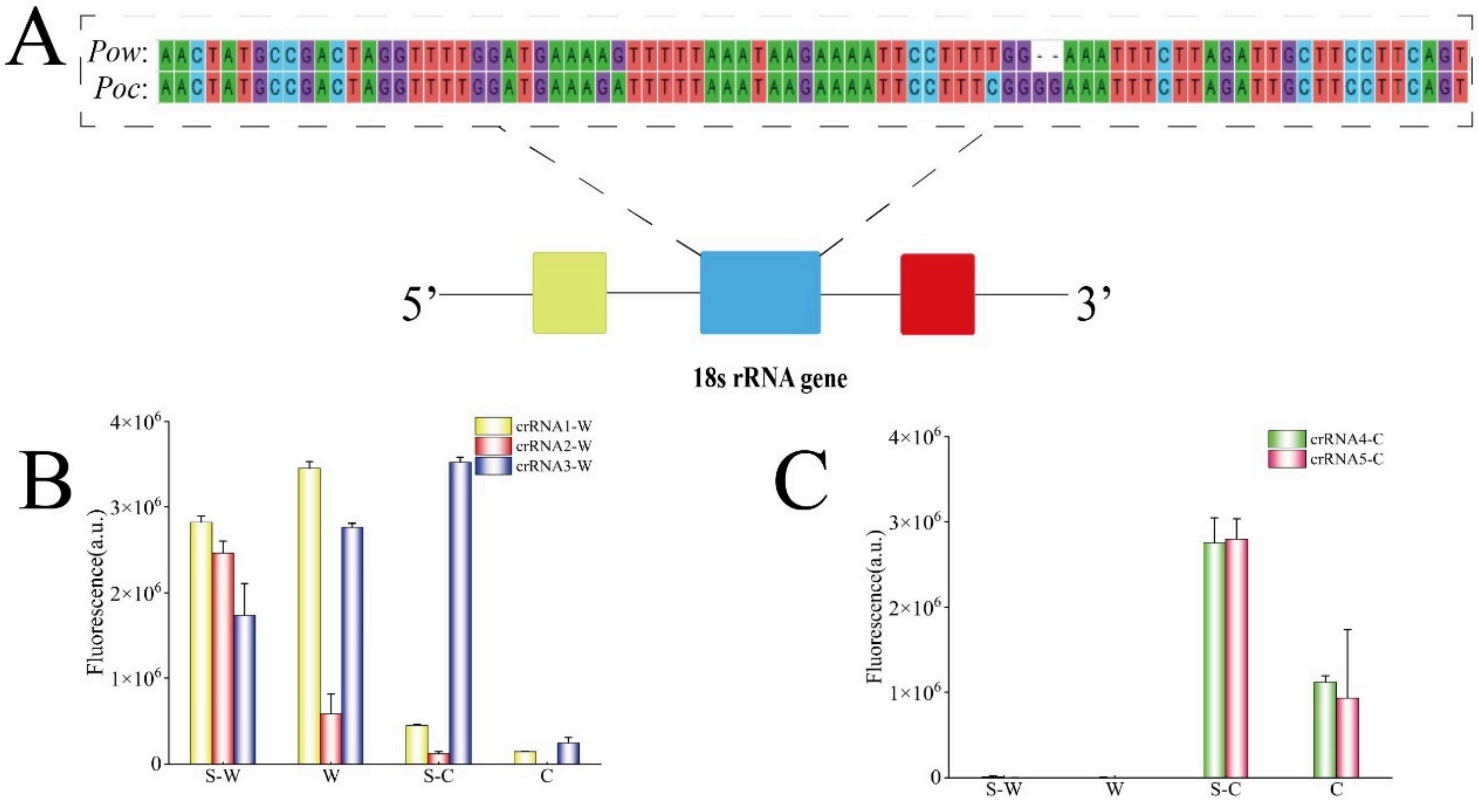


Figure 1

Discrimination viability between *Pow* and *Poc*. (A) Schematic representation of the target amplification sites. (B) Evaluation of crRNA1, crRNA2, and crRNA3 designed for *Pow* detection using *Pow* and *Poc* samples. (C) Evaluation of crRNA4 and crRNA5 designed for *Poc* detection using *Pow*and *Poc* samples. S-W: standard sample *Pow*. W: clinical sample *Pow*. S-C: standard sample *Poc*. C: clinical sample *Poc*. Error bars represent the standard deviation from three independent replicates.

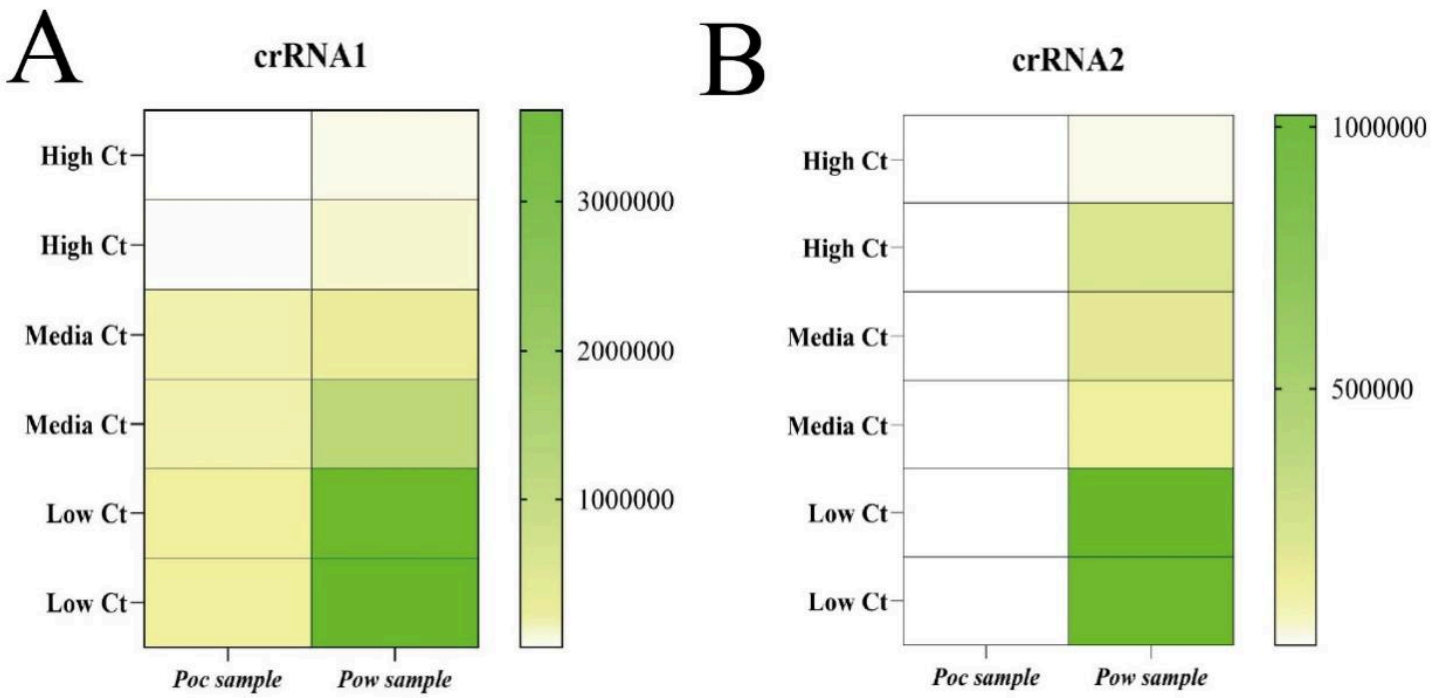


Figure 2

Screening optimal crRNA for *Pow* detection system. (A) Fluorescence intensity of six *Pow*and *Poc* samples with crRNA1. (B) Fluorescence intensity of six *Pow*and *Poc* samples with crRNA1. High Ct: The sample Ct value exceeds 30. Media Ct: The sample Ct value is between 25 and 30. Low Ct: The sample Ct value is between 20 and 25.

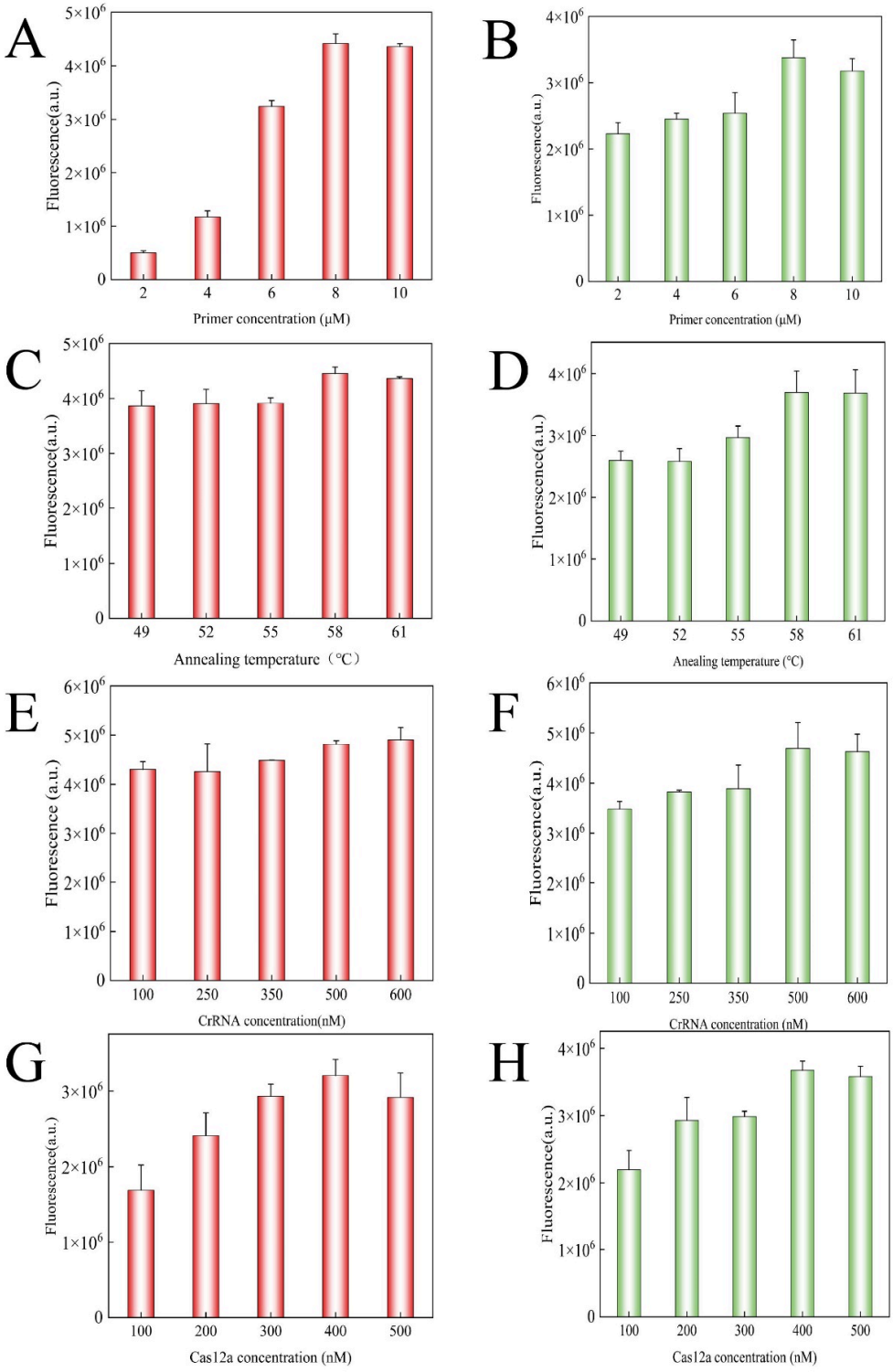


Figure 3

Optimization of assay reaction conditions. (A-D) PCR parameters: primer concentration for *Pow* (A) and *Poc*(B), and annealing temperature for *Pow* (C) and *Poc* (D). (E-H) CRISPR/Cas12a reaction parameters: crRNA concentration for *Pow* (E) and *Poc*(F), and Cas12a nuclease concentration for *Pow* (G) and *Poc* (H). Error bars represent the standard deviation from three independent replicates.

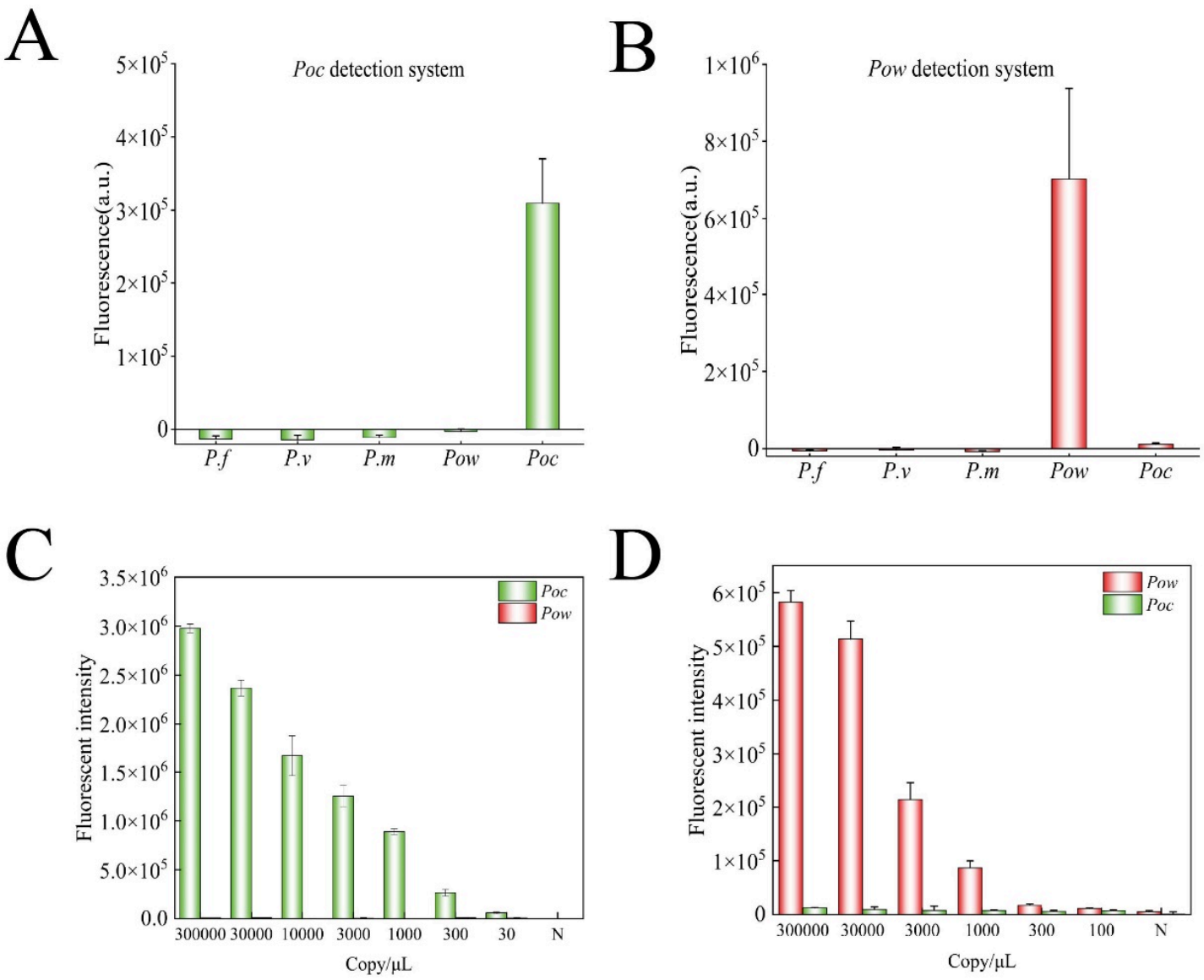


Figure 4

Analytical performance of the CRISPR/Cas12a-based method. (A) Specificity evaluation of the *Poc*detection system. (B) Specificity evaluation of the *Pow* detection system. (C, D) Fluorescence signals obtained for *Poc* and *Pow* samples with different concentrations.

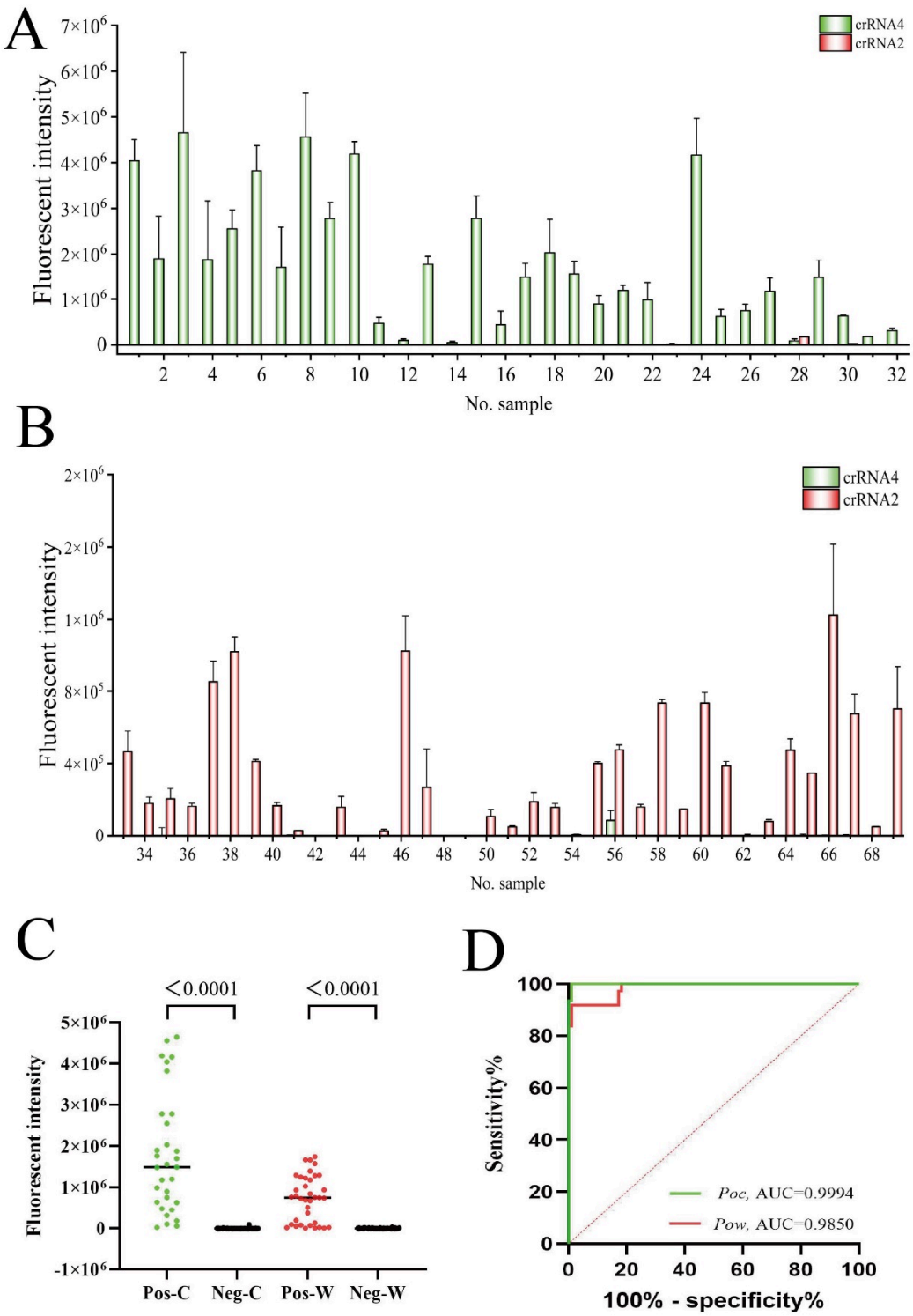


Figure 5

Application in different types of malaria samples. (A) CRISPR/Cas12a-based method applied in *Poc* samples. (B) CRISPR/Cas12a-based method applied in *Pow* samples. (C) Scatter plot demonstrating the CRIPSR/Cas12a-based method signals of negative and positive clinical samples for *Poc* and *Pow*, respectively. (D) ROC curve analysis of CRISPR/Cas12a-based methods.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- floatimage1.jpeg
- Supplementaryinformation.docx