Photoactivatable CRISPR/Cas12a Strategy for One-Pot DETECTR Molecular Diagnosis

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Photoactivatable CRISPR/Cas12a Strategy for One-Pot DETECTR Molecular Diagnosis
Yong Chen, Xiaoling Xu, Jiachun Wang, Yibin Zhang, Wentao Zeng, Yizhen Liu, and Xueji Zhang
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ABSTRACT: As a golden partner of recombinase polymerase amplification (RPA), CRISPR/Cas12a has been proven to solve the false-positive problem caused by nonspecific amplification perfectly; meanwhile, its trans-cleave activity has further enhanced the sensitivity. However, the solution transfer operation after tube cap opening greatly increases the risk of aerosol contamination of amplicon, which is inconsistent with point-of-care (POC) diagnostics requirements. This study proposes a photoactivated CRISPR/Cas12a strategy to achieve one-pot high-sensitivity nucleic acid detection. Using photocleavable complementary ssDNA to block crRNA, RPA amplification can smoothly pass through the exponential interval without being affected by
![Amplicons\nCas12a\nPhotoCleavage\ntrans-cleavage\n](5/eeSnBYxHk3lOr6r5YKOawH/pdf_d52a49c73ca3df75afa97d4b293af708_e9d5c482e2eed2478a94c782ff07d0667508518051523035/tmp_zip_img/a4b98cba-e4de-4b40-b9ec-65d585c509b9.png)
activated Cas12a in the critical early stage. After enough amplicons were produced, the Cas12a test was activated by short bursts of ultraviolet radiation at 365 nm. This one-pot method achieved a sensitivity of 2.5 copies within 40 min. This simple and sensitive one-pot method can effectively avoid amplicon contamination and lower the threshold for molecular diagnostics in POC.
\blacksquare INTRODUCTION
High-sensitivity and high-specificity nucleic acid detection are crucial in clinical detection, food safety, and environmental monitoring, such as pathogen detection, genotyping, species identification, genetic research, and so on.1,2 In recent years, the RNA-guided CRISPR/Cas nucleic acid detection technology has attracted widespread attention due to the single-base specificity and point-of-care compatibility.3,4 The CRISPR/Cas system is developed from the antiviral defense system of prokaryotes and has always been regarded as a powerful gene-editing tool.5,6 Interestingly, when the CRISPR/Cas nucleases, including RNA-guided RNase (Cas13a and Cas13b) and RNA-guided DNase (Cas12a and Cas14), recognize the target nucleic acid, they not only cis-cleave the target nucleic acid but also indiscriminately trans-cleave the nearby single-stranded non-targeted nucleic acids.7−9 Taking advantage of this feature,

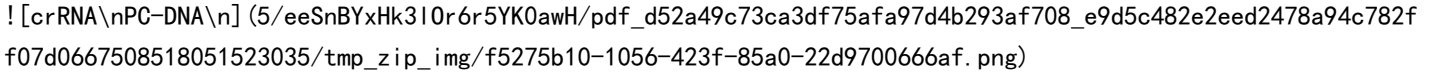
Zhang et al. combined Cas13a RNase with recombinase polymerase amplification (RPA) and proposed the Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHER-LOCK) technology for rapid detection of ZIKA virus,10 while Doudna et al. proposed DNA Endonuclease-Target CRISPR Trans reporter (DETECTR) technology based on the Cas12a DNase to achieve the specific detection of human papillomavirus in the clinical sample.11

The CRISPR/Cas nuclease system with extraordinary specificity and reliability has shown perfect complementarity with RPA amplification: CRISPR/Cas eliminates nonspecific signals from RPA nonspecific amplification, while RPA increases the sensitivity of the CRISPR/Cas system.12−15

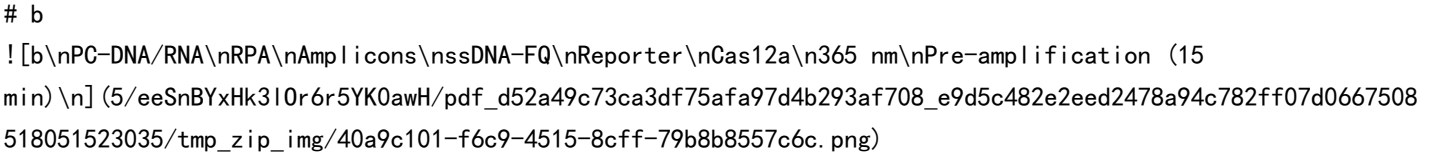
However, when researchers attempted to integrate CRISPR/Cas12a detection and nucleic acid amplification into a single reaction, they found that the reported sensitivity was typically low (~104 aM).16,17 The well-designed and optimized integrated Cas12a-assisted RPA assay achieved high sensitivity. However, the problems of excessively long detection time (60−90 min) and low endpoint signal were still inevitable in trace nucleic acid analysis.4,18,19 The main reason for these problems is that Cas12a nuclease continuously digested amplicons and primers, resulting in low amplification efficiency.16,17 As a compromise, most Cas12a-assisted RPA assays separated the amplification from the CRISPR/Cas readout, which contained the transfer of amplicons and multiple manual operations, complicating the testing process and increasing the contamination risk.20,21 More elaborate, the "spatial isolation" strategy was reported for one-pot separated RPA-Cas12a assay for ultrasensitive molecular diagnoses, such as Cas12a protein in the cap of PCR tube and dynamic aqueous multiphase.17,22 However, these solutions are not suitable for the current POCT diagnostic due to the high

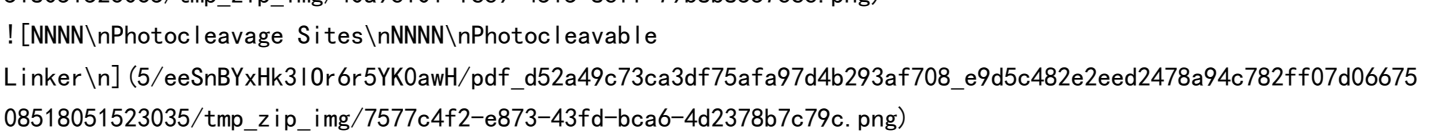
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a



b





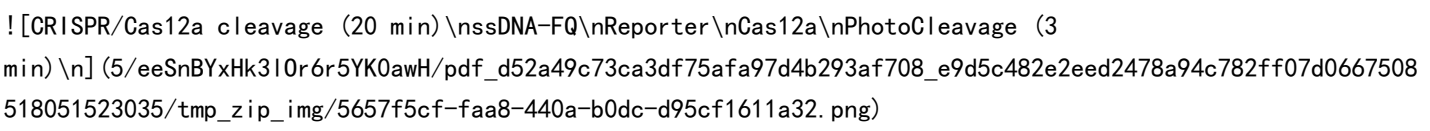


Figure 1. Schematic of photoactivatable CRISPR/Cas12a-assisted RPA assay. (a) Photocleavable PC−DNA/RNA hybrid and structure of the photocleavable linker. (b) Photoactivatable CRISPR/Cas12a-assisted RPA assay for one-pot nucleic acid detection.

technical requirements of the operator and the difficulty in automation.

This study developed a photoactivatable CRISPR/Cas12a-assisted RPA assay for ultrasensitive one-pot nucleic acid detection. As a noninvasive, fast, high-spatial-resolution biochemical reaction switch, photoactivation strategies have long been used as a time controller for gene editing in vivo via photocleavable linkers.23,24 For example, a photoactivatable CRISPR/Cas9 system includes light-responsive

Cas9 analogues, light-activatable transcription factors, gRNA-blocking oligonucleotides, and so on.25-27 These systems were activated by UV (365 nm), blue (450–470 nm), cyan (500 nm), red (650 nm), far-red (730 nm), or near-infrared light (980 nm).28-30 In our experiment, crRNA is bound by complementary photocleavage ssDNA (PC–DNA) to prevent its assembly with CRISPR/Cas12a, thus keeping Cas12a's cleavage function inactive.31,32 This system effectively ensures that RPA amplification is not interfered with by CRISPR/Cas systems and provides compatibility between the two systems. Therefore, RPA and CRISPR/Cas12a reagents can be mixed directly in one tube, after 15 min of preamplification at 37 ° C, the CRISPR/Cas12a reaction can be activated using 365 nm UV light. The ssDNA-FQ reporter gene was then rapidly cleaved by Cas12a nuclease within 20 min to produce a bright fluorescent signal. In this sealed one-pot system, the method realized a step-by-step process of RPA amplification and then photoactivatable CRISPR/Cas12a detection, which solved the problems of sensitivity reduction (10^4 copies vs 2.5 copies) and detection time delay (90 vs 40 min) caused by the competitive reaction in traditional integrated RPA/Cas12a detection. The reliable one-pot photoactivatable CRISPR/Cas12a-assisted RPA assay maintains a reliable block against external forces and avoids the risk of amplicon contamination associated with open cover testing operations, which has excellent potential for the POC field diagnostics.

\blacksquare MATERIALS AND METHODS

Materials and Instruments. Lachnospiraceae bacterium Cas12a (LbCas12a) protein was from Shanghai Tolo Biotechnology. TwistAmp Basic kit was from TwistDx Limited. The DNA/RNA nucleic acid sequences (Table S1) and the RNase inhibitor were from Shanghai Sangon Biotechnology.

The plasmid (pUC57-p72) was from Beijing Tsingke Biotechnology. The SYBR Green I was from Thermo Scientific, Inc., and the other chemical reagents were from Shanghai Macklin, Inc.

The pH value was measured by a Mettler Toedo FE28-Five Easy pH (Mettler Toedo, Switzerland). The nucleic acid concentration was determined by a GENESYS 10S UV–vis spectrophotometer (Thermo Scientific). Electrophoresis was carried out using a Bio-Rad electrophoresis system. An LED lamp with a 365 nm wavelength carried out the photocleavage of PC–DNA. A Tanon gel imager system acquired the endpoint fluorescent images and electropherograms. The real-time fluorescence was measured by a QuantStudio 6 Flex qPCR system (Applied Biosystems, Massachusetts). The fluorescence spectra were measured by the Tecan Spark 20M microplate reader.

Preparation of PC-RNA/DNA. crRNA (1 L, 100 M) and PC-ssDNA (2 L, 100 M) were added to 97 L of 1× optimized one-pot buffer containing 50 mM Tris-HCl, 10 mM MgCl, 100 mM KCl, 2 mM DTT, 100 g/mL BSA, 5% PEG. The mixture was heated to 95° C for 5 min, and then slowly cooled to room temperature at 0.8 ° C/min for complete annealing. Finally, the PC-RNA/DNA was stored in a refrigerator at 4 ° C.

Photoactivatable CRISPR/Cas12a. The photoactivatable CRISPR/Cas12a system is identical to those described in the reference, requiring only the substitution of crRNA with PC-DNA/RNA hybrid.33,34 Briefly, 25 L of a photoactivatable CRISPR/Cas12a reaction solution consisted of 60 nM PC-DNA/RNA, 60 nM LbCas12a, 300 nM DNA-FQ reporter, 2 U/L RNase inhibitor, and a varying amount of target DNA. Unlike conventional CRISPR/Cas12a assays, the photo-activatable CRISPR/Cas12a assay does not generate fluorescence signals. When irradiated with 365 nm UV light for 3 min, the blocked crRNA would be released and activate the CRISPR/Cas12a system to generate a fluorescence signal.

Photoactivatable Cas12a-Assisted RPA Assay. The reaction of RPA amplification and Cas12a nuclease detection were integrated with one pot. Briefly, 25 L of the one-pot reaction solution consisted of 360 nM forward/reverse primer, 750 nM DNA-FQ reporter, 1.6 U/L RNase inhibitor, 1× optimized one-pot buffer, 60 nM LbCas12a nuclease, 60 nM PC–DNA/RNA hybrid, 16 mM MgOAc, and a varying amount of target DNA. Reactions first proceed for 15 min at 37 ° C for RPA amplification, then photoactivated with UV light at 365 nm for 3 min, and finally, fluorescent kinetic curves were obtained by a microplate reader or real-time qPCR system.

Separated RPA-Cas12a Assay. The reagent (20 L) of RPA amplification was dropped to the bottom of the PCR tube, containing an RPA reaction pellet, 360 nM forward/reverse primer, 1× optimized one-pot buffer, 16 mM MgOAc, and a varying amount of target DNA. The 5 L reagent of 5×CRISPR/Cas12a was dropped into the PCR tube cap (careful handing would not drop to the bottom of the PCR tube), containing 3.75 M DNA-FQ reporter, 8 U/L RNase inhibitor, 1× optimized one-pot buffer, 300 nM LbCas12a nuclease, 300 nM PC-DNA/RNA hybrid.

The reaction was performed at 39 ° C for 15 min for RPA preamplification. The PCR tube was then placed in a centrifuged, and the CRISPR/Cas12a reagent on the cap of the PCR tube was dropped into the bottom of the PCR tube. In this way, the amplicon was exposed to the CRISPR/Cas12a and activated the trans-cleavage activity of Cas12a nuclease, and finally, the fluorescent kinetic curves were obtained by a microplate reader or real-time qPCR system for 20 min.

\blacksquare RESULTS AND DISCUSSION

Scheme of Photoactivatable CRISPR/Cas12a-Assisted RPA Assay. The principle of photoactivatable CRISPR/Cas12a-assisted RPA assay is described in Figure 1. To establish the photoactivatable CRISPR/Cas12a, we designed 40 nt of photocleavable ssDNA oligonucleotides (PC-DNA), which complements crRNA and consists of four 9 - 10 nt oligonucleotides connected by three photocleavable linkers (Figure 1a). As shown in Figure 1b, the hybrid structure generated by complete complementation of PC-DNA and crRNA is more stable, preventing crRNA from forming the hairpin structure and binding with Cas12a nuclease^{32,35 In this way, the deactivated CRISPR/Cas12a system neither competes with RPA amplification for template DNA nor consumes primers so that the RPA reaction can produce sufficient amplicons within 15 min for subsequent CRISPR/Cas12a reaction. Upon photolysis, under a 365 nm UV lamp, the short fragments of the cleaved PC-DNA will have a reduced binding affinity for crRNA, so crRNA is released and forms the hairpin structure. Then, the crRNA with a hairpin structure binds to Cas12a protein to form riboprotein (RNP), which is activated by the target amplicons to rapidly cleave the ssDNA-FQ reporter and generate a bright fluorescent signal within 20 min.

Photoactivatable CRISPR/Cas12a Cleavage. As shown in Figure 2a, three kinds of PC - DNA were designed: the first 40 nt of PC - DNA was utterly complementary to crRNA; the second 20 nt of F-PC - DNA only complementary to the hairpin structure of crRNA; the third 20 nt of R-PC - DNA only complementary to the recognition region of crRNA. The three PC - DNA were used for the photoactivated CRISPR/Cas12a test. The real-time fluorescence kinetics curve is shown in Figure S1, and the fluorescence contrast histogram of the endpoint is shown in Figure 2a. The results showed that 40 nt of PC - DNA achieved the expected results: preventing the crRNA-bonded Cas12a nuclease and no fluorescence signal produced in the absence of UV light; PC - DNA photolysis and crRNA activate the CRISPR/Cas system to produce fluorescence signal under UV light; no fluorescence signal was generated when no target DNA added. In contrast, F-PC - DNA generated fluorescence signals without UV light because the target DNA bound to crRNA through the chain replacement process and activated the CRISPR/Cas reaction. The R-PC - DNA cannot prevent crRNA binding to Cas nuclease because the hairpin structure is not blocked, and signals were generated in the absence of target DNA because R-PC - DNA complementary to the crRNA recognition region can directly activate the Cas12a system. To confirm the effectiveness of the photoactivatable CRISPR/Cas12a strategy, we demonstrate the breaking of ssDNA indicator (ssDNA of 30 nt) and generation of fluorescent signal from the ssDNA-FQ reporter by the gel electrophoresis images and fluorescent spectrometer. As shown in Figure 2b, the "marker" lane contains standard dsDNA with different lengths. The "turn off" and "turn on" lanes indicate changes in the nucleic acid size before and after being photoactivated under 365 nm UV light in the photoactivatable CRISPR/Cas12a system. The "normal" lane indicates the size information of nucleic acid in the conventional CRISPR/

Figure 2. Comparison of photoactivation with conventional CRISPR/Cas12a detection. (a) Optimization experiments of PC－DNA. (b) Gel electrophoresis image confirms that the photoactivatable CRISPR/Cas12a reaction cleaves target DNA and ssDNA indicator. (c) Photoactivatable CRISPR/Cas12a achieves the same effect as the conventional CRISPR/Cas12a reaction and eliminates cap opening. NTC: negative test control.

Cas12a system after the reaction is completed. The "reference" lane indicates the 65 bp of target dsDNA. As we expected, the bands of 40 bp of PC-DNA/RNA hybridization, 65 bp of target dsDNA, and 30 nt ssDNA indicator were visible in the reaction system before the photoactivable CRISPR/Cas12a reaction irradiated with 365 nm UV light, indicating that the CRISPR/Cas12a system was not activated. However, after 3 min of irradiation with UV light and incubation at 37 ° C for 1 h, the photocleavable PC-DNA broke and released crRNA, which activated the CRISPR/Cas12a system and completed the cleavage of dsDNA and ssDNA indicator, so no bands of nucleic acid are indicated in the image. These results were consistent with the conventional CRISPR/Cas12a system as the "normal" lane.

Unlike the above gel electrophoresis images, the ssDNA-FQ reporter replaced the 30 nt ssDNA indicator in the fluorescent spectrum experiment. The "Cas12a-added NTC" and "Cas12a-added" curves, respectively, represent the fluorescent intensity of conventional CRISPR/Cas12a reaction before and after activation, and the "photoactivated NTC" and "photo-activated" curves, respectively, represent the fluorescent intensity of photoactivated CRISPR/Cas12a reaction before and after activation by 365 UV light (NTC: Negative Test Control). As shown in Figure 2c, the photoactivitable CRISPR/Cas12a reaction generated the same fluorescent intensity as the conventional CRISPR/Cas12a reaction, and the fluorescent background interference before activation was

the same, indicating that the photoactivatable CRISPR/Cas12a strategy did not affect the detection performance for target DNA and signal amplification. In addition, it is worth noting that the conventional CRISPR/Cas12a reaction started immediately after the reagents were added and cannot be precisely controlled, while the photoactivatable CRISPR/Cas12a reaction can be activated through light irradiation in the closed tube.

Optimized Photoactivatable CRISPR/Cas12a Reaction. To improve the signal enhancement rate and shorten the detection time, we further optimized the ratio of PC－DNA and crRNA in PC－DNA /RNA hybridization and studied the time requisition for complete photoactivated the CRISPR/Cas12a. In the CRISPR/Cas12a system, the activated Cas12a nuclease would indiscriminately cleave all ssDNA in the reaction. Considering that the 9－10 nt of PC－DNA fragments from photolyzed would compete with the ssDNA-FQ reporter for the cleaving activity of Cas12a nuclease, slowing down the enhancement rate of fluorescent signal, we need to minimize the dosage of PC－DNA while ensuring that the background signal was completely shielded. As shown in Figure 3a, when

a

![Fluorescence\n\nStandard\n\n→ 1.2:1 (PC-DNA:crRNA)\n\n→ 1.5:1 (PC-DNA:crRNA)\n\n→ 2.0:1 (PC-DNA:crRNA)\n\n→ NTC\n\nTime (min)](5/eeSnBYxHk3lOr6r5YK0awH/pdf_d52a49c73ca3df75afa97d4b293af708_e9d5c482e2eed2478a94c782ff07d0667508518051523035/tmp_zip_img/1a3aad6a-02ab-4d47-a941-87e64bf44c1b.png)

![|PC-DNA:crRNA|Photoactivate NTC|Photoactivate|\n|:---:|:---:|:---:|\n|1:1.2|500|12500|\n|1:1.5|500|12500|\n|1:2|100|13000|](5/eeSnBYxHk3lOr6r5YK0awH/pdf_d52a49c73ca3df75afa97d4b293af708_e9d5c482e2eed2478a94c782ff07d0667508518051523035/tmp_zip_img/d441841e-78ce-4b72-827d-7477a77aaf7d.png)

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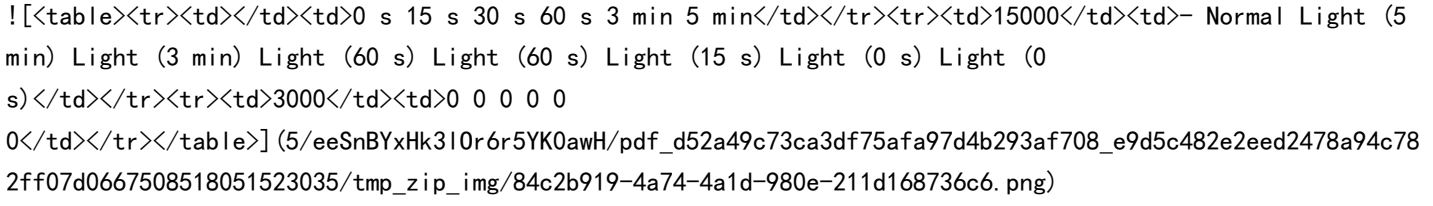


Figure 3. Dosage and time optimization of photoactivatable CRISPR/Cas12a reaction. (a) Optimization of the ratio of PC-DNA and crRNA to reduce background interference and improve detection speed. (b) Optimization of the photocleavage time of PC-DNA/RNA to achieve the best detection effect.

the hybridization ratio of PC-DNA to crRNA was 1.2:1 and 1.5:1, the background signal both increased slightly. The reason is that Cas12a nuclease was activated by free crRNA in the reaction due to insufficient PC-DNA dose, interfering with the accuracy of trace detection. However, when the dosage of PC-DNA was increased to twice that of crRNA, the fluorescent kinetics curve was utterly consistent with that of the negative group, indicating that the background interference signal of the photoactivatable CRISPR/Cas12a reaction

disappeared completely. In addition, when the CRISPR/Cas12a system was activated again with 365 nm UV light, all of the experimental groups with different doses of PC-DNA obtained the same endpoint fluorescent signal intensity, suggesting that the twice amount of PC-DNA did not affect the claving efficiency of the activated CRISPR/Cas12a system.

The more crRNA released from PC-DNA/RNA hybridization after 365 nm UV irradiation, the more Cas12a nuclease activated and the faster the signal enhancement rate. However, an overly long irradiation time is inefficient and may lead to DNA damage, so the minimum time required for PC-DNA/RNA to fully release crRNA was studied in detail through gel electrophoresis images and the enhancement rate of the fluorescent signal after different activation times. As shown in Figure 3b, the two leftmost lanes indicate the location of crRNA and PC-DNA, respectively. When PC-DNA/RNA was not cleaved by UV irradiation, 40 bp of hybridization bands and faint excess bands of PC-DNA can be observed. With the extension of illumination time, PC-DNA/RNA bands gradually decreased and disappeared, while the 40 nt PC-DNA would break into 9−10 nt fragments, and the amount of crRNA released steadily increased. After UV irradiation for 1 min, the bands of PC-DNA almost disappeared, while the bands of crRNA became very clear. Photoactivated CRISPR/Cas12a reactions were performed with different UV irradiation times in a further study. The corresponding fluorescence kinetics curves and endpoint fluorescence images showed that when the light activation time was 3 and 5 min, the accumulation rate and endpoint intensity of the fluorescent signal was consistent with that of the standard CRISPR/Cas12a reaction system, indicating that sufficient crRNA had been released to activate the CRISPR/Cas12a system. Finally, considering the detection time, we set the light activation time as 3 min.

One-Pot CRISPR/Cas12a-Assisted RPA Assay. After the photoactivatable CRISPR/Cas12a reaction was confirmed to be available, we needed to establish the corresponding RPA amplification system. The focus of RPA amplification lies in the design of primers. The optimal primers can reduce nonspecific amplification and

improve the detection sensitivity, especially for low concentrations of nucleic acid samples. In this study, the p72 gene in the conserved region of the African Swine Fever (ASF) virus sequence was selected as the template DNA, and Premier 5 software was used to design primers. The OligoAnalyzer Tool of Integrated DNA Technologies investigated the secondary structure and primers dimer. Finally, four pairs of primers were selected (Table S1). Then, the amplification curve and melting curve of four pairs of primers were measured by the qPCR system to evaluate the amplification efficiency and nonspecific amplification. As shown in Figure S2, all four pairs of primers could quickly generate obvious fluorescence signals when 10,000 copies of template DNA were added. The corresponding melting curves of the four pairs of primers all have peak values at 55 ° C (primers dimer) and 88 ° C (target amplicons) (Figure S3). As shown in Figure S4, the AFSV-P1 primer had the lowest nonspecific product peak relative strength at 55 ° C, so it would be used for subsequent one-pot photoactivatable CRISPR/Cas12a-assisted RPA detection.

The commercial TwistAmp Basic kit and CRISPR/Cas12a reaction reagent are equipped with corresponding reaction buffers. To be more compatible with RPA amplification and CRISPR-Cas12a detection, we optimized the one-pot photo-activatable CRISPR/ Cas12a-assisted RPA buffer and named it "one-pot buffer" (Table S2). Compared with the original RPA buffer and CRISPR buffer, we found that the optimized one-pot buffer showed better results: the same RPA amplified efficiency and faster CRISPR/Cas12a detection (Figure 4a).

![|Time (min)|One-pot Buffer|RPA Buffer|\n|:---:|:---:|:---:|\n|0|600|600|\n|5|900|750|\n|10|1500|1200|\n|15|6000|5400|\n|20|10500|9000|\n|25|12000|10500|\n|30|12600|11100|\n|35|12900|11400|\n|40|13200|11700|\n|45|13500|11850|\n|50|13800|12000|](5/eeSnBYxHk3lOr6r5YK0awH/pdf_d52a49c73ca3df75afa97d4b293af708_e9d5c482e2eed2478a94c782ff07d0667508518051523035/tmp_zip_img/d39bc85c-0a81-4c36-8339-bf92b89bcc98.png)

![|Time (min)|One-pot Buffer|CRISPR Buffer|\n|:---:|:---:|:---:|\n|0|0|0|\n|5|1500|1500|\n|10|4500|4500|\n|15|7500|7500|\n|20|10500|9000|\n|25|12000|10500|\n|30|13500|11500|\n|35|13500|12000|\n|40|13500|12500|\n|45|13500|12500|\n|50|13500|13500|](5/eeSnBYxHk3lOr6r5YK0awH/pdf_d52a49c73ca3df75afa97d4b293af708_e9d5c482e2eed2478a94c782ff07d0667508518051523035/tmp_zip_img/f3a4aa13-07ad-4b05-b6f0-48d94bc95b59.png)

![|浓度类型|数值|荧光值|\n|:---:|:---:|:---:|\n|MgOAc Concentration|14mM|10500|\n|MgOAc Concentration|16mM|13000|\n|MgOAc Concentration|18mM|11500|\n|Primer Concentration|0.12uM|6500|\n|Primer Concentration|0.24uM|12500|\n|Primer Concentration|0.36uM|12500|\n|ssDNA-FQ Concentration|0.5uM|1500|\n|ssDNA-FQ Concentration|1uM|3500|\n|ssDNA-FQ Concentration|1.5uM|12500|\n|ssDNA-FQ Concentration|3uM|13500|](5/eeSnBYxHk3lOr6r5YK0awH/pdf_d52a49c73ca3df75afa97d4b293af708_e9d5c482e2eed2478a94c782ff07d0667508518051523035/tmp_zip_img/ab9b0279-bbd0-483d-be7e-48ab9860ddb6.png)

Figure 4. Optimization of photoactivatable CRISPR/Cas12a-assisted RPA detection. (a) Optimization of the one-pot buffer for RPA amplification and CRISPR/Cas12a detection. (b) Optimized the concentration of MgOAc, primers, and DNA-FQ reporter.

Furthermore, we optimized the concentration of primers, MgOAc, and ssDNA-FQ reporter in the one-pot photo-activatable CRISPR/Cas12a-assisted RPA system by monitoring the growth of fluorescent signal (Figure S5). The results showed that the optimized primer concentration of 240 nM and MgOAc concentration of 16 mM could slightly improve the detection performance, while increasing the concentration of ssDNA-FQ reporter could significantly improve the enhancement rate of the fluorescent signal and the final fluorescent signal intensity (Figure 4b).

The detection sensitivity of the CRISPR/Cas12a system is only pM level, so the focus of the CRISPR/Cas12a-assisted RPA assay is to generate sufficient amplicons before CRISPR/Cas12a cleavage is activated.36,37 As shown in Figure 5a, when a large amount of template DNA (2.5 × 105 copies) was added to the detection system, the CRISPR/Cas12a reaction was activated after different preamplification times (0, 5, 10, 15, 20, 30 min), there was no significant difference in the enhancement rate of fluorescence signal. The reason is that the amplified efficiency of RPA is very high, which only takes a few minutes to

consume the raw material and produce a large number of amplicons when the template concentration is very high,38 so extending the preamplification time does not improve the detection system. However, when the template concentration was low (25 copies), the short amplification time was insufficient to exhaust the raw material. The number of amplicons produced was too small, thus prematurely activating the CRISPR/Cas12a reaction, resulting in a prolonged accumulation of fluorescent signals. Extending the preamplification time is necessary to obtain a better detection effect. As shown in Figure 5b, when the preamplification time

|Time (min)|0 min Pre-amplification|5 min Pre-amplification|10 min Pre-amplification|15 min Pre-amplification|20 min Pre-amplification|\n|:---:|:---:|:---:|:---:|:---:|:---:|\n|0|0|0|0|0|0|\n|10|5000|40000|60000|55000|35000|\n|20|5000|95000|110000|100000|80000|\n|30|5000|160000|165000|160000|120000|](5/eeSnBYxHk3lOr6r5YK0awH/pdf_d52a49c73ca3df75afa97d4b293af708_e9d5c482e2eed2478a94c782ff07d0667508518051523035/tmp_zip_img/f0e43c43-af1d-4024-85a9-0f6ff6e51dac.png)

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|时间 (min)|0 min Pre-amplification|5 min Pre-amplification|10 min Pre-amplification|15 min Pre-amplification|20 min Pre-amplification|30 min Pre-amplification|\n|:---:|:---:|:---:|:---:|:---:|:---:|:---:|\n|0|5000|5000|5000|5000|5000|5000|\n|10|5000|8000|10000|25000|30000|50000|\n|20|5000|10000|15000|40000|50000|70000|\n|30|5000|12000|20000|50000|60000|80000|](5/eeSnBYxHk3lOr6r5YK0awH/pdf_d52a49c73ca3df75afa97d4b293af708_e9d5c482e2eed2478a94c782ff07d0667508518051523035/tmp_zip_img/076aef42-8056-4221-b62f-9b8a2c948740.png)

Figure 5. Effect of different preamplification times on fluorescent signal enhancement rate. (a) Shorter preamplification time did not affect the detection sensitivity for the high-concentration template DNA (2.5 × 105 copies). (b) Sufficient preamplification time can significantly improve the detection sensitivity for low-concentration template DNA (25 copies).

is extended to 15 min, the enhancement rate of the fluorescent signal is significantly accelerated. When the preamplification time was further extended, the enhancement rate of the fluorescent signal only increased slightly. The reason is that the raw materials had been consumed after 15 min of amplification, resulting in decreased amplification rate. The real-time fluorescent kinetics curves for template DNA amplification at different concentrations are shown in Figure S6. The results showed that no matter how much template DNA was added, the RPA reaction produced many amplicons products within 15 min. Therefore, after 15 min of RPA amplification, 365 nm UV light can activate the CRISPR/Cas12a reaction, which shortens the testing time and ensures high sensitivity. Notably, NTC still produced a significant fluorescence signal because SYBR staining could not distinguish the nonspecific amplification products from RPA reaction, which further confirms the necessity of using the CRISPR/Cas technology for specific detection of amplicons.

Sensitivity and Selectivity of One-Pot Photoactivatable CRISPR/Cas12a-Assisted RPA Assay. Finally, we tested the sensitivity and selectivity of the one-pot photoactivatable CRISPR/Cas12a-assisted RPA method under the above optimal conditions. For selective validation tests, we tested both PRV (Pseudorabies virus) and PCV2 (porcine circovirus type 2) samples using the ASFV test system. As shown in Figure S7, the reaction could only generate a bright fluorescence signal when ASFV samples were added. This method that combines RPA amplification with CRISPR technology has ultrahigh sensitivity due to the dual specificity of RPA primers and CRISPR/Cas technology. For the sensitivity tests, the target DNA samples with different concentrations were added to 25 L of the system and incubated at 37 ° C for 15 min to complete the preamplification process. Then, the reaction was irradiated to 365 nm UV light

for 3 min and the fluorescent intensity was tested after incubation for 20 min. The results in Figure 6a confirm that

a

b

Figure 6. Photoactivatable CRISPR/Cas12a-assisted RPA assay for one-pot nucleic acid detection. (a) Sensitivity of one-pot photoactivatable Cas12a-assisted RPA assay. (b) Sensitivity of the one-pot photoactivatable Cas12a-assisted RPA assay was consistent with that of the separated RPA-Cas12a detection.

the one-pot photoactivatable CRISPR/Cas12a-assisted RPA method has a sensitivity of 2.5 copies in each reaction. The fluorescence images of the endpoints also show a visual signal difference between the blank group and the test group. Furthermore, the results in Figure 6b confirm that the one-pot photoactivatable CRISPR/Cas12a-assisted RPA assay has the same sensitivity as the conventional separated RPA-Cas12a reaction but is simpler to use the open-free operation that avoids aerosol contamination.

\blacksquare CONCLUSIONS

This paper established a photoactivatable CRISPR/Cas12a-assisted RPA assay for one-pot nucleic acid detection. The method uses ssDNA complementary to crRNA to block CRISPR/Cas12a cleavage and restores CRISPR/Cas12a activity under 365 nm UV light by coupling with the photolabile linker. In this sealed one-pot system, the method realized a step-by-step process of RPA amplification and then photoactivatable CRISPR/Cas12a detection, which solved the problems of sensitivity reduction and detection time delay caused by the competitive reaction in traditional integrated RPA-Cas12a detection. The whole detection only took 40 min, and the detection sensitivity was 2.5 copies. In addition, compared with the current separation RPA-Cas12a detection, this method is simpler to operate and more accurate to control and avoids the problem of aerosol contamination caused by amplicons transfer. We think that the photoactivatable CRISPR/Cas12a-assisted RPA assay will play an essential role in POC nucleic acid detection with high sensitivity and specificity.

\blacksquare ASSOCIATED CONTENT

\blacksquare Supporting Information

The Supporting Information is available free of charge at
https://pubs.acs.org/doi/10.1021/acs.analchem.2c01193.

Additional characterization data and sequence information; optimization experiments of PC-ssDNA (Figure S1); primer screening for RPA amplification (Figure S2); melting curves of RPA amplicons (Figure S3); analysis of RPA amplicons of four primers (Figure S4); optimization for one-pot photoactivatable CRISPR/Cas12a-assisted RPA system (Figure S5); sensitivity test (Figure S6); selectivity test (Figure S7); sequence information (Table S1); and composition of one-pot buffer (Table S2) (PDF)

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Notes
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