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Technical Note

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Cas12aVDet: a CRISPR/Cas12a-based platform for rapid and visual nucleic acid detection

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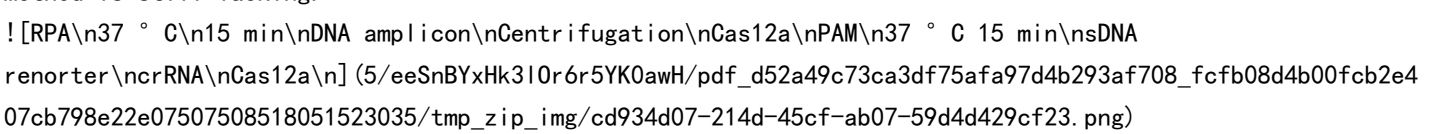
ABSTRACT: Rapid and sensitive method is crucial for nucleic acid detection. Recently, RNA-guided CRISPR/Cas12a nuclease-based methods present great promise for nucleic acid detection. In the present methods, however, DNA amplification and subsequent Cas12a cleavage is separated and the whole process takes as long as two hours. Most importantly, the uncapping operation increases the risk of aerosol contamination. In this study, we propose a CRISPR/Cas12a-based method named "Cas12aVDt" for rapid nucleic acid detection. By integrating recombinant polymerase amplification (RPA) with Cas12a cleavage in a single reaction system, the detection can be accomplished in 30 minutes and uncapping contamination can be

avoided. The detection signal can be observed by the naked eye under blue light. This method could detect DNA at single molecule level and demonstrated 100% accuracy for mycoplasma contamination detection, presenting great potential for a variety of nucleic acid detection applications.

Rapid and sensitive nucleic acid detection is critical for a variety of applications, such as point-of-care testing (POCT), pathogen detection, genotyping and general laboratory tasks.1-5 Recently, RNA-guided CRISPR/Cas nuclease-based nucleic acid detection methods have shown great promise for highly sensitive and rapid nucleic acid detection.6,7

The CRISPR/Cas nucleases, including two RNA-guided RNases (Cas13a and Cas13b) and two RNA-guided DNases (Cas12a and Cas14), display collateral cleavage activity.8-13 Upon recognition of their RNA or DNA targets, activated Cas nucleases indiscriminately cleave nearby single-stranded nontargeted nucleic acids.8,11 This property is exploited to detect the presence of a specific nucleic acid in vitro by non-specific degradation of reporter nucleic acids. Reporter nucleic acids are labeled with both a fluorophore and quecher. Collateral cleavage of the reporter nucleic acids results in release of the fluorophore from the quencher, leading to an increase in fluorescent signal. During nucleic acid detection, a small amount of the target sequence in the sample is first PCR-amplified, and then digested by Cas nucleases. Gootenberg et al. combined recombinase polymerase amplification (RPA) with Cas13a and established an integrated one-step diagnostic called SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) that allows highly sensitive and specific nucleic acid detection.8 As a RNase, Cas13a-based detection requires conversion of the amplified DNA to RNA by T7 RNA polymerase transcription. In contrast, Cas12a and Cas14 can directly digest DNA. Combined with nucleic acid amplification, Cas12a has been used to develop diagnostics called HOLMES (one-HOur Low-cost Multipurpose highly Efficient System) and DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter).11,12 However, in both systems, the amplification and detection processes are separated. More recently, Li et al.
integrated Cas12a and LAMP amplification into a single reaction system. However this method requires ~two hours for completion with a need to optimize the sensitivity as well 14.

Visual detection methods are vital for routine applications in nucleic acids detection. Gootenberg et al. developed "SHERLOCKv2", where they used lateral flow strips for visual readout. However, this system requires an additional step of transferring the sample to the test strip.9 Spoelstra et al. developed a turbidity-based visual readout method, allowing direct observation of the result in the tube. In this method, cleavage of long oligonucleotides caused the solution to change from turbid to transparent with the detection sensitivity limited at a micro-molar level.15 A more sensitive in-tube visual detection method is still lacking.



Scheme 1 Schematic of Cas12aVDet for rapid and visual nucleic acid detection. All reagents are mixed in a single reaction, except Cas12a enzyme, which is on the tube wall. After 15 minutes of RPA reaction, the Cas12a enzyme is centrifuged into the reaction mix for target cleavage for an additional 15 minutes. The fluorescent signals can be observed by the naked eye under blue light.

Here, we proposed a rapid and visual detection method that we named Cas12aVDet (Cas12a-based Visual Detection) by combination of RPA with Cas12a in a one-pot reaction. In this system, optimized RPA reagents and Cas12a enzyme are

put in one tube, but they are physically separated (Scheme 1). After 15 minutes of RPA reaction, Cas12a is added into the reaction to start the cleavage. The whole process takes ~30 minutes. Bright fluorescence generated by Cas12a digestion can be observed by the naked eye under blue light.

EXPERIMENTAL SECTION

Materials.

All primers were synthesized by GENEWIZ (Suzhou, China). And ssDNA (quenched fluorescent DNA reporter FAM-TTATT-BHQ1) was synthesized by Sangon Biotech (Shanghai, China). RNase inhibitor was purchased from TaKaRa Bio Inc. (Dalian, China). EnGenLba Cas12a, NEBuffer 2.1, NEBuffer 3.1 and HiScribeTM T7 High Yield RNA Synthesis Kit were purchased from New England Biolabs (Ipswich, MA UK). The TwistAmp Basic kit was purchased from TwistDx Ltd. (Hertfordshire, AL UK). The 2×SYBR Green qPCR Master Mix kit was purchased from Bimake (Houston, TX USA). RNAXP clean beads were purchased from Beckman Coulter Inc. (Indianpolis, IN USA). Other regents used in this study were purchased from Thermo Fisher Scientific Inc. (Waltham, MA USA).

dsRNA target and crRNAs preparation

Primers (target-T1-F and Target-T1-R) were annealed and cloned into the T-vector to form a target plasmid template. For crRNA preparation, the synthesized oligonucleotides containing T7 promoter, repeat and spacer sequences were annealed with a short T7 primer (T7-crRNA-F) to form the in vitro transcription templates. Then, the crRNAs were synthesized by incubating at 37 ° C for 4 h with the High Yield RNA Synthesis Kit. Synthesized crRNAs were treated with DNase and purified using RNAXP clean beads according to the manufacturer's protocol. All DNA sequences used in this assay were shown in table S1, ESI.

Standard RPA reaction assay

Primers for RPA reaction were designed with Primer Premier 5.0. The primer length was between 28 nt and 35 nt, and the expected amplicon size was under 500 bp. Standard RPA reactions was performed according to the instructions of the Twist-Amp basic kit. Each reaction contained 14.75 L of rehydration buffer, one pellet, 0.36 M forward and reverse primers, an appropriate amount of purified plasmid, 1.25 L of magnesium acetate (MgAc), and sterile water up to 25 L. The mixture was incubated in a conventional water bath at 37 ° C for RPA reaction. The amplified products were extracted using phenol/chloroform and electrophoresed on 2% agarose gel.

Cas12a-mediated cleavage assay

The Cas12a-mediated cleavage assay contained 500 nM crRNA, 250 nM Cas12a, 200 nM quenched fluorescent ssDNA reporter, 10 U of RNase inhibitor and 2 L of NEBuffer 3.1 with 3 L of amplified products in a 20-L reaction volume. The reaction was performed at 37 ° C for 30 min on a qPCR machine with fluorescence measurements taken every one minute.

Cas12aVDet method

Cas12aVDet combined the RPA reaction with Cas12a digestion in a one-pot reaction system. Briefly, the 25-L one-pot reaction assay consisted of 14.75 L of rehydration buffer, one pellet, 0.36 M forward and reverse primers, 500 nM crRNA, 200 nM quenched fluorescent ssDNA reporter, 10 U of RNase inhibitor, 2 L of NEBuffer 2.1, 0.53 L of MgAc, 1
L of target DNA, 250 nM Cas12a and sterile water up to 25 L. Cas12a was initially added on the tube wall. After RPA reaction for 15 min, Cas12a was centrifuged into the reaction solution, followed by incubation for another 15 min. Bright-green fluorescence signals could be observed by the naked eye under blue light using a Blue Light Gel Imager (Sangon Biotech. Inc., Shanghai, China; 440~485 nm).

Detection of mycoplasma contamination in cell culture with Cas12aVDt

1 mL of cell culture supernatant was collected in a 1.5-mL microcentrifuge tube and heated at 98 ° C for 2 min. After centrifugation, 2 L of supernatant at the bottom was collected and added into the prepared one-pot reaction system. The RPA reaction was performed at 37 ° C for 15 min, followed by Cas12a digestion at 37 ° C for another 15 min. The fluorescence signals were examined by the naked eye under blue light.

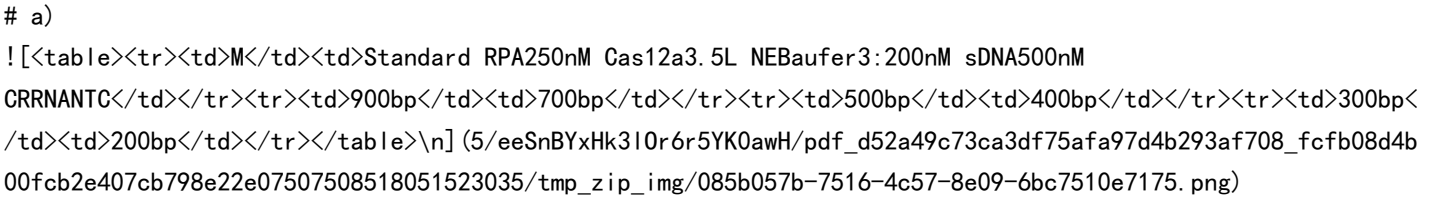
Real-time PCR detection

Primers of q-myco-F (CACCATCTGTCACTCTGTTAA) and q-myco-R (GGAGCAAACAGGATTAGATAC) were designed for qPCR amplification. The 25-L total reaction volume was made up of 12.5 L of 2×qPCR buffer, 400 nM of each primer, 2 L of heat-inactivated supernatant and sterile water up to 25 L.

RESULTS AND DISCUSSION

To prevent the uncapping operation, we attempted to integrate the RPA reaction with Cas12a-mediated cleavage in a single reaction system. Reagents of the RPA reaction include an enzyme pellet, rehydration buffer, primers and MgAc buffer, while reagents of Cas12a reaction include Cas12a enzyme, crRNA and labeled ssDNA. A previous study has shown that the detection signals could be observed in 15 minutes when amplified products were added in to Cas12a solution in NEBuffer 3.1.11 Therefore, we initially chose NEBuffer 3.1 for the one-pot detection system. One nanogram (equal to ~108 aM) of template DNA was used. However, after incubation of the RPA with Cas12a at 37 ° C for 30 minutes, there were no fluorescent signals (Figure S1a,ESI). Agarose gel electrophoresis revealed that there were no amplified products (Figure S1b,ESI).

a)



b)

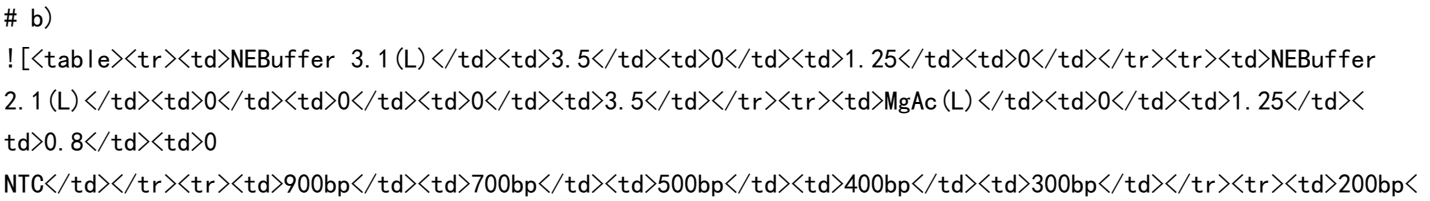
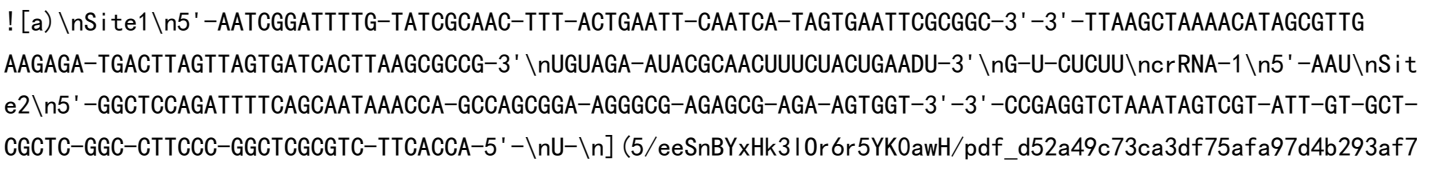


Figure 1 a) Agarose gel electrophoresis of RPA products with different components from Cas12a reaction. One nanogram of target plasmid was used as template. b) Gel electrophoresis of RPA products with different amounts of NEBuffer 3.1 or NEBuffer 2.1. One nanogram of target plasmid was used as template. NTC, negative control.

To find which components in the Cas12a reagents inhibited RPA, we added individual components of Cas12a reagents, including Cas12a, ssDNA and crRNA, into the standard RPA reaction system, one at a time. Agarose gel electrophoresis revealed that all samples contained the

expected amplified DNA bands (Figure 1a). Next, we investigated the effects of NEBuffer 3.1 on the RPA reaction. The RPA kit contained MgAcBuffer, where the Mg 2+ was essential for the initiation of the RPA reaction. Mg 2+ also existed in NEBuffer 3.1. To ensure a normal concentration of Mg 2+ was used in the RPA reaction, we reduced the MgAc buffer when NEBuffer 3.1 was used. Agarose gel electrophoresis revealed that there were no amplified DNA bands, indicating that NEBuffer 3.1 inhibited the RPA reaction (Figure 1a). Next, we reduced the NEBuffer 3.1 from 3.5 L to 1.25 L, resulting in amplified DNA bands (Figure 1b). When NEBuffer 2.1 was used, amplified DNA bands were also observed (Figure 1b). Therefore, we used the NEBuffer 2.1 in the following experiments.

Next, we optimized the amount of the NEBuffer 2.1 with a consistent concentration of Mg^{2+ supplement for the RPA reaction. A very "low" amount of plasmid (10^{-4 ng) was used as template so that the variation of amplified products could be readily observed. After RPA amplification at 37 ° C for 15 minutes, amplified products of each sample were electrophoresed. The results showed that the optimal amount of NEBuffer 2.1 was 2 L in a 25 L reaction volume (Figure S2,ESI).



08_fcfb08d4b00fcb2e407cb798e22e07507508518051523035/tmp_zip_img/6904e789-4df4-4c3a-b63f-3a2fcad43be0.png)
Figure 2 Two examples of Cas12aVDet detection. a) The sequences of crRNA1 and crRNA2 for the two target sites. The target sites are highlighted in blue, and PAM sequences are marked in red. b) Top row: quantification of maximum fluorescent signals generated after adding Cas12a into the reaction solution by centrifugation. (n=3, error bars showed mean± SEM). Bottom row: visual detection under blue light, photographed by smart phone.

Next, we integrated the optimized RPA reaction with Cas12a cleavage in a one-pot reaction system. Four concentrations of DNA template were used (10^{-3, 10^{-2, 10^{-1 and 10^{0 ng). Reactions were incubated in a PCR machine for up to 150 minutes at 37 ° C with fluorescence measurements taken every one minute. However, very weak fluorescent signals were generated in the three samples with lower amounts of DNA. When 10^{-1 ng of DNA was used, it took ~90 minutes to generate ~500 RFU, which was the minimal signal that could be visualized (Figure S3,ESI). When 10^{-2 or 10^{-3 ng of DNA was used, the RFU values were less than 500 after 150 minutes of incubation (Figure S3,ESI). We hypothesized that
the template DNA was digested by Cas12a, leading to inefficient RPA reaction. To solve this problem, we added Cas12a on the tube wall. After 15 minutes of RPA reaction, Cas12a was centrifuged to mix it into the reaction, and the sample was incubated for an additional 15 minutes. When 10^{-4 ng of template DNA was used, robust fluorescent signals were visualized (Figure S4a, ESI). Agarose gel electrophoresis revealed that RPA generated the amplified products, which were cleaved into two bands (Figure S4b, ESI). We chose two additional target sites on the plasmid to test this detection method (Figure 2a). When 10^{-4 ng of DNA was used, robust fluorescent signals were visualized for both target sites (Figure 2b). Hereafter this detection strategy was named Cas12aVDet.

Next, we compared the sensitivity of the Cas12aVDet method with the RPA-only method and the DETECTR method. In the DETECTR assay, the samples were first amplified by RPA, and then the amplified products were transferred into the Cas12a solution for cleavage. The fluorescent signals were monitored by a fluorescence plate reader. We employed 10-fold serial dilutions of plasmid DNA as templates. Agarose electrophoresis revealed that the limit of detection (LOD) of the RPA was 10^3 aM (Figure 3a). The DETECTR method was much more sensitive than only RPA, with a LOD of 10 aM (Figure 3b). The sensitivity of the Cas12aVDet method was equal to the DETECTR method (Figure 3c, Figure S5,ESI). The concentration of plasmid dilutions was verified by digital-droplet PCR (ddPCR) (Figure S6,ESI)).

a)

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b)

![|DNA concentration
(aM)|Fluorescence|\n|:---:|:---:|\n|10^7|4500|\n|10^6|4100|\n|10^5|4400|\n|10^4|3000|\n|10^3|2200|\n|10^2|2300|\n|10^1|1700|\n|10^0|300|\n|N/C|0|](5/eeSnBYxHk3lOr6r5YK0awH/pdf_d52a49c73ca3df75afa97d4b293af708_fcfb08d4b00fcb2e407cb798e22e07507508518051523035/tmp_zip_img/733065f6-f71a-4097-b2e7-60cd5be8c51e.png)

c)

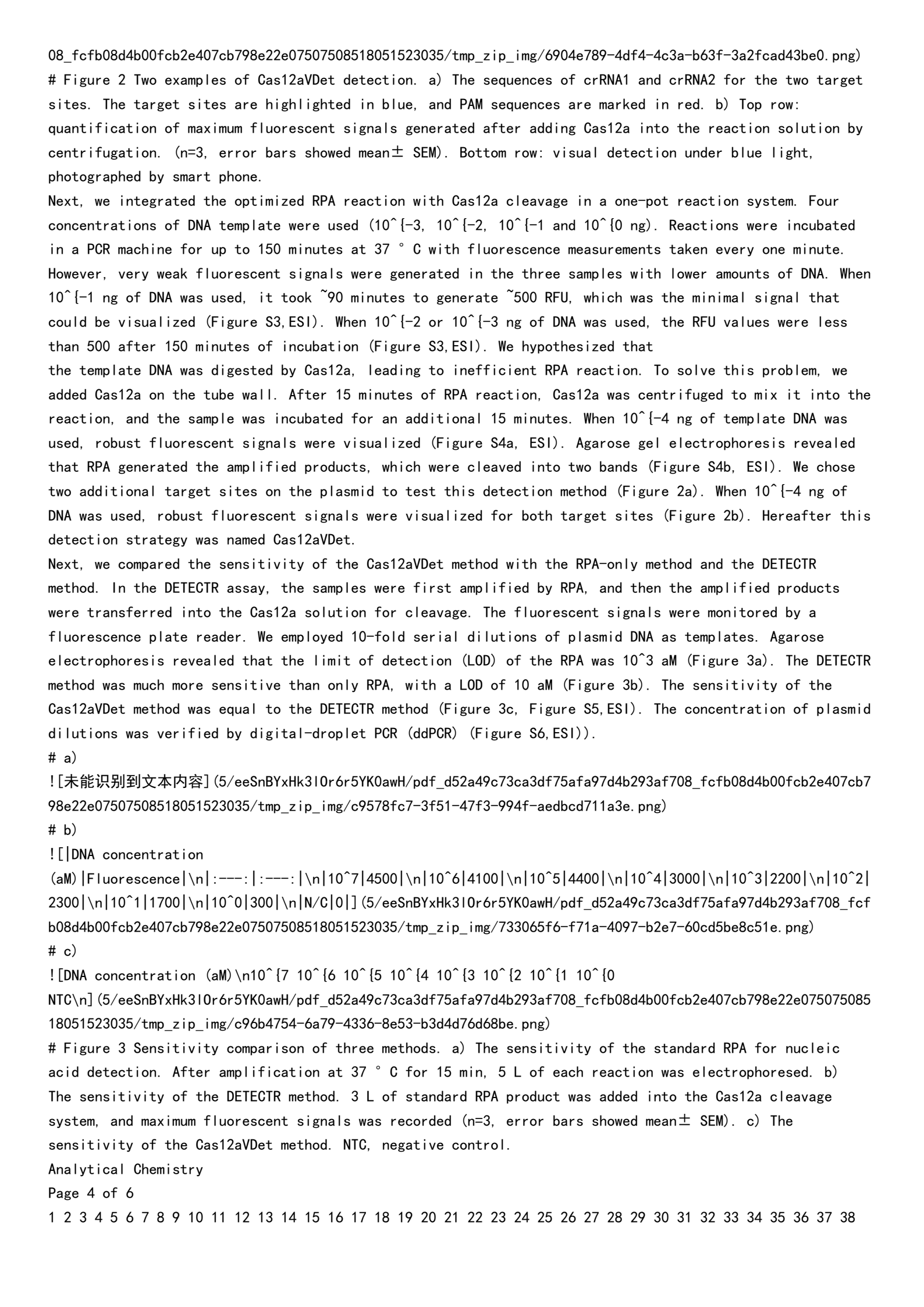


Figure 3 Sensitivity comparison of three methods. a) The sensitivity of the standard RPA for nucleic acid detection. After amplification at 37 ° C for 15 min, 5 L of each reaction was electrophoresed. b) The sensitivity of the DETECTR method. 3 L of standard RPA product was added into the Cas12a cleavage system, and maximum fluorescent signals was recorded (n=3, error bars showed mean± SEM). c) The sensitivity of the Cas12aVDet method. NTC, negative control.

39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

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Figure 4 Mycoplasma detection in 20 potentially contaminated cell culture samples with the Cas12aVDet and real-time PCR.

Then, we compared the sensitivity of fluorescence-based readout to that of the turbidity-based readout. Turbidity arises from coacervates of positively charged polyelectrolytes with long poly(dT) or poly(U) oligonucleotides. In the presence of a target sequence, long oligonucleotides are progressively shortened, causing the solution to go from turbid to transparent.15 Initially, we incubated ten-fold serial dilutions of target DNA with Cas12a for one hour, but the turbidity change could not be detected (Figure S7a, ESI). We subsequently extended the cleavage duration to four hours, and observed a turbidity change with 10 M of target DNA (Figure S7b,ESI). In contrast, fluorescence was detectable with only 10-3 M target DNA within 30 minutes of cleavage (Figure S7c,ESI). These data demonstrated that fluorescence-based readout is significantly more sensitive than the turbidity-based readout.

Finally, we tested Cas12aVDet for detection of mycoplasma contamination in cell culture. Mycoplasma contamination remains a major problem in cell culture. The contamination rate has been reported to be between 15-30% and could be as high as 70%.16-18 It alters the cellular characteristics, resulting in spurious experimental data and potentially unsafe biological products.19,20 Therefore, a rapid and convenient mycoplasma detection method would be desirable.

We first employed standard PCR to screen out one positive sample as a positive control (named P), and the screen

result was furthered verified by DNA sequencing (Figure S8a-b, ESI). We next designed four pairs of primers targeting the conserved region of the 16s rRNA gene of mycoplasma for RPA, and three of them resulted in strong amplified DNA bands (Figure S9a, ESI). We chose one pair of primers for the Cas12aVDet. Cas12a target sequences require the TTTN PAM sequence, which does not exist in the tested region. We introduced the PAM sequence in the primers. The Cas12aVDet detection resulted in a very strong fluorescent signals for the sample P, demonstrating that the conditions used here worked well for mycoplasma detection (Figure S9b, ESI).

We collected 20 potentially contaminated samples of cell culture supernatant, and tested the mycoplasma contamination by both Cas12aVDet and real-time PCR. The Cas12aVDet results were consistent with the real-time PCR results, with 11 of 20 samples showing contamination (Figure 4, Figure S10,ESI). At the same time, standard PCR amplification was carried out as control (Figure S11,ESI). The advantage of the Cas12aVDet for mycoplasma detection is that it is very convenient and rapid. Users add the samples into the tube, and the results can be visualized in half an hour.

Both RPA and Cas12a reagents are commercially available. RPA reagent is stable for half a year at -20 ° C, while Cas12a reagent is stable for two years at -20 ° C. crRNA and ssDNA reporter are stable for at least half a year at -80 ° C.

After storage period of 6 months, crRNA and ssDNA reporter remained effective for mycoplasma detection in our hands.

To render the proposed method feasible for practical application, a portable and contamination preventing cartridge was used for RPA amplification and visual detection.21 Cas12a and RPA reaction system were pre-added in two connected tubes. After RPA amplification, the RPA reaction system and Cas12a is mixed homogenously and incubated for another 15 mins to allow for Cas12a cleavage. Fluorescent results are directly visible by the naked eye under blue light. As displayed in Figure S12, fluorescence signals were

generated in the positive samples but not in negative controls.

In summary, we developed a rapid and visual method for nucleic acid detection. By integrating RPA with Cas12a cleavage in a single reaction, the fluorescent signals can be visualized in half an hour. The sensitivity of our method is comparable to the DETECTR method.10 Cas12aVDet only requires a portable heater for the reaction and a blue light for visualization.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Keywords: Cas12a; recombinantase polymerase amplification (RPA); visual detection; nucleic acid detection