Rapid detection of multiple phytoplasma with an All-In-One Dual (AIOD) CRISPR assay

Peng Chen[1], Qicheng Li[1], Jian Jiao[1], Qiqi Yang[1], Sicheng Guo[1], Jia Yao[1], yun Chen[1], Xia Ye[1], Jun Cheng[1], Bin Tan[1], Xianbo Zheng[1], Yingxia Wu[1], Hong Cai[2], Yunfeng Wu[3], Huiyu Wang[4], Jidong Li[1], and Jiancan Feng[1]

[1]Henan Agricultural University
[2]Yunnan Agricultural University
[3]Northwest Agriculture and Forestry University
[4]Zhengzhou Normal University

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Abstract

Phytoplasma can infect thousands of plants and caused huge economic losses around the world. The large-scale spread and serious lethality of phytoplasma prompt the urgent need for sensitive, accurate, visual and rapid detection of these pathogens. Current molecular assays used for detecting phytoplasma are expensive and time consuming. Here, we established a novel All-In-One Dual (AIOD) CRISPR detection platform based on CRISPR/LbCas12a technology and Recombinase Polymerase Amplification (RPA) for the diagnosis of multiple phytoplasma. The protocol is simple, requiring one vessel, rapid and sensitive, and the output is visual. Cas12a/crRNAs complexes are added into a reaction containing RPA Mix, RPA Primers and single-stranded DNA fluorophore-quencher (ssDNA-FQ). All components, including 1 μL of sample DNA, are added together and then incubated in one tube at 37 °C. Phytoplasma was detected after 15 min or less from leaf harvest. Positive results can be observed by the naked eye via fluorescent signals. We optimized the amounts of crRNA, LbCas12a and the ssDNA fluorophore in the detection system. Finally, an optimized system was established containing 1,000 nM ssDNA-FQ and a 2:1:1 ratio of LbCas12a/crRNA1/crRNA2 complex with a 0.8 μM concentration as 1. In the optimized reaction, the AIOD-CRISPR detection system exhibited high sensitivity, with limits of detection reaching 3.37E + 2 copies of phytoplasma DNA per reaction. Field tests indicated the AIOD-CRISPR detection system possessed high specificity and reached the 100% accuracy when compared with PCR detection. In conclusion, the AIOD-CRISPR detection system is a ideal selection with high specificity and sensitivity for phytoplasma detection. Our work provides a technique that can be potentially used to rapidly and simultaneously detect more pathogens.

Peng Chen[1+], Qicheng Li[1+], Jian Jiao[1+], Qiqi Yang[1], Sicheng Guo[1], Jia Yao[1], yun Chen[1], Xia Ye[1], Jun Cheng[1], Bin Tan[1], Xianbo Zheng[1], Yingxia Wu[1], Hong Cai[2], Yunfeng Wu[3], Huiyu Wang[4], Jidong Li[1*], Jiancan Feng[1*].

1 Henan Agricultural University, 95 Wenhua Road, Zhengzhou 450002, China; *515162318@qq.com* (P.C.); *513342648@qq.com* (Q.L.);*jiaojian@henau.edu.cn (J.J.); 505816969@qq.com* (Q.Y.);*guofourx@163.com* (S.G.); *205239413@qq.com (J.Y.);sweetchenyun@163.com* (Y.C.); *xye@henau.edu.cn*

(X.Y.);*jcheng2007@163.com* (J.C.); *tanbin166@163.com* (B.T.);*XianboZ@163.com* (X.Z.); *wyxgwy@163.com* (Y.W.);

2 Yunnan Agricultural University, 452 Fengyuan Road, Kunming 65227165, China; *caihong0623@126.com* (H.C.) 3 Northwest A&F University, 3 Taicheng Road, Yangling 712100, China;*wuyf@nwsuaf.edu.cn* (Y.W.)4 Zhengzhou Normal University, 6 Yingcai Street, Zhengzhou 450044, China; *714347658@qq.com* (H.W.)+ Peng Chen, Qicheng Li and Jian Jiao contributed equally to this work. * Correspondence: lijidong@henau.edu.cn; Tel.: +86-371-63558070; jcfeng@henau.edu.cn; Tel.: +86-371-63579621;

Running title: All-In-One Dual CRISPR detection of phytoplasma

Abstract

Phytoplasma can infect thousands of plants and caused huge economic losses around the world. The large-scale spread and serious lethality of phytoplasma prompt the urgent need for sensitive, accurate, visual and rapid detection of these pathogens. Current molecular assays used for detecting phytoplasma are expensive and time consuming. Here, we established a novel All-In-One Dual (AIOD) CRISPR detection platform based on CRISPR/LbCas12a technology and Recombinase Polymerase Amplification (RPA) for the diagnosis of multiple phytoplasma. The protocol is simple, requiring one vessel, rapid and sensitive, and the output is visual. Cas12a/crRNAs complexes are added into a reaction containing RPA Mix, RPA Primers and single-stranded DNA fluorophore-quencher (ssDNA-FQ). All components, including 1 μL of sample DNA, are added together and then incubated in one tube at 37 °C. Phytoplasma was detected after 15 min or less from leaf harvest. Positive results can be observed by the naked eye via fluorescent signals. We optimized the amounts of crRNA, LbCas12a and the ssDNA fluorophore in the detection system. Finally, an optimized system was established containing 1,000 nM ssDNA-FQ and a 2:1:1 ratio of LbCas12a/crRNA1/crRNA2 complex with a 0.8 μM concentration as 1. In the optimized reaction, the AIOD-CRISPR detection system exhibited high sensitivity, with limits of detection reaching $3.37E + 2$ copies of phytoplasma DNA per reaction. Field tests indicated the AIOD-CRISPR detection system possessed high specificity and reached the 100% accuracy when compared with PCR detection. In conclusion, the AIOD-CRISPR detection system is a ideal selection with high specificity and sensitivity for phytoplasma detection. Our work provides a technique that can be potentially used to rapidly and simultaneously detect more pathogens.

Key words: All in one; CRISPR; Limit of Detection; Phytoplasma; ssDNA-FQ; Visual detection;

1 Introduction

Phytoplasmas are wall-less, obligate bacterial pathoens that belong to the class Mollicutes. Phytoplasmas threaten more than 1,000 cultivated plant species, causing serious economic losses worldwide (Bertaccini et al. 2014; Hoshi et al. 2009; Namba et al. 2019). Phytoplasma-infected plants show multiple abnormal phenotypes such as witches' broom, phyllody, dwarfism and produce more smaller leaves (Chen et al., 2022). Phytoplasma are exclusively parasitic in plant phloem, where they absorb essential compounds, and are difficult to culture artificially. Although some studies have shown that tetracycline can effectively eliminate phytoplasma, for instance in diseased jujube (*Ziziphus jujuba*) seedlings grown *in vitro* (Wang et al., 2018), there is still no effective treatment for phytoplasma-infected plants, which are generally destroyed. Research has proved that phytoplasma can migrate regularly in host plants (Zhao et al., 2006). A large number of phytoplasmas have colonized in the plant before the symptoms emerge(Chen et al., 2019; Ye et al., 2017; Wang et al., 2018). Therefore, establishment of a rapid and accurate detection system for phytoplasma can aid in the control of these microorganisms.

Phytoplasma are difficult to culture artificially, thus cannot be characterized by the traditional techniques used for bacterial classification (Bove and Garnier, 1998). Current strategies in detection, classification, and phylogenetic analysis of phytoplasma are mainly based on nucleic acid-based detection methods, such as Restriction Fragment Length Polymorphism (RFLP) marker analysis and sequence analysis of the 16SrRNA

gene, which is highly conserved in different phytoplasma (Hodgetts et al., 2008; Lee et al., 1998). The existing nucleic acid detection methods for phytoplasma are highly sensitive. However, most of the diagnostic assays are time consuming, expensive and being inhibited by compounds in crude plant extracts and susceptibility to nonspecific amplification (Schrader et al., 2012). In addition to traditional PCR methods, isothermal amplification techniques, such as Recombinase Polymerase Amplification (RPA, Lee et al. 2021) and loop-mediated isothermal amplification (LAMP, Sui et al. 2018), do not require expensive equipment, but may produce false positive results under constant temperature amplification conditions.

Recently, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) nuclease-based nucleic acid detection has been used as a novel molecular diagnostics technology to perform pathogen diagnostics (Chertow et al. 2018; Li et al. 2019; Myhrvold et al., 2018). Several Cas endoribonuclease systems, such as LbCas12a, Cas13a and Cas14, can be used for nucleic acid detection. LbCas12a possesses DNase activity and can be activated by DNA targets. LbCas12a can cut arbitrary ssDNA indiscriminately when the LbCas12a/CrRNA/target DNA ternary complex is formed. Chen established the DETECTR (DNA endonuclease targeted CRISPR trans reporter) method, by integrating Cas12a ssDNase activation with RPA reaction to sensitively detect DNA (Chen et al., 2018). Matthew successfully detected the 'Candidatus Phytoplasma trifolli' with Cas12a-based diagnostics (Matthew et al., 2022). A specific and sensitive method for the diagnosis of the five most-prevalent RNA viruses/viroids in apple uses a CRISPR/LbCas12a system (Jiao et al., 2020). Cas13a also possesses a similar, nonspecific cleavage activity. Unlike Cas12, Cas13 cleaves RNA after recognizing the specific RNA targets. The researchers combined these enzymes with quenched fluorescent reporters and nucleic acid amplification steps to establish a nucleic acid detection platform called SHERLOCK (Gootenberg et al., 2017). The Cas14 protein is the smallest Cas protein. A Cas14a-mediated nucleic acid detection platform (CMP) proved to be highly sensitive and specific and uses fluorescence for bacterial detection (Ge et al. 2021).

Using CRISPR-based nucleic acid detection shows great prospects in the molecular diagnosis of viral and bacterial diseases. An All-In-One Dual (AIOD) CRISPR-LbCas12a assay method was established for simple, rapid, ultrasensitive, single vessel, visual detection of coronavirus SARS-CoV-2 and HIV virus in 2020 (Ding et al. 2020). Although CRISPR technology has been applied to phytoplasma diagnosis, these systems have only been developed for the detection of 'Candidatus Phytoplasma trifolli' (Wheatley et al., 2022). In this study, a specific and sensitive AIOD-CRISPR system for diagnosis of multiple phytoplasma species was established by combining with RPA. The limit of detection (LOD) of our AIOD-CRISPR detection system can reach 3.37E+2 copies per reaction, and takes no more than fifteen minutes, results are observe with the naked eye. Our work contributes to a better understanding of how to design and develop CRISPR/LbCas12a assays for phytoplasma detection and directly provides a technique that can be used to rapidly and simultaneously detect multiple phytoplasma.

2 Experimental methods

2.1 Plant collection and DNA extraction

Plant materials suspected to carry phytoplasma, including *Trema tomentosa* , *Crotalaria juncea,Crotalaria pallida* ,*Cajanus scarabaeoides,Justicia gendarussa,Vigna unguiculata,Arachis hypogaea,Lycoperisicon esculentum,Raphanus sativus,Parthenium hysterophorus* and *Catharanthus roseus* , were collected in Kunming, Yunnan Province, China, *Triticum aestivum* was collected in Hancheng, Shannxi Province, China, *Ziziphus jujuba* ,*Paulownia fortunei, Prunus persica* and *Bambusoideae* were collected in Henan Province, China.

Total DNA of the above samples was extracted with CTAB methods (Chen et al., 2019). Conventional PCR with universal primers (R16F2n/R16R2) of phytoplasma 16S rDNA genes (Gundersen et al., 1996; Lee et al., 1995) was performed to detect the presence of phytoplasma in each sample.

2.2 RPA primer and crRNA design

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We generate an AIOD-CRISPR assay system according to previous studies (Yang et al. 2016; Ding et al. 2020). The spacer sequences of the crRNAs used for specific recognition of phytoplasmas were designed according to the highly conserved domain of the phytoplasma 16S rDNA sequences (Fig. S1). The 16SrRNA gene of jujube witches' broom (JWB) phytoplasma was used as the probe for BLAST in NCBI database (https://preview.ncbi.nlm.nih.gov/). A total of 74 sequences were downloaded and aligned with DNAMAN software. RPA primers were designed from the conversed nucleotide region according to the multiple sequence alignment (Fig. S1).

The 200-bp conserved gene fragment was selected for designing the RPA primers and the crRNAs for the AIOD-CRISPR assay (Fig. S2). The conserved fragment was used as probe to perform BLAST search in the NCBI database to confirm the specificity of the phytoplasma sequence. This domain must be included in the amplification region of the RPA primers (Fig. S2). The designed crRNA sequences were also checked against the available sequences to ensure that it was only belong to the phytoplasma sequence. All of them were annotated as phytoplasma 16SrRNA sequences.

2.3 Generation of phytoplasma crRNA and DNA standards

The method for crRNA synthesis followed Jiao et al. (Jiao et al., 2020). To prepare the crRNA, DNA templates encoding crRNA were prepared using a crRNA-F/crRNA-R primers (Table S1) that gradiently annealed (1 per minute) from 95 to 25 to form a 40-nt target dsDNA. The annealed products were transcribed using a TranscriptAid T7 High Yield Transcription Kit, according to the manufacturer's protocol (Thermo Fisher Scientific). crRNA was then purified with RNA Clean & Concentrator™-5 Kit. Nucleotide sequences harboring the targets of the RPA primers and the crRNA were first PCR-amplified from the total DNA of phytoplasma-infected jujube leaves with the RPA primers then fused to the pEASY-T vector (TransGen Biotech Ltd. Beijing, CHINA) and verified by sequencing.

2.4 All-in-one CRISPR assay

The All-in-one Dual (AIOD) CRISPR system was designed and performed as previously reported (Ding et al. 2020). The detection system consisted of part A, part B and part C. Part A was the reaction solution for Recombinase Polymerase Amplification (RPA), Part B and Part C were mainly responsible for recognition, trans-cleavage and visual detection.

Part A contained RPA Primers (Table S1) , reaction Buffer, Basic E-mix , dNTPs and MgOAc. Part B consisted of ssDNA fluorophore-quencher (ssDNA-FQ) reporter and 1× core Reaction Mix. Part C consisted of crRNAs (crRNA2 and crRNA3; Table S1), Cas12a and target solution. Finally, solutions A, B, and C were mixed to form a 25-μL reaction system. The reactions were performed at 37 °C before fluorescence observation by endpoint imaging and UV visual detection.

2.5 Optimization of the experimental condition for AIOD-CRISPR visual detection system

The effects of different crRNAs on the detection efficiency of the AIOD-CRISPR system were determined in this assay. Four reaction systems (test 1 - 4) with various crRNA combinations were generated. In test1, the RPA assay was carried out with the PRA-F1/RPA-R1 primer pair, and the recognition site of crRNA1 was not adjacent to the RPA primers site. Test 2, 3 and 4 contained the RPA-F2/ PRA-R2 primer pair. Two crRNA (crRNA2 and crRNA3) were added in test 2, while crRNA2 or crRNA3 were added alone in test 3 and test 4, separately. After incubation at 37 °C for 15 min and 25 min, the fluorescence signal was observed with the naked eye and the fluorescence intensity were recorded with a SpectraMax M2 microplate reader (Molecular Devices, Shanghai).

The fluorescent ssDNA, LbCas12a and crRNAs are essential components in the detection system and their concentrations were optimized to improve the sensitivity and accuracy of the detection system. Eight AIOD-CRISPR reactions with different concentrations of ssDNA-FQ (4000 nM, 2000 nM, 1000 nM, 500 nM, 250 nM, 125 nM, 62.5 nM and 0 μM) were tested. The ratio between LbCas12a, crRNA1 and crRNA2 was set at 2:1:1 according to previous reports (Ding et al., 2020). A series of AIOD-CRISPR reactions with different quantities of LbCas12a/crRNA1/crRNA2 complex were tested, with LbCas12a/crRNA1/crRNA2

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concentrations of 2.24 µM:1.12 µM:1.12 µM, 1.92 µM:0.96 µM:0.96 µM, 1.6 µM:0.8 µM:0.8 µM, 1.28 µM:0.64 µM:0.64 µM, 0.96 µM:0.48 µM:0.48 µM, 0.68 µM:0.34 µM:0.34, µM, and 0.34 µM:0.17 µM:0.17 µM.

2.6 Sensitivity and specificity assay of the AIOD-CRIPSR phytoplasma detection system

The sensitivity of the AIOD-CRISPR detection system was determined using different concentrations of pEASY-DNA plasmid templates. The recombinant pEASY-DNA-16SRNA plasmid was serially diluted to concentrations ranging from 3.37E+8 to 3.37E+2 copy number. Then the AIOD-CRISPR assay was carried out using the optimized reaction conditions. To further evaluate the limit of detection (LOD) with the AIOD-CRISPR detection system, different concentrations of plasmid templates were also detected by PCR and fluorescence microplate reader.

To determine the specificity of the detection system, sixteen plant samples suspected of carrying phytoplasma and healthy plant tissues were collected for AIOD-CRISPR detection using the optimized reaction conditions. All of the plant samples were also tested by PCR. To further evaluate the practicality of AIOD-CRISPR phytoplasma detection in the field, forty jujube seedlings were randomly sampled and tested using both the AIOD-CRISPR system and PCR assay.

3 Results

3.1 Validation of AIOD-CRISPR detection system
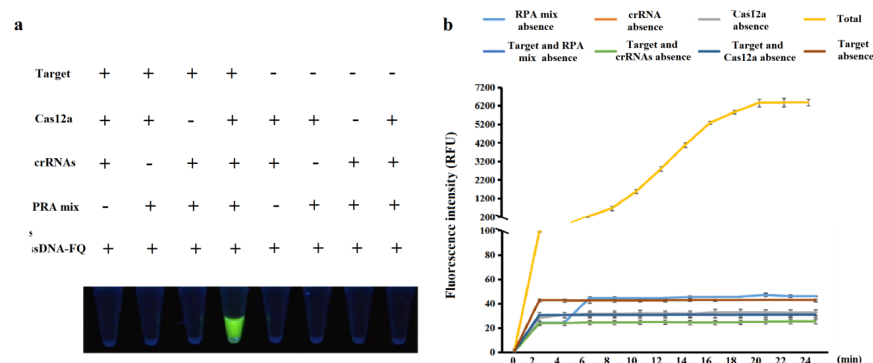
To evaluate our AIOD-CRISPR assay, we prepared and tested different reaction systems with various components (Fig. 1). A plasmid containing a 1,200-bp fragment of the JWB phytoplasma *16SrRNA* gene was used as the target sequence. The activity and visual signal of the ssDNA-FQ were detected. Results showed that only reactions containing all components (target sequence, Cas12a, crRNAs and RPA Mix) produced a visibly obvious, bright fluorescence signal after incubation at 37 (Fig. 1). The fluorescence signal was almost invisible when the detection system lacked any one component, such as LbCas12a, crRNA or target DNA (Fig. 1a). Real-time fluorescence showed a significantly increased fluorescence signal within 5 min only when all components were added to the reaction system (Fig. 1b). Results suggested that this AIOD-CRISPR assay can successfully detect the phytoplasma target nucleic acid within 15 min.

Fig. 1. Evaluation of AIOD-CRISPR reaction components containing various components. Reactions were tracked using **(a)** endpoint visual fluorescence detection and imaging and **(b)** real-time fluorescence detection. Reaction components as listed in Fig.1a.

3.2 Optimization of AIOD-CRISPR assay

We first evaluated the effect of different recognition sites on the phytoplasma-detecting efficiency. Four reactions (Test 1-4) containing various RPA primers and crRNAs were prepared (Fig. 2a). In test 1, the

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RPA assay was carried out with the PRA-F1/RPA-R1 primer pair and the recognition of crRNA1 was not adjacent to the RPA primers recognition sites in the target sequence, so the crRNA1 then requires a PAM motif in the target sequence to active the Cas12a protein. Fluorescence signal was detected in this test, but a crRNA primer not dependent on a PAM motif is preferred. If the crRNA is adjacent to the RPA primer, no PAM motif is needed, as shown design for test 2, 3, and 4 in Fig. 2a. Test 2 contained two crRNAs adjacent to the RPA primers, test 3 and 4 each contained the single crRNA2 or crRNA3, respectively. All the sequences of crRNAs and their targets were shown in Fig. S2.

After incubation at 37 °C for 15 min, test 2 produced a visible and bright fluorescence signal, directly observable under UV light. The fluorescence signals of test 3 and 4 was difficult to distinguish from reactions lacking the target. All assays showed a change from colorless to green that was visible by the naked eyes 25 min after reaction, but test 2 showed an elevated fluorescence signal compared to test 3 and 4 (Fig. 2b) which was consistent with the visual detection results (Fig. 2c). These results indicated that the AIOD-CRISPR assay system can detect the phytoplasma target sequence and that the use of two crRNAs can greatly improve the intensity of the reaction when compared with those containing a single crRNA. Interestingly, test 1 showed a florescence intensity compared to test 3 and 4 (Fig. 2b), reaching 1500 RFU within 15 min and exceeding 4000 RFU within 25 min (Fig. 2c). Although the AIOD-CRISPR system can detect the phytoplasma successfully without the PAM sequence (Test 2), the PAM sequence does improve the detection efficiency by enhancing the activity of Cas12a when only one crRNA is used. In sum, test 2 offered the best detection system.
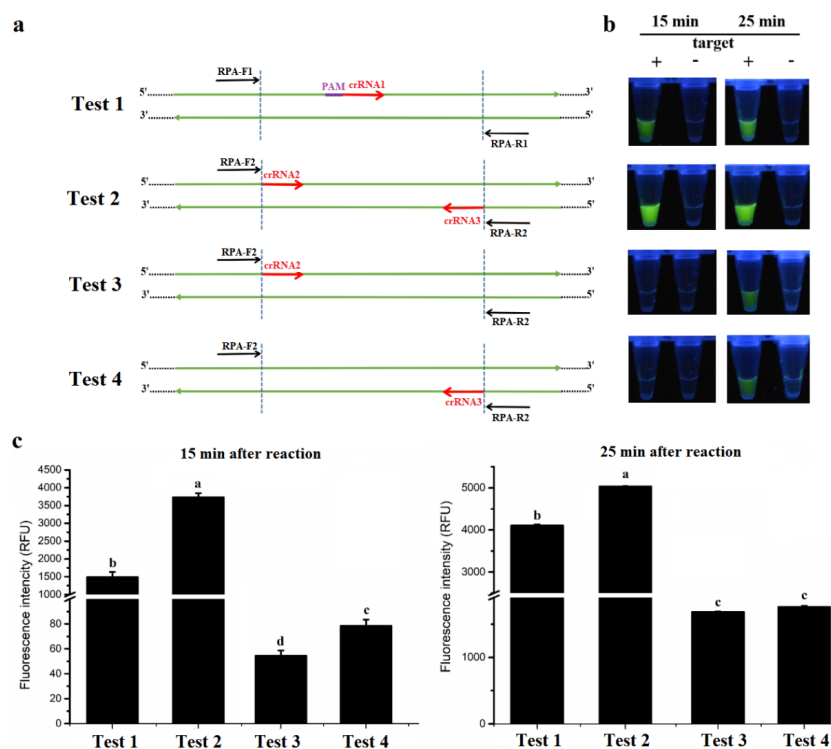

Fig. 2. The efficiency of different primer and crRNA combinations on AIOD-CRISPR reaction

(a) Four AIOD-CRISPR reactions were prepared . Test 1, a single crRNA targeting a site with a PAM motif in the middle of the RPA amplification product. Test 2, two crRNAs without PAM motifs are closely adjacent to the recognition sites of the RPA primers. Test 3 and Test 4, single crRNA without PAM motif, the same individuals as in Test 2.**(b)** Visual fluorescence detection of the AIOD-CRISPR reactions.**(c)** Real

time fluorescence detection for phytoplasma.

Based on the detection system with two crRNAs, we further explored the optimal contents of ssDNA-FQ, LbCas12a and crRNAs (Fig. 3). We determined the dosage of ssDNA-FQ that would create a visual fluorescence intensity detectable by both LED and UV visual detection and real-time fluorescence detection (Fig. 3a, 3b). The higher the concentration of the ssDNA-FQ reporter, the shorter the threshold time in real-time fluorescence detection (Fig. 3b). Consistent with the changes in absorbance, the color transition was distinctly observed when the dosage of ssDNA-FQ was 4,000 nM, 2,000 nM and 1,000 nM (Fig. 3a). The fluorescence signal can still be easily distinguished with the naked eye when the concentration of ssDNA-FQ was 1,000 nM. For better visual colorimetric detection, 1,000 nM ssDNA-FQ was the best choice.

After optimizing the concentration of the ssDNA-FQ, the concentration of the LbCas12a/crRNA complex was optimized in assays using ssDNA-FQ at the optimized dose (1,000 nM). For endpoint imaging and UV visual detection, the fluorescence brightness was remarkably changed when the ratio of LbCas12a/crRNA2/crRNA3 complex was 1.6 μM: 0.8 μM: 0.8 μM (Fig. 3c). The result was further confirmed by real-time fluorescence detection (Fig. 3d). Therefore, an optimized system was established containing 1,000 nM ssDNA-FQ and a 2:1:1 ratio of LbCas12a/crRNA1/crRNA2 complex with a 0.8 μM concentration as 1.
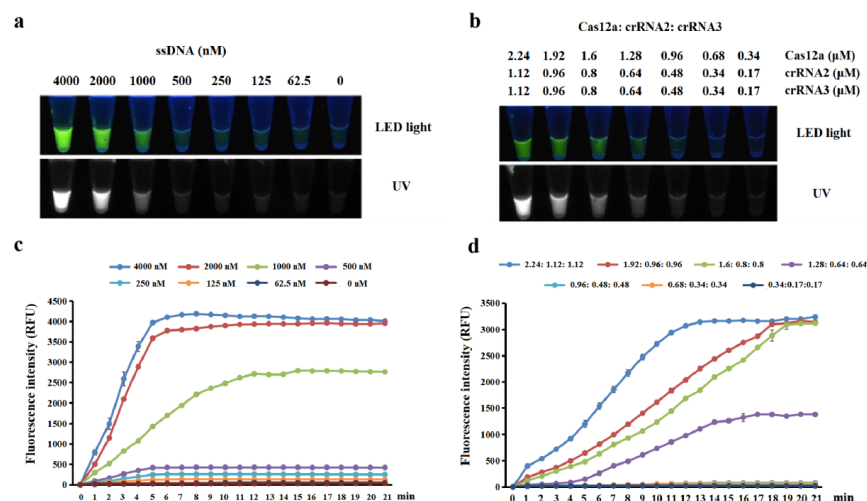

Fig. 3. Concentration optimization of ssDNA-FQ fluorescent molecules and Lb-Cas12a/crRNA1/crRNA2 complex in AIOD-CRISPR detection system

(**a**) Concentration gradient of ssDNA-FQ by endpoint imaging (at 15 min) under blue LED and UV light. (**b**) Concentration gradient of ssDNA-FQ by real-time fluorescence detection. (**c**)Concentration gradient of LbCas12a/crRNA1/crRNA2 complex as detected by endpoint imaging under blue LED and UV light. (**d**)Concentration gradient of LbCas12a/crRNA1/crRNA2 complex by real-time fluorescence detection.

3.3 Sensitivity of the AIOD-CRISPR system

The minimum detection limit of the LbCas12a/crRNA assay was initially determined using 10-fold gradient dilutions of pEASY-*16SrRNA*clones. At the optimum reaction conditions, visible fluorescence signals were observed with around 3.37E+2 copies per reaction (Fig. 4b), reach the same level as PCR detection (Fig. 4a). Consistent with the color transition in the endpoint imaging, the fluorescence intensity was significantly higher than control when the copy number of the pEASY-*16SrRNA* clone was above 3.37E+2 in real-time fluorescence detection (Fig. 4c). In sum, we confirmed that the LOD of the AIOD-CRISPR assay for phytoplasma detection was as low as 3.37E+2 copies per reaction when using the optimized detection system.

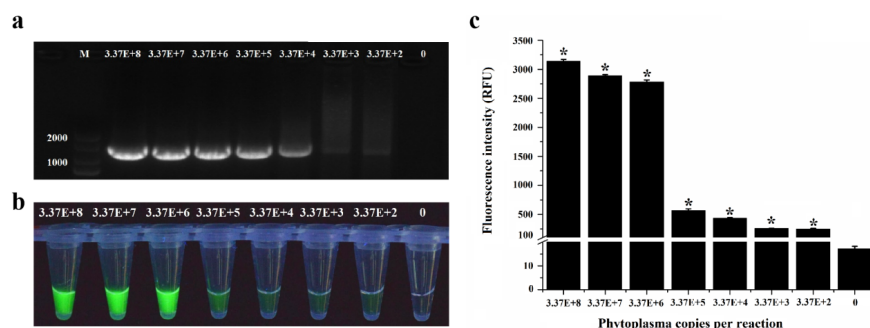**Fig. 4. Limit of Detection (LOD) of PCR and AIOD-CRISPR system for plasmid containing phytoplasma *16SrRNA* detection**

a: LOD of PCR assay for phytoplasma 16SrRNA detection.**b:** LOD of AIOD-CRISPR system for phytoplasma 16SrRNA detection.**c:** LOD of AIOD-CRISPR system for phytoplasma detection by real-time fluorescence assay.

3.4 Specificity and accuracy analysis of AIOD-CRISPR system for phytoplasma detection

To confirm the specificity of the AIOD-CRISPR detection assay, DNA samples suspected to carry phytoplasma from sixteen species (*Trema tomentosa* , *Crotalaria juncea,Crotalaria pallida* ,*Cajanus scarabaeoides,Justicia gendarussa,Vigna unguiculata,Arachis hypogaea,Lycoperisicon esculentum,Raphanus sativus,Parthenium hysterophorus, Catharanthus roseus, Triticum aestivum, Ziziphus jujuba, Paulownia fortunei, Prunus persica* and *Bambusoideae*) were prepared for AIOD-CRISPR detection using the optimized reaction conditions. Healthy plant samples of these sixteen species were also collected as negative controls for AIOD-CRISPR detection. First, the DNA of the plant samples were used as templates for PCR to confirm the presence of phytoplasma. Twelve of the samples amplified a target fragment (1,300 bp) (S1, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S14 and S16), while no amplified products were observed in their negative controls (Fig. 5a). All the amplification products were recovered for sequencing, and all of fragments corresponded to the phytoplasma 16sRNA sequence, indicating that they were infected with phytoplasma (data not shown). Then each DNA sample was subjected to the AIOD-CRISPR assay. All samples except S2, S13 and S15 showed obvious fluorescence in the optimal reaction system, while the fluorescence signal was almost invisible for the corresponding negative controls (Fig. 5b). The results were consistent with those of PCR detection. Results showed that the specificity of AIOD-CRISPR system for phytoplasma detection was very high and can be used to specifically detect phytoplasma in plant DNA extracts.
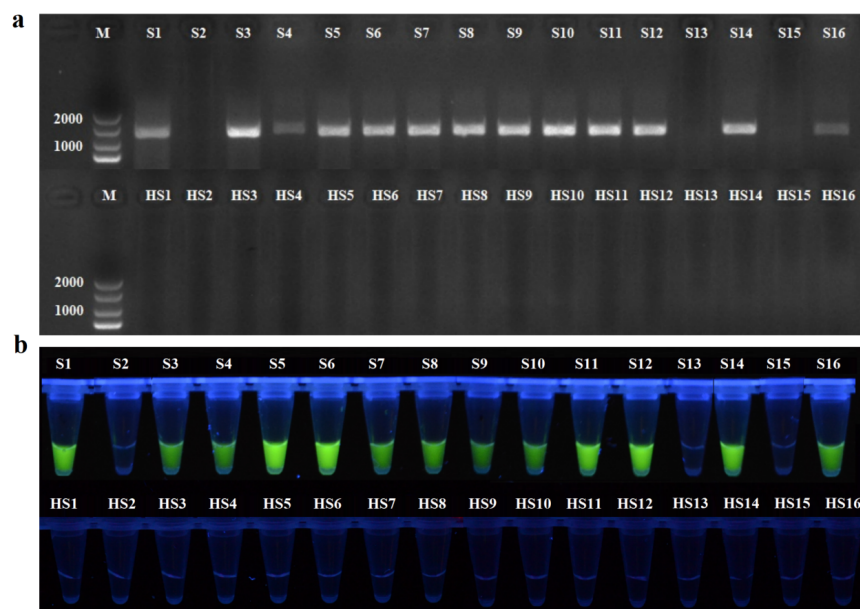
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Fig. 5. Specificity analysis of AIOD-CRISPR system for phytoplasma detection

a: PCR detection for plant samples by PCR-gel electrophoresis.**b:** Visual detection of phytoplasma DNA in plant samples corresponding to Fig. 5a by endpoint imaging. S1: *Trema tomentosa;* S2: *Crotalaria juncea;* S3*: Crotalaria pallida* ; S4: *Cajanus scarabaeoides;* S5: *Justicia gendaruss;* S6:*Vigna unguiculata;* S7: *Arachis hypogaea;* S8:*Lycopersicon esculentum;* S9: *Raphanus sativus;* S10:*Parthenium hysterophorus* ; S11: *Ziziphus jujuba;* S12:*Paulownia fortunei;* S13: *Catharanthus roseus;* S14:*Triticum aestivum;* S15: *Bambusoideae;* S16: *Prunus persica.* HS represent healthy samples of the species.

To evaluate the accuracy of the AIOD-CRISPR phytoplasma detection in the field, forty jujube seedlings were randomly sampled. The total DNA extracts were subjected to the AIOD-CRISPR system and PCR assay. For PCR-gel electrophoresis assay, the phytoplasma 16SrRNA universal primers were used (Chen et al., 2022). Thirty-one samples were confirmed that to be infected by JWB phytoplasma (Fig. 6a), while samples 2, 3, 16, 18, 21, 33, 36, 37 and 38 were negative for phytoplasma *16SrRNA* gene fragment. These thirty-one JWB phytoplasma-infection samples were selected to further evaluate the accuracy of the AIOD-CRISPR system, all of the samples showed powerful fluorescence in the optimal reaction system (Fig. 6b), which was perfect consistent with that of PCR detection . The results indicated that the accuracy of AIOD-CRISPR system reached 100% and can be used for the accurate detection of phytoplasma in the field.
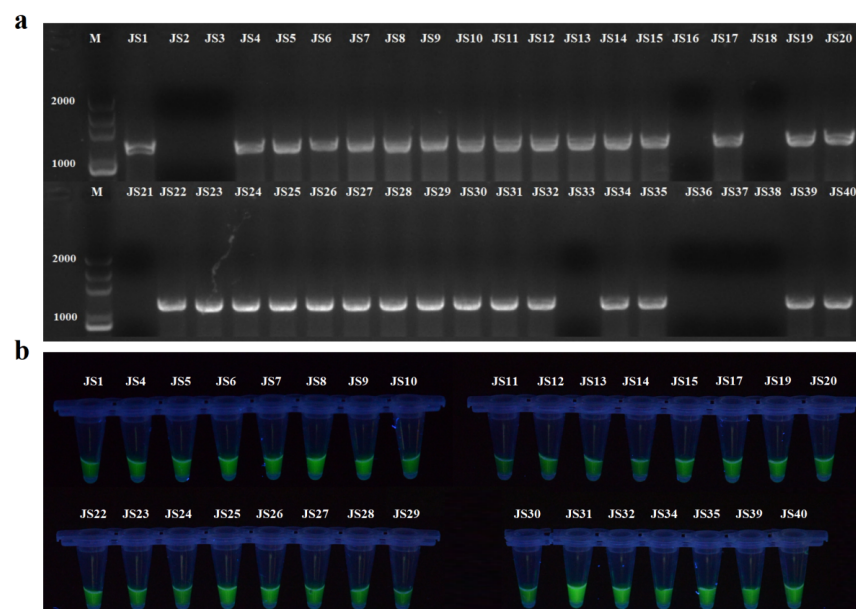
Fig. 6. Specificity analysis of AIOD-CRISPR system for JWB phytoplasma detection

a: Detection of JWB phytoplasma DNA with PCR-gel electrophoresis. b: JWB phytoplasma detection with AIOD-CRISPR assay. Forty samples of jujube seedlings were tested. JS represent jujube samples.

3.5 Diagram of AIOD-CRISPR detection system of phytoplasma

A schematic of the visual detection system for phytoplasma in plant tissues and based on an All-in-one dual (AIOD)-CRISPR/LbCas12a platform is generated (Fig.7). Within the AIOD-CRISPR platform, the Cas12a-crRNA complexes are prepared prior to being adding into the reaction solution containing RPA primers, single-stranded DNA fluorophore-quencher (ssDNA-FQ) reporter, RPA Mix and sample DNA. All components were added together and incubated in one tube at 37 °C. On one hand, once the phytoplasma is existed in the reaction, the crRNAs immediately bind the target DNA, which activates the Cas12a endonuclease that cleaves the ssDNA-FQ reporters to produce fluorescence. RPA products also provide additional binding sites for the Cas12a-crRNA complexes, effectively amplifying the process and the fluorophore signal through continuous triggering of CRISPR-Cas12a-based collateral cleavage activity. On the other hand, if there is no phytoplasma in the reaction, crRNAs loss the target, which will not activates the Cas12a endonuclease and the ssDNA-FQ keeps stable. No fluorescence is produced in these reactions (Fig. 7).
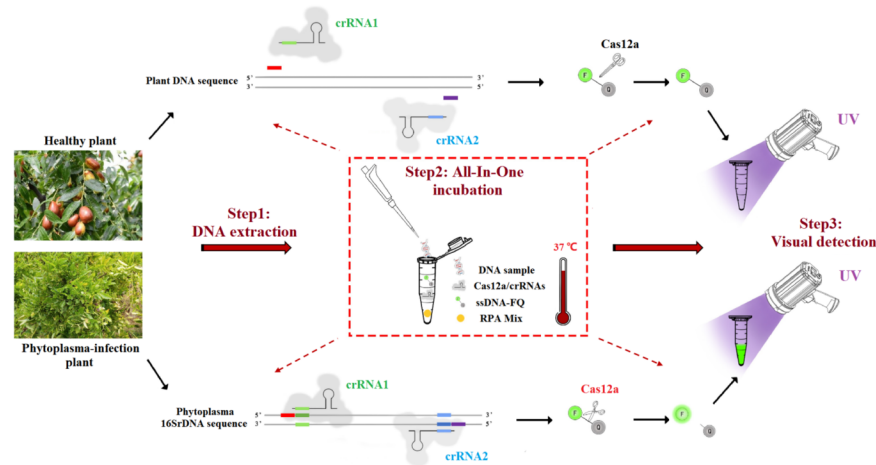
Fig. 7. Schematic of the phytoplasma visual detection system based on AIOD-CRISPR/LbCas12a platform.

4 Discussion

Phytoplasma can infect thousands of plants and caused huge economic losses around the world (Bertaccini et al. 2014; Namba et al. 2019). The large-scale spread and serious lethality of these pathogens creates the urgent need for sensitive, accurate, visual and rapid detection methods to prevent further spread of this disease. In this study, we established an AIOD-CRISPR system based on CRISPR technology. In this detection system, two crRNAs are introduced to markedly improve the sensitivity of the assay. The ssDNA-FQ reporter is added to produce the fluorescence, so the detection results can be easily distinguished with naked eye based on the fluorescence change. In addition, this AIOD-CRISPR system is not limited by the Cas12a PAM motif, thus greatly reduce the detection time. This study showed that the system is not only accurate but also reach the same sensitive level than traditional PCR methods. This AIOD-CRISPR system provided a simple, rapid, ultrasensitive, one-tube, and visually readable method for phytoplasma detection. The availability of the AIOD-CRISPR system will greatly enhance the detection efficiency of phytoplasma.

CRISPR-based methods are well established in plant disease detection (Ding et al. 2020; Jiao et al. 2020; Safari et al. 2021; Zhang et al. 2021). Compared to previously reported CRISPR-based nucleic acid detection, our AIOD-CRISPR system possesses a series of distinctive advantages. The crRNA in the system was designed according to the conserved domain of the phytoplasma 16SrRNA segment. This broad specificity of our detection system can detect multiple phytoplasmas with published 16SrRNA sequences, unlike other systems that can only detect one or several 'Candidatus phytoplasma' species. At the same time, AIOD-CRISPR is a true detection system capable of field operation, because the whole process is carried out at 37 and the detection result can be clearly distinguished with the naked eye. AIOD-CRISPR eliminates the need of a high temperature for the initial denaturing of dsDNA targets in isothermal nucleic acid amplification techniques such as LAMP (Notomi et al. 2000) and SDA (Crannell et al. 2014). What's more, in our AIOD-CRISPR system, each component is prepared as three parts (part A, part B and part C) before the reaction beginning, there are no more reagent are added to the reaction once the test start up. The feature of one-step greatly reduces the risk of potential sample contamination. Finally, the AIOD-CRISPR detection system is really fast and highly sensitive. In the study, the fluorescence of ssDNA-FQ was rapidly readable after being cleaved by LbCas12a by a microplate reader. In the optimized reaction system, the fluorescence signal increases rapidly after reaction for 1 min and reaches its maximum value within 5 min (Fig. 3b, 3d). The detection results can be observed by the naked eye within only 15 minutes. This detection system is also highly sensitive, able to detect as few as 3.37E+2 copies of the DNA target, which reached

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the same level of PCR detection. High sensitivity means that the detection system can correctly detect the presence of phytoplasma during the early stage of infection, which is of great significance for preventing and controlling the disease. In summary, AIOD-CRISPR is a sensitive and accurate system with high specificity and possesses great potential in phytoplasma detection for both quantitation and digital analysis.

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Author contributions

PC, QCL and JJ performed experiments and led the writing of the manuscript. JDL and JCF conceived the ideas and designed methodology. QQY, SCG, JY, YC and YXW analyzed the data. HC and YFW contributed plant materials. XY, JC, BT, XBZ and HYW reviewed the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data availability

All data needed to evaluate the conclusions in this paper are present in the paper and/or the Supplementary Materials.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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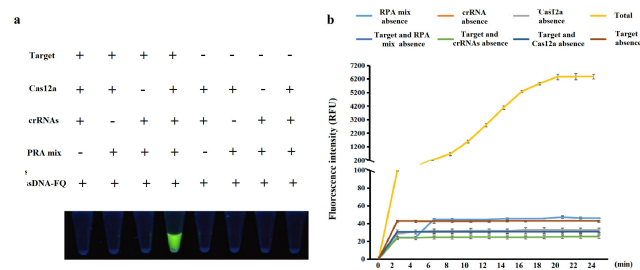
Fig. 1. Evaluation of AIOD-CRISPR reaction components containing various components. Reactions were

tracked using **(a)** endpoint visual fluorescence detection and imaging and **(b)** real-time fluorescence detection.
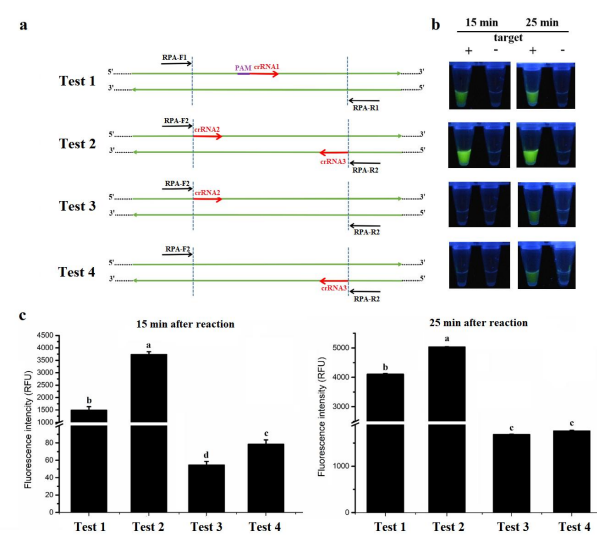
Reaction components as listed in figures.

Fig. 2. The efficiency of different primer and crRNA combinations on AIOD-CRISPR reaction

(a) Four AIOD-CRISPR reactions were prepared . Test 1, a single crRNA targeting a site with a PAM motif in the middle of the RPA amplification product. Test 2, two crRNAs without PAM motifs are closely adjacent to the recognition sites of the RPA primers. Test 3 and Test 4, single crRNA without PAM motif, the same individuals as in Test 2. **(b)** Visual fluorescence detection of the AIOD-CRISPR reactions. **(c)** Real time fluorescence detection for phytoplasma.

Fig. 3. Concentration optimization of ssDNA-FQ fluorescent molecules and LbCas12a/crRNA1/crRNA2 complex in AIOD-CRISPR detection system

(a) Concentration gradient of ssDNA-FQ by endpoint imaging (at 15 min) under blue LED and UV light. **b:** Concentration gradient of ssDNA-FQ by real-time fluorescence detection. **c:** Concentration gradient of LbCas12a/crRNA1/crRNA2 complex as detected by endpoint imaging under blue LED and UV light. **d:** Concentration gradient of LbCas12a/crRNA1/crRNA2 complex by real-time fluorescence detection.
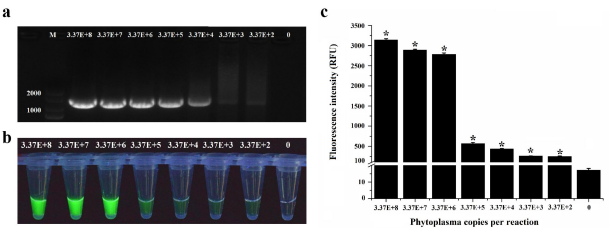
Fig. 4. Limit of Detection (LOD) of PCR and AIOD-CRISPR system for plasmid containing phytoplasma

16SrRNA **detection**

a: LOD of PCR assay for phytoplasma 16SrRNA detection. **b:** LOD of AIOD-CRISPR system for phytoplasma

16SrRNA detection. **c:** LOD of AIOD-CRISPR system for phytoplasma detection by real-time fluorescence assay.
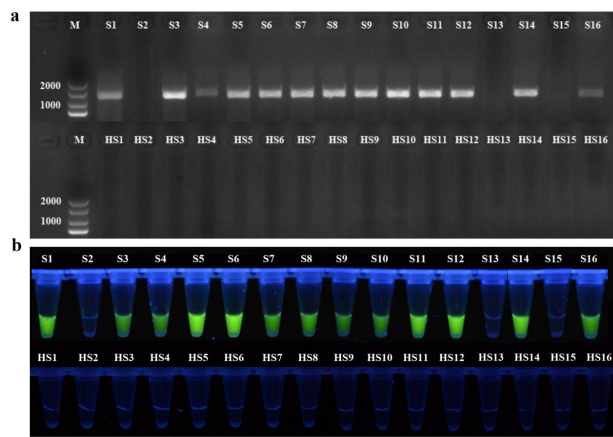
Fig. 5. Specificity analysis of AIOD-CRISPR system for phytoplasma detection

a: PCR detection for plant samples by PCR-gel electrophoresis. **b:** Visual detection of phytoplasma DNA in plant samples corresponding to Fig. 5a by endpoint imaging. S1: *Trema tomentosa;* S2: *Crotalaria juncea;* S3: *Crotalaria pallida*; S4: *Cajanus scarabaeoides;* S5: *Justicia gendaruss;* S6: *Vigna unguiculata;* S7: *Arachis hypogaea;* S8: *Lycopersicon esculentum;* S9: *Raphanus sativus;* S10: *Parthenium hysterophorus;* S11: *Ziziphus jujuba;* S12: *Paulownia fortunei;* S13: *Catharanthus roseus;* S14: *Triticum aestivum;* S15: *Bambusoideae;* S16: *Prunus persica.* HS represent healthy samples of the species.
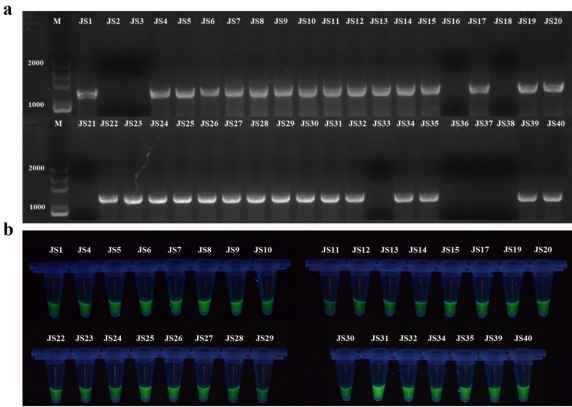
Fig. 6. Specificity analysis of AIOD-CRISPR system for JWB phytoplasma detection

a: Detection of JWB phytoplasma DNA with PCR-gel electrophoresis. b: JWB phytoplasma detection with

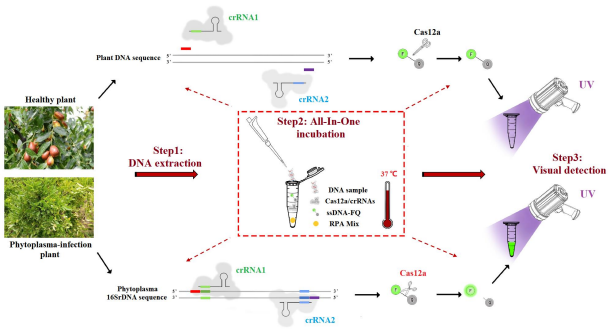AIOD-CRISPR assay. Forty samples of jujube seedlings were tested. JS represent jujube samples.

Fig. 7. Schematic of the phytoplasma visual detection system based on AIOD-CRISPR/LbCas12a platform.