Project Title: A field-deployable diagnosis of XDP based on optimized Cas12a method

Report Type: Final Project Report

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Project Duration: 2-Year

Total Project Request for Year 1 Funding: $ 183,029

Total Project Request for Year 1 Funding: $ 93,786
Total Project Request for Year 2 Funding: $ 89,243

Other related/associated funding sources: None

WTFRC Collaborative Costs: None

Budget 1

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Item	1/3/2025	1/3/2026
Salaries[1]	$20,925.00	$21,762.00
Benefits[1]	$8,563.00	$8,905.00
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies[2]	$14,398.00	$15,080.00
Travel	$2,000.00	$2,000.00
Plot Fees		
Miscellaneous-equipment[3] (ge	$8,000.00	
Total	$53,886.00	$47,747.00

Footnotes: 4% inflation for year 2. [1]Postdoc salary for 4.5 months and postdoc benefit rate at 40.9%. [2]Including budget for co-PI Scott Harper: $6000 for year 1 and $6240 for year 2. [3]Equipment cost.

Budget 2

Co PI 2: Dr. Yinong Yang
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Item	1/3/2024	1/3/2025
Salaries	$24,311.00	$25,283.00
Benefits	$8,752.00	$9,102.00
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies	$4,837.00	$5,111.00
Travel	$2,000.00	$2,000.00
Plot Fees		
Miscellaneous		
Total	$39,900.00	$41,496.00

Footnotes: 4% inflation for year 2.

Budget 3 & 4

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Footnotes: Scott and Amit's budget is included in PI Zhao's budget 1. Amit resigned in May.

Objectives:

1. **To optimize one-pot RPA/Cas12a assay for field-deployable diagnosis using Genie III device or visual detection method.**

2. **To validate, apply and recommend the field-deployable RPA/Cas12a assay for field sample diagnosis.**

Significant Findings:

- A one-pot RPA/Cas12a assay was developed for specific detection of XDP.
- Optimized a one-pot DETECTR assay using a Genie III device.
- Optimized a 30-minute one-pot visual detection (VDet) under UV/blue light.
- Designed a method using plant sap without DNA isolation for detection of XDP.
- Detection of various strains of XDP using one-pot and VDet assays.
- Finalized a protocol for a field-deployable one-pot RPA/Cas12a assay for XDP diagnosis.
- Demonstration of the one-pot and VDet assays to growers and consultants.

Methods

Procedures and Methodology. Previously, a two-pot RPA/Cas12a method was developed for specific, sensitive, and rapid detection of cherry X-disease phytoplasma (XDP) (**Tables 1-3**). The first step of the method is RPA reaction (**Table 1**); followed by the second step: cas12a detection reaction (**Tables 2-3**) and fluorescens signal reading using fluorescence microplate reader. In this project, we developed and optimized a one-pot assay using a less expensive portable Genie III platform (OptiGene) and/or visual detection (VDet) (**Table 4**).

Table 1 RPA reaction

Pre-Master Mix:	1x rxn	5x rxn
2x reaction buffer: dNTPs (10 mM): Primer A (10 uM) Primer B (10 uM): 10x Basic E-mix:	10 ul (1x) 3 ul (1.5 mM) 1 ul (0.5 uM) 1 ul (0.5 uM) 2 ul (1x)	50 ul 15 ul 5 ul 5 ul 10 ul
Total	17 ul	85 ul

Table 2 Pre-detection reaction

Pre-Det Mix	10x rxn	Final conc
ddH2O: NEBuffer r2.1 (10x) Lba Cas12a (10 uM) crRNA (10 uM) FAM-reporter (10 uM)	14.75 ul 2 ul 1 ul 1.25 ul 1 ul	N/A 1x 500 nM 625 nM 500 nM
Total	20 ul	

Table 3 Cas12a Detection reaction

Detection Rxn Mix	10x rxn	Final Conc
NEBuffer r2.1 (10x) Pre-Det Mix (Cas12a; crRNA; FAM reporter) ddH2O	20 ul 20 ul 140 ul	1x 50/62.5/50 nM N/A
Total	180 ul	

1. One-pot RPA/Cas12a DETECTR assay using Genie III device

The one-pot assay combined a two-step process into a single step by merging the isothermal RPA and CRISPR-Cas12a reactions in a single tube. Briefly, the concentration of all ingredients, including rehydration buffer, RPA forward/reverse primers, MgOAc, CrRNA, FAM-reporter, NEBuffer2.1, and DNA template (**Table 4**), were optimized and added into a PCR tube except LbaCas12a nuclease.

LbaCas12a nuclease was placed on the cap of PCR tube. Initial reaction was incubated at 37°C for 15 min. After incubation, vortex and short spin the tube to mix the Cas12a nuclease into the reaction to initiate cleavage of fluorescent oligos at 37°C for 15 min. Fluorescent signals were then recorded at 37°C within 30 minutes by using Genie III device (**Fig. 1**).

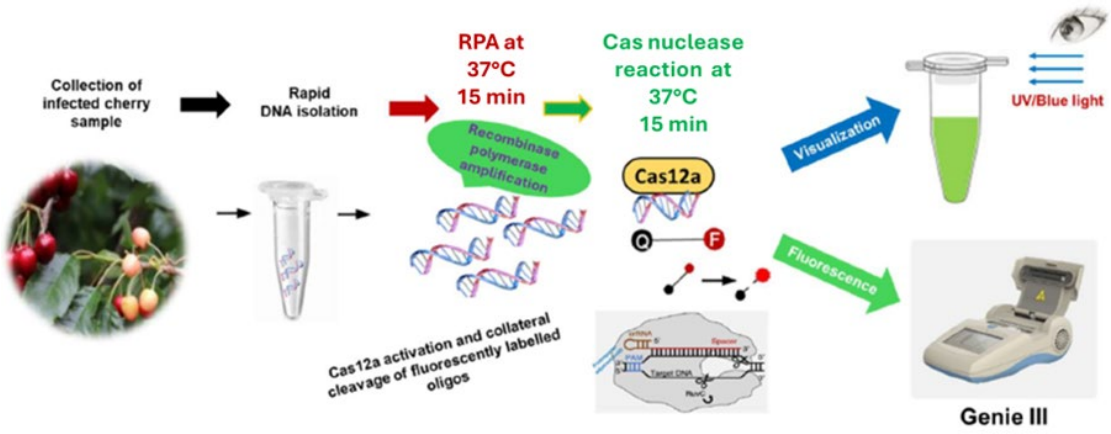


Fig 1. Illustration of one-pot DETECTR and visual detection (VDet) assay. The assay combines rapid DNA extraction, XDP amplification using RPA, followed by Cas12a detection using Genie III device or visual detection with UV/blue light (362-595 nm).

2. One-pot RPA/CRISPR-Cas12a-based visual detection (VDet)

Alternatively, fluorescent signals were checked under UV/blue light (362 to 595 nm) and visualized by naked eye (**Fig. 1**).

Table 4. One-pot DETECTR/visual detection reaction

Ingredients	Reaction (1x)	Reaction (10x)
Rehydration buffer	11.80 µl	118.0 µl
Primer A (360 nM)	0.72 µl	7.2 µl
Primer B (360 nM)	0.72 µl	7.2 µl
CrRNA (250 nM)	0.50 µl	5.00 µl
5-mer FAM reporter (200 nM)	0.40 µl	4.0 µl
NEBuffer 2.1	1.60 µl	16.0 µl
MgOAc (4.725 mM)	0.42 µl	4.2 µl
LbCas12a (250 nM)	0.50 µl	5.0 µl
Nuclease free water	1.34 µl	13.4 µl
Template	2.00 µl	20 µl
Total	20.00 µl	200.00 µl

Stock: Primers:10 µM; FAM reporter:10 µM; Magnesium Acetate (MgOAc):280 mM; LbCas12a:10 µM.

3. Using plant sap for one-pot RPA/Cas12a DETECTR assay without DNA isolation

A method was used to obtain cherry sap directly for the one-pot RPA/Cas12a DETECTR or VDet assay (**Fig. 2**). Briefly, petioles of cherry leaves of known phytoplasma positive or negative plants were punched using a needle. The tip of the needle was then dipped into the one-pot reaction to mix the sap with the RPA reaction. Fluorescence signals were recorded either by Genie III device or visually checked under UV/Blue light (362-595nm) (**Fig.2**).

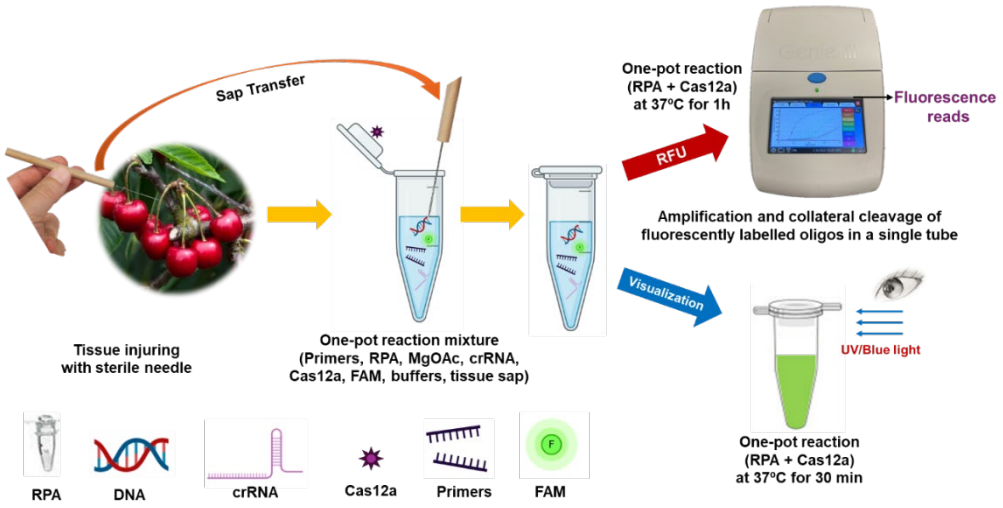


Fig. 2 Illustration of using sap for one-pot DETECTR and visual detection (VDet) assays. The assay used plant sap without DNA isolation and Genie III device or VDet using UV/blue light.

Types and timing of anticipated results. We optimized the one-pot RPA/Cas12a method with Genie III detection or VDet combined with either rapid DNA extract method or sap without DNA isolation or tissue destruction for detection of XDP in a field-deployable way. We finalized our protocols for the field-deployable one-pot RPA/Cas12a-based assay.

Potential problems or limitations. The potential problem for the two-pot RPAS/Cas12a method is false negative when bacterial titer is low in a sample (with CT value >35), especially for young nursery trees and the risk of cross contamination, which may lead to false positive results. By using a one-pot assay, we largely eliminate the risk of cross-contamination but still have similar sensitivities. However, the sensitivity for visual detection might not be as high as using one-pot DETECTR by Genie III device. Another advantage for using sap in one-pot assay is to eliminate DNA isolation, thus it is suitable for field deployable diagnosis. However, the method still requires professional handling of all the reagents (**Table 4**). Therefore, in the future, it will be ideal to develop an end-user diagnostic tool/kit that contains most of the reagents except the enzyme and sample DNA or sap, which should be easy enough for everyone to perform the assay.

Results and Discussion:

1. **Sensitivity of one-pot/two-pot DETECTR assays**

The sensitivity of the one-pot DETECTR assay was evaluated with estimated copy number of pure DNA dilutions of XDP. One pM (picomolar) DNA per µl were diluted up to 1aM (attomolar) DNA per µl to obtain one copy DNA per µl. These dilutions were used for one-pot RPA/Cas12a nuclease reactions using Genie III device. Time course of one-pot DETECTR assay indicated that this assay could detect fluorescence signals with ~one copy DNA (1aM) of XDP and provided the minimum fluorescence ≥ 6,500 RFU, while reached ≥ 37000 RFU with 1pM DNA (**Fig. 3A**). However, the RFU value for 1aM DNA was very close to that of negative control (**Fig. 3A**).

When we compared the one-pot assay with two-pot DETECTR assay, similar levels of sensitivity were obtained at one pM (picomolar) DNA, except the RFU value at this level of DNA was higher for two-pot assay than that of one-pot assay due to detection thresholds of the instruments used (fluorescence microplate reader for two-pot assay versus Genie III device for one-pot assay)(**Fig. 3B**). This result suggests that the Genie III device can detect fluorescence signals using the smallest amount of XDP DNA *i.e.,* one attomolar (10^{-18}) or one copy DNA, but fluorescence signal is

relatively low. Therefore, interpretation of the low titer DNA result should be cautious, otherwise it will lead to either false positive or false negative results.

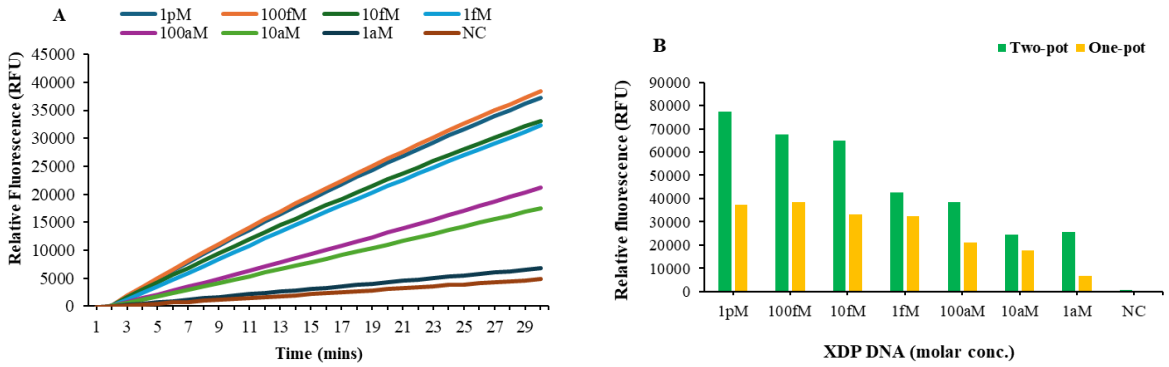


Fig. 3 Sensitivity of one-pot DETECTR assay. Dilutions of quantified DNA of the XDP *secY* gene were used for testing sensitivity of the assay. **A)** Time course of one-pot DETECTR using Genie III device. **B)** Comparing one-pot to two-pot DETECTR assay using same dilutions of XDP DNA. One-pot assay provides similar sensitivity of XDP detection as compared to two-pot DETECTR assay at one attomolar level (10^{-18} or one copy DNA/µl). NC, negative control. pM: picomolar; fM: femtomolar; aM: attomolar. RFU: Relative Fluorescence Unit.

2. Sensitivity of one-pot RPA/CRISPR-Cas12a-based visual detection (VDet)

The sensitivity of visual detection (VDet) assay was also evaluated using estimated copy number of pure DNA dilutions of XDP *i.e.,* 1pM (picomolar) to 1aM (attomolar). The reactions were incubated at 37°C for 30 minutes to visualize the fluorescence under UV/blue light (362 to 595 nm). Green fluorescence (UV/Blue light) or whitish background (UV) could be seen for dilutions of the XDP up to 1aM level, which is similar to those of the one-pot and two-pot DETECTR assays (**Fig. 4**). However, interpretation of the low titer DNA (1aM) result may be difficult in practice due to that visual detection is inherently subjective. Experience may help in interpretation of the results when you compare signals at 1aM versus control (**Fig. 4**). It should also be noted that the VDet assay requires only 30 mins to complete, which is about half of the time required for the one-pot or two-pot assays using fluorescence microplate reader or Genie III device. Therefore, the sensitivity of VDet might be slightly lower than that of one-pot using Genie III device.

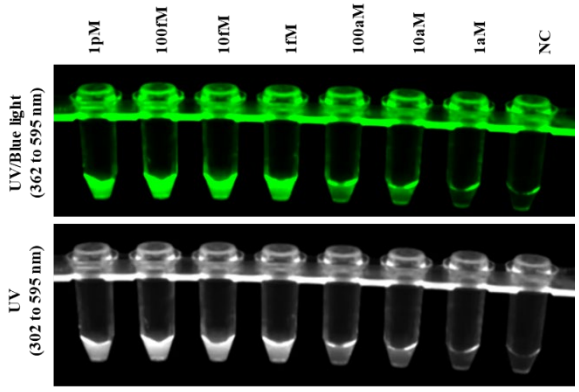


Fig. 4 Sensitivity of visual detection of XDP using one-pot DETECTR assay. Dilutions of quantified DNA of XDP *secY* gene were used for testing sensitivity of the assay. The assay could detect one attomolar level (10^{-18} or one copy DNA) within 30 minutes. The gel-imager instrument was used for visualization of green fluorescence (UV/Blue light) or whitish background (UV) using UV/blue spectrum from 362 to 595 nm. NC, negative control.

3. One-pot, two-pot and visual detection (VDet) assays using DNA samples

To determine the effectiveness of the detection assays, we isolated DNA samples from six one-year-old nursery sweet cherry trees grown in the greenhouse and a total of seven samples per tree with each sample about 10 cm apart were collected. These DNA samples were subject to detection of XDP by one-pot, two-pot, and visual detection assays. As shown in **Table 5** and **Fig. 5**, all six trees were tested positive for XDP. However, there are discrepancies among the different assays and not all samples were tested positive with one, two-pot RPA/Cas12a based detection method and visual detection. These results suggested that due to uneven distribution of XDP and low-titer samples of young trees, multiple samples from each tree are needed for accurate diagnosis of XDP infection.

Table 5. Detection of XDP by one-pot and two-pot assays using DNA

Assay	Tree 1		Tree 2		Tree 3		Tree 4		Tree 5		Tree 6	
Sample	**Two**	**One**	**Two**	**One**	**Two**	**One**	**Two**	**One**	**Two**	**One**	**Two**	**One**
A	-	-	-	-	+	+	+	+	-	-	+	+
B	+	+	+	+	+	+	-	-	+	+	+	+
C	-	-	+	+	-	-	+	+	+	+	-	-
D	-	-	+	+	+	+	+	+	-	-	-	-
E	+	-	-	-	-	-	+	+	-	-	+	+
F	+	+	+	-	+	+	-	-	+	+	-	+
R	-	-	-	+	-	-	-	-	-	-	-	+

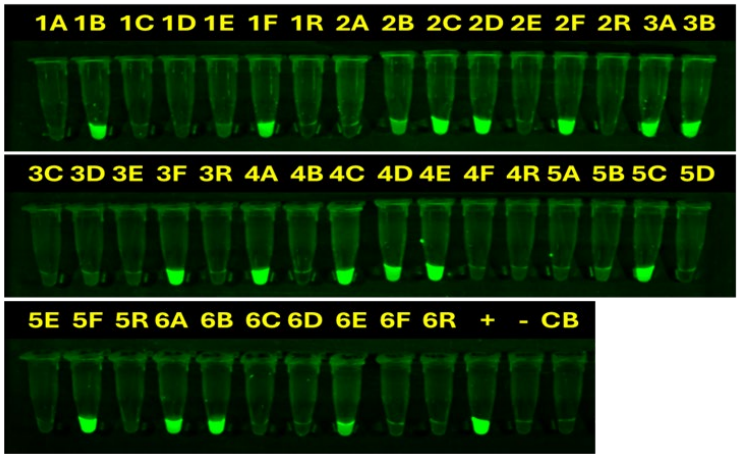


Fig. 5 Visual detection of XDP of tree samples using isolated DNA from six trees, each with seven samples (A to R). +, positive control; -, negative control.

4. Detection of various X-disease phytoplasma strains using one-pot/VDet.

Instead of isolating DNAs from cherry samples, we detected XDP directly using cherry plant saps without DNA isolation. One-pot DETECTR and visual detection assays were used to detect the various strains of XDP (X1-3; EX, WX, CC2) as well as other subgroups (CFB and POIBI) using collected sap from infected plants. Results showed that one-pot VDet assay detected all tested XDP strains and closely related phytoplasmas, demonstrating broad applicability across different 16SrIII subgroups (**Fig. 6**).

5. One-pot, two-pot, and VDet assays using plant saps

Three cherry trees (two-years-old) and one apple tree from an undisclosed commercial source were tested for XDP infection. Saps from petioles of five different spots per cherry tree were obtained using dissection needles and dipped into the one-pot, two-pot, and VDet reactions (**Fig. 7**). The

reactions were incubated at 37°C (98.6°F) for 1h using Genie III device for one-pot DETECTR assay to obtain the fluorescence signals and 30 mins for VDet assay to visualize the detection with UV/blue light, respectively. Our results showed all four trees were tested positive for XDP and the titers of XDP were relatively high in these trees as shown in the visual detection assay (**Table 6, Fig. 7**). Therefore, the results were much more uniform among the three assays. These results suggested that, when the titer is relatively high, one pot and two-pot assay as well as VDet assay could achieve similar accurate diagnosis of XDP infection. Our findings also indicate that using sap directly for XDP diagnosis without DNA isolation is feasible, which can reduce the risk of cross-contamination and save-time. This also makes the assay field-deployable one step-further.

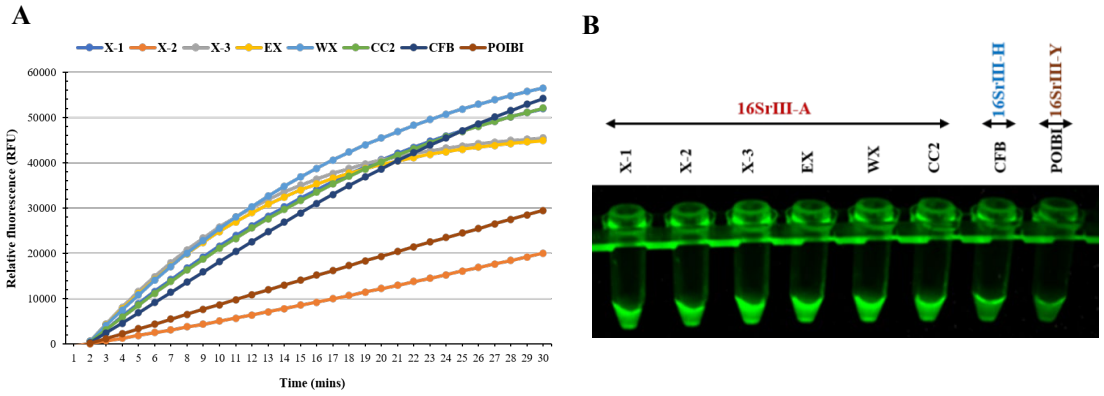


Fig. 6 One-pot and visual detection (VDet) of XDP strains from 16SrIII subgroup A, Y and H infected plants. A) Sap samples were used to detect various strains of XDP and close related subgroups using Genie III device. X-disease phytoplasma (XDP): 16SrIII subgroup A – X-1, X-2, X-3, Eastern X, and Western X:, CC2: *Ca.* phytoplasma pruni; subgroup Y – CFB: *Ca.* phytoplasma pruni; subgroup H – POIBI (Poinsettia branch-inducing phytoplasma JR1); B) Visual detection of XDP strains using UV/blue light (362-595 nm).

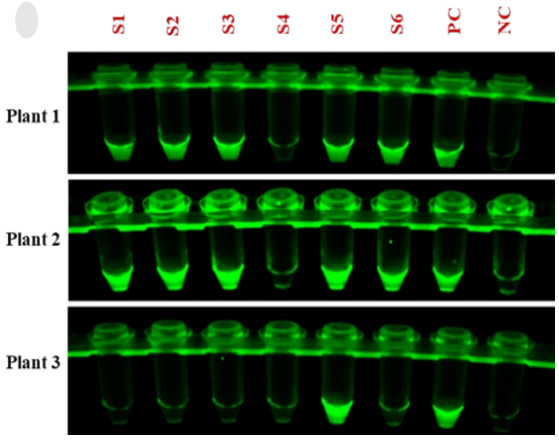


Fig. 7. One pot VDet detection of XDP using plant saps. Signal visualized using UV/blue light (362-595 nm). PC: positive control; NC: negative control. S1-S5 - sap samples from the same tree (Plants 1 to 3); S6- apple samples.

Table 6. Detection of XDP using one-pot and two-pot assays using tree sap

Assay	Plant 1		Plant 2		Plant 3	
Sample	**Two**	**One**	**Two**	**One**	**Two**	**One**
S1	-	+	+	+	-	-
S2	+	+	+	+	-	-
S3	+	+	+	+	-	-
S4	-	-	-	-	-	-
S5	+	+	+	+	+	+
S6 (Apple)	+	+	+	+	-	-

6. Protocol for one-pot and VDet assays

Based on our results, we finalized our protocol for the assays and summarized steps below.

1. Prepared the master mix of eight reactions by adding all the ingredients (**Table 4**) in a 1.5ml sterile Eppendorf tube except template and LbCas12a.
2. Add Twist Amp RPA reaction powder (8x) and mix the suspension with vortex.
3. Aliquot 17.50 µl of reaction mix into PCR tubes and add template DNA (2 ul).
4. Add LbCas12a (0.5 ul) on the cap of the PCR tube.
5. Incubate at 108°F (37°C) for 15 minutes in Genie III device.
6. Vortex and incubate for 15 minutes and then read the relative fluorescence for 30 minutes.

Genie III protocol settings:

1. Turn on the machine, select Lamp and Anneal option
2. Set preheat at 108°F (37°C) for 30 minutes.
3. Set amplification at 108°F (37°C) for 30 minutes.
4. Check the Relative fluorescence graph.
5. Export the results by selecting the export file.

UV/Blue Light: Visualize the one-pot reaction using UV torch by naked eyes or with Gel Doc machine at 362 to 595 nM.

Significance to the industry and potential economic benefits.

A reliable and inexpensive method for early disease detection in non-fruit-bearing trees and in non-symptomatic trees/rootstocks in both orchards and nurseries is critically needed for the cherry industry, as this would not only save growers and nurseries diagnostic costs, but also reduce economic losses due to tree removal and replacement. However, the major challenge for early detection of XDP in non-fruit-bearing trees and in non-symptomatic trees/rootstocks is due to its low titer and uneven distribution of the pathogen within infected plants. This will potentially lead to false positive and false negative results due to the DNA volume tested in each reaction (1-2 µl) if copy number is below the limits of detection. The RPA/Cas12a-based one-pot and two-pot assays as well as VDet methods we have developed can detect small amount of DNA in a sample and is not as susceptible to producing false negatives due to inhibition of its chemistry by compounds present in cherry. This also allowed us to use sap directly without DNA isolation in the assay, which has its advantage by not destructively sampling trees and can be performed in orchard environment. In addition, this method also allows users to skip DNA isolation process, saving time and money and allowing-in field diagnosis of XDP infection. However, the method in its current form still requires professional handling of all the reagents. Therefore, in the future, it will be ideal to develop an end-user diagnostic tool/kit that contains most of the reagents except the enzyme and sample DNA or sap, which then should be easy enough for everyone to perform the assay.

Literature Review:

Since 2015, the X-disease phytoplasma (*Candidatus* Phytoplasma pruni) (XDP) became the predominant pathogen in Washington, increased from less than 10% in 2017 to 36% in 2020 (Clarke et al. 2024; Davis et al. 2013; Harper et al. 2020; Wright et al. 2021, 2022). The outbreak has caused severe damage to the cherry and stone fruit industry in Washington and in northern Oregon. Between 2015 and 2020, more than 238,856 cherry tress (974 acres) and 33082 stone fruits trees (>81 acres) were removed due to X-phytoplasma disease, a total of $95 million lost revenue for the industry (Harper et al. 2020). Estimated lost revenue and re-establishment costs were about $115 million over the seven-year period (Molnar et al. 2022). The emergence of new phytoplasma genotypes, increased host range, and abundant vector species makes management of XDP even challenging (Clark et al. 2024; Molnar et al. 2024; Wright et al. 2022). Besides cherry or stone fruits, surveys found that 52 out of 77 plant species were tested positive for XDP (https://treefruit.wsu.edu/ article/alternative-weedy-hosts-for-x-disease-phytoplasma/). In addition, young active growing trees may easily get reinfected if the pathogen or vector presents nearby. These findings suggest that early detection of XDP infections using fast and easy methods is critical to the cherry industry as well as prevention of the disease, thus reducing the economic losses to growers and the cherry industry.

The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas12a system is an emerging biosensor technology where CRISPR-associated proteins (Cas protein) utilize the distinct guide RNA (gRNA) to target a specific site of the nucleic acid (DNA). Upon initial cleavage of specific target DNA, activated Cas12a gains the ability to trans-cleave the fluorescence oligo reporter, which is detectable by a fluorescence microplate reader (Chen et al., 2018; Kaminski et al. 2021; Mao et al. 2022). One of the great advantages of the Cas12a-based detection is its high specificity at the single nucleotide level and super-sensitivity at the attomolar level or even lower concentration (Kaminski et al. 2021). Typically, Cas12a-based detection is coupled with target DNA amplification by using isothermal methods such as recombinase polymerase amplification (RPA) or loop-mediated isothermal amplification (LAMP) for improved specificity and sensitivity. A number of CRISPR/Cas-based detection strategies such as DETECTR (DNA endonuclease-targeted CRISPR trans reporter), have been demonstrated as point of care diagnostic tools for ultrasensitive and specific detection of pathogen nucleic acids in biomedical samples (Chen et al. 2018; Gootenberg et al. 2017; Jiao et al. 2021; Li et al. 2019). The use of RPA/Cas12a DETECTR assay for supersensitive and specific detection of low titer bacterial pathogens such as *Candidatus* Liberibacter asiaticus (Wheatley et al. 2021) and phytoplasmas (Wheatley et al. 2022) has also been previously reported. We also successfully developed a two-pot RPA/Cas12a assay, which has been used in the laboratory setting for detection of XDP in cherry samples, weeds, and insect vectors with a 384-well fluorescence microplate reader. However, our two-pot RPA/Cas12a assay system for XDP detection requires an expensive instrument (microplate reader, > $25,000) and is not field-deployable.

The isothermal RPA/Cas12a-based one-pot assay has been used for the detection of SARS-Cov-2 and plant RNA viruses (Aman et al. 2020; Ding et al. 2020; Malci et al. 2022; Sun et al. 2021). Cas12a has also been combined with loop-mediated isothermal amplification (LAMP) for DNA detection (Wang et al. 2019). However, this LAMP/Cas12a method requires at least 2 hours to complete, and its sensitivity needed to be optimized. More recently, advances have been made in developing a CRISPR/Cas12a-based platform to visually detect nucleic acids and pathogens (Kanitchinda et al. 2022; Qin et al. 2022; Wang et al. 2019). As compared to LAMP/Cas12a, RPA/Cas12a visual detection method requires only 30 min to complete. In our current study, our goal is to make the one-pot RPA/Cas12a assay field-deployable and practically useful to diagnosticians, crop consultants and even growers. Therefore, we optimized a one-pot RPA/Cas12a method with Genie III detection or VDet combined with either rapid DNA extract method or sap without DNA isolation for detection of XDP in a field-deployable way. We also finalized our protocol for the field-deployable one-pot RPA/Cas12a-based assay.

References:

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Executive Summary

Project Title: A field-deployable diagnosis of XDP based on optimized Cas12a method

Key words: X-disease Phytoplasma, Early Detection, Field Diagnosis, Management

Abstract: The cherry X-disease is at epidemic levels in the Pacific Northwest and has caused significant economic losses. A reliable and inexpensive method for early disease detection in non-fruit-bearing trees and in non-symptomatic trees/rootstocks in both orchards and nurseries is critically needed for the cherry industry, as this would not only save growers and nurseries diagnostic costs, but also reduce economic losses due to tree removal and replacement. However, the major challenge for early detection of XDP in non-fruit-bearing trees and in non-symptomatic trees/rootstocks is due to its low titer and uneven distribution of the pathogen within infected plants. A two-pot recombinase polymerase amplification (RPA)/Cas12a diagnostic assay for detection of XDP has been previously established, which is capable of specific, sensitive and rapid detection of XDP-infected samples from the field. However, the assay requires an expensive fluorescence microplate reader and has a high risk of cross-contamination. In this study, we developed and optimized a one-pot RPA/Cas12a assay using a less-expensive portable Genie III platform and a 30-minute one-pot visual detection (VDet) assay under UV/blue light. This method also allows us to apply plant sap directly without DNA isolation for detection of XDP, thus permitting in field diagnosis of XDP infection. Finally, we finalized a protocol for a field-deployable one-pot RPA/Cas12a assay for XDP diagnosis. In summary, the RPA/Cas12a assays for XDP detection not only provide the industry with a much-needed platform for fast, supersensitive, and accurate diagnosis, but also save time and money for both growers and nurseries. However, the method in its current form still requires professional handling of all the reagents. Future studies should focus on developing an end-user diagnostic tool/kit that contains most of the reagents except the enzyme and sample DNA or sap, which should be easy for everyone to perform.