Winter 2023

Developing Onsite Detection Methods for Grapevine Vein Clearing Virus and Grapevine Red Blotch Virus

Gregory Bryce Goodlett
Missouri State University, gbg44s@MissouriState.edu

DEVELOPING ONSITE DETECTION METHODS FOR GRAPEVINE VEIN CLEARING VIRUS AND GRAPEVINE RED BLOTCH VIRUS

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Plant Science

By

Gregory Bryce Goodlett

December 2023

DEVELOPING ONSITE DETECTION METHODS FOR GRAPEVINE VEIN CLEARING VIRUS AND GRAPEVINE RED BLOTCH VIRUS

William H. Darr College of Agriculture

Missouri State University, December 2023

Master of Science

Gregory Bryce Goodlett

ABSTRACT

Both grapevine vein clearing virus and grapevine red blotch virus are detrimental pathogens on grapevines in the Midwestern United States. The most effective method of control for these viruses is early detection and removal of infected vines. A rapid and equipment-free method of detecting grapevine vein clearing virus and grapevine red blotch virus was developed using the isothermal DNA amplification technique, recombinase polymerase amplification, in conjunction with lateral flow strips. The method yields testing results under 35 minutes and can be performed onsite, and thus provides a rapid and grower-friendly diagnostic tool for preventing diseases caused by the two viruses.

KEYWORDS: recombinase polymerase amplification, grapevine vein clearing virus, grapevine red blotch virus, lateral flow, onsite testing, rapid diagnostic

DEVELOPING ONSITE DETECTION METHODS FOR GRAPEVINE VEIN CLEARING VIRUS AND GRAPEVINE RED BLOTCH VIRUS

By

Gregory Bryce Goodlett

A Master's Thesis
Submitted to the Graduate College
Of Missouri State University
In Partial Fulfillment of the Requirements
For the Degree of Master of Science, Plant Science

December 2023

Approved:

Wenping Qiu, PhD., Thesis Committee Chair

Jason Streubel, PhD., Committee Member

Steven Schneider, MS., Committee Member

Julie Masterson, Ph.D., Dean of Graduate College

ACKNOWLEDGEMENTS

I thank Dr. Wenping Qiu for welcoming me into his lab and giving me this amazing opportunity. His wisdom was invaluable in writing this thesis. I also thank him for always challenging me to think critically and use good science in everything that I do in the lab. I am a better scientist because of it.

I thank Dr. Jason Streubel for setting me on this path. His passion and guidance throughout my undergrad are the reason that I entered higher academia. After the first lecture of his that I heard, I knew that I wanted to use science to help people.

I thank Jacob Schneider for answering the call at the last minute. His willingness to stand in on my committee is greatly appreciated and why I am able to graduate on time.

I thank Sylvia Petersen for all of her help in the lab. I learned so much from her teachings in the lab. I also thank her for her help with dealing with false positives and detection limits.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW

Grapevine Vein Clearing Virus

In 2004, reports of a series of symptoms resembling a viral infection in *Vitis vinifera* 'Chardonnay' were submitted to researchers at Missouri State University (Qiu et al. 2007). These symptoms included vein clearing, chlorosis of leaf tissue, stunted growth, and decreased berry cluster and size. Over the next three years, these symptoms were continually observed in the vines infected and researchers began to investigate the causal pathogens associated with the symptoms. Infected vines were grafted onto multiple varieties of grapevine and symptoms showed on the indicator plants, providing evidence that the pathogens could spread via grafting. The infected plants were tested using enzyme-linked immunosorbent assay for several viruses that are known to infect grapevines. All of these tests came back negative (Qiu et al. 2007). Next, reverse transcription polymerase chain reaction (RT-PCR) was used to amplify DNA from RNA templates to test for a number of viruses. These tests showed positive results for *Tomato ringspot virus* (ToRSV), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), and *Grapevine fanleaf virus* (GFLV) (Zhang et al. 2011). Unfortunately, these viruses did not cause symptoms that match the symptoms of the emergent disease (Zhang et al. 2011).

The lack of association of these viruses with the symptoms present in the diseased vines led researchers to conclude that perhaps an unknown virus was responsible (Zhang et al. 2011). They immediately began work to uncover the suspected virus. To do this, the researchers began using deep sequencing of small interfering RNA (siRNA). The premise of this methodology takes advantage of RNA interference (RNAi), a phenomenon observed in many organisms that scans for unwanted RNA segments using siRNAs and breaks them down into small RNA

(sRNA) and more siRNA (Ding and Voinnet 2007). By isolating these sRNAs, fragments of viral genomes were collected and then reconstructed through sequencing (Zhang et al. 2011). Over half of the fragments generated were closely related to the virus family *Caulimoviridae* with 99% of them associated with the genus *Badnavirus*. Based on the large amount of small RNAs that are homologous to *Badnavirus* genomes, the researchers focused their efforts on assembling a *Badnavirus* genome. By using highly conserved regions for designing primers, the researchers assembled a genome with three open reading frames (ORFs) and a size of 7,753 bp. They named this new virus *Grapevine vein clearing virus* (GVCV). After performing phylogenetic analysis on several viral genera, they determined that GVCV is a member of the genus *Badnavirus.* To provide evidence that GVCV was associated with the mysterious vein-clearing and vine decline syndrome, PCR was performed on symptomatic and asymptomatic vines with results showing positive in symptomatic plants and negative in asymptomatic plants. The PCR tests suggested that GVCV existed as a DNA virus, the first in grapevines (Zhang et al. 2011).

The advent of a novel DNA virus in grapevines, the first of its kind, sparked a litany of discoveries about GVCV in the following decade. The first area of discovery was the genetic diversity of GVCV. Researchers at the Center for Grapevine Biotechnology of Missouri State University isolated GVCV samples from a number of grapevine varieties across multiple states (Guo et al. 2014). These samples were amplified using PCR to create a fragment 2,580 bp in length and subjected to a restriction digest using multiple restriction enzymes. The fragments of different samples were broken down into multiple sizes significantly different from each other, suggesting heterozygosity in GVCV (Guo et al. 2014). This heterozygosity did not show close association to grape variety or state of origin. The study also performed sequencing on the GVCV samples' zinc finger (ZF) and reverse transcriptase (RT) regions, two distinct regions

vital to the lifecycle of the virus. This sequencing again indicated a genetic diversity but a lack of association with state of origin or grape variety (Guo et al. 2014).

Further research into the genetic diversity of GVCV was published in 2017 by Beach et al. They investigated GVCV's presence in *Vitis rupestris,* a grape species native to the Eastern and Midwestern United States. It is widely used as rootstock and was vital in curbing a European phylloxera epidemic. The study gathered samples from various wild populations of *V. rupestris* and tested for GVCV using PCR. Recombinant pCR8/GW/TOPO clones were created from the DNA of GVCV infected *V. rupestris* (GVCV-VRU1 and GVCV-VRU2). These clones were then sequenced and compared to the GVCV-CHA reference sequence, the first GVCV isolate. The comparisons found significant differences in both size and content of the three GVCV genomes, most notably a 9-nt insert in GVCV-VRU1. The genetic variability of GVCV isolates is consistent with other badnaviruses. The presence of GVCV in wild *V. rupestris* populations suggest a potential natural reservoir for the virus (Beach et al. 2017).

Another natural reservoir of GVCV was found serendipitously while surveying wild *Vitis* spp. (Petersen et al. 2019). While in the field, researchers noticed mild vein clearing symptoms in wild *Ampelopsis cordata* plants. Tissue samples were taken from these plants and labeled AMP1, AMP2, and AMP3. AMP3 was sampled at the edge of a vineyard and a nearby symptomatic 'Chardonel' vine was also sampled, labeled CHA2. PCR was performed on these samples and a reconstructed genome from each sample was created. All four of these samples were found to be positive for GVCV. Based on the proposed classification of Beach et al. that marks ORF II similarities less than 90% as the criterion for GVCV variants, AMP1, AMP2, and AMP3 are each a unique variant of GVCV. However, AMP3 and CHA2 were found to be the same variant, suggesting a natural transmission of GVCV between wild *A. cordata* and

cultivated *Vitis* spp.. The researchers then performed a survey of *A. cordata* to estimate the population of GVCV in the plants. Of the 113 plants sampled, 35 tested positive for GVCV. This 31% infection rate suggests a sizable reservoir for GVCV and a potential source for the disease. This finding also laid the groundwork for further studies into a possible insect vector for the virus: *Aphis illinoisensis* (Petersen et al. 2019).

A. illinoisensis, commonly known as the grape aphid, feeds on *A. cordata* and cultivated *Vitis* spp. (Petersen et al. 2019). In the study by Petersen et al., *A. illinoisensis* were collected from wild *A. cordata* vines and PCR was performed to test for the presence of GVCV. In four out of the ten aphid colonies sampled, GVCV was detected. This study also performed a transmission assay to attempt to provide evidence that *A. illinoisensis* is an insect vector for GVCV. In the test, *A. illinoisensis* were placed on *A. cordata* plants that tested positive for GVCV and allowed to feed for two days. These aphids were then transferred to three GVCV-negative 'Chardonel' vines and after two months, all three 'Chardonel' vines tested positive for GVCV. This evidence supports that *A. cordata* is a natural reservoir for GVCV and that *A. illinoisensis* is an insect vector for the spread of GVCV to cultivated grapevines (Petersen et al. 2019). In later surveys, it was found that 42-44% of *A. illinoisensis* carry GVCV (Uhls et al. 2021). GVCV is also genetically diverse in *A. illinoisensis* populations, again consistent with the genetic diversity of other badnaviruses (Uhls et al. 2021).

GVCV is a critical threat to grapevine cultivation in the Midwest (Qiu and Schoelz 2017). It is therefore advantageous to find resistant varieties of grapevines. Researchers reported their discoveries in a 2020 publication (Qiu et al.). Looking for a GVCV-resistant grapevine, the researchers chose 'Norton' (Qiu et al. 2020). 'Norton' is known to be resistant to multiple plant diseases (Fung et al., 2008). It is because of this resistance along with its historical significance

in the Midwest that the researchers chose ‘Norton’ (Qiu et al., 2020). After using grafting as the mode of inoculation, no ‘Norton’ plants were found to have GVCV based on PCR testing (Qiu et al. 2020). Using viral small RNAs (vsRNAs), the byproduct of RNA interference, resulted in similar results, showing only 4.7-9.4 GVCV reads per million (RPM) in ‘Norton’ vines compared to 3,398-33,092 GVCV RPM in infected vines (Qiu et al. 2020). A survey of ‘Norton’ vines in a vineyard further supported the lab results with a complete lack of GVCV even though GVCV was present and spreading in the vineyard in susceptible vines (Qiu et al. 2020).

GVCV induces a variety of symptoms with different degrees of severities based on the cultivar of grapevine where the virus is present (Qiu and Schoelz 2017). Symptoms range from mosaic patterns of chlorosis to loss of berry quality and yield, and potentially vine death (Qiu and Schoelz 2017). The symptom that remains consistent throughout grapevine varieties is its namesake, vein clearing (Qiu and Schoelz 2017). Because of this, the distinct chlorosis of the veins is the primary visual diagnostic of GVCV (Qiu and Schoelz 2017). Badnaviruses reproduce through converting their dsDNA into RNA that is used as template for making viral proteins and then is reserve-transcribed to form dsDNA (Zhang et al. 2015). They do this by using a single promoter to create a terminally redundant pregenomic RNA molecule (Zhang et al. 2015). The promoter for GVCV has been found to be located at nt 7571 (Zhang et al. 2015). PCR is currently the only detection method for GVCV (Qiu and Schoelz 2017).

Grapevine Red Blotch Virus

For some time, grapevines have experienced a disease that symptomatically resembles an infection by grapevine leafroll-associated viruses (GLRaV). but when tested indicates a lack of infection by GLRaV (Rwahnih et al. 2013). In 2008, researchers at the University of California,

Davis found vines in an experimental vineyard that matched this description (Rwahnih et al. 2013). In 2013 researchers published a paper detailing their extraction of RNA and deep sequencing of a single stranded DNA virus (Rwahnih et al. 2013). The virus contains a nonanucleotide sequence in the intergenic region (IR) that is found in a number of geminiviruses, leading the researchers to tentatively place the virus into the family *Geminiviridae* (Rwahnih et al. 2013). They named the virus Grapevine red botch-associated virus (GRBaV) (Rwahnih et al. 2013). This would be the first single stranded DNA virus and the second DNA virus found in grapes, unfortunately for the researchers, a team on the other side of the continent beat them to the punch (Rwahnih et al. 2013).

A team of researchers at Cornell University performed rolling circle amplification (RCA) and then restriction fragment length polymorphism on DNA extracted from *V. vinifera* 'Cabernet Franc' in the hopes of finding a geminivirus (Krenz et al. 2012). RCA is a form of amplification that specifically amplifies circular ssDNA using DNA polymerase from *Bacillus subtilis* bacteriophage Φ 29 (Haible et al. 2006). Because of the circular single stranded nature of geminiviruses, RCA is a way of amplifying them without needing to know a sequence for the design of primers (Haible et al. 2006). The Cornell scientists were able to isolate and sequence a unique virus that had ORFs and a nonanucleotide sequence in the IR that were consistent with other geminiviruses (Krenz et al. 2012). The virus was 3,206 nucleotides (nt) in length (Krenz et al. 2012). Naming this new virus grapevine Cabernet Franc-associated virus (GCFaV), they published their findings in 2012 (Krenz et al. 2012).

GRBV's genetic diversity has been broken up into two clades based on a study of its presence in feral *Vitis* species in California (Cieniewicz et al. 2018). The majority of isolates were found within clade 2, only two samples from the study were found in clade 1 (Cieniewicz et

al. 2018). Within the clades, there is a range of genetic diversity (Cieniewicz et al. 2018). Isolates from clade 1 were found to have 98.3% similarity in their genomic diversity fragment while isolates in clade 2 had similarities from 100% down to 96.9% (Cieniewicz et al. 2018). Similarity between the clades was between 88.3% and 90.9% (Cieniewicz et al. 2018). The presence of GRBV in feral *Vitis* indicates a potential reservoir for the virus, especially with the findings that GRBV was more prevalent in feral plant populations in counties with intensive grapevine production (Cieniewicz et al. 2018).

Feral, sometimes called free-living or uncultivated, *Vitis* has been suggested as a reservoir for GRBV on multiple occasions (Bahder et al. 2016; Perry et al. 2016). Bahder et al. further suggests that feral *Vitis* may be one of the only reservoirs for GRBV (Bahder et al. 2016). Their study involved sampling 13 species of plants near vineyards that had known infections of GRBV (Bahder et al. 2016). The testing showed only feral *Vitis* and *Rubus armeniacus* to contain GRBV, and *R. armeniacus* only tested positive in winter and spring whereas *Vitis* tested positive year round (Bahder et al. 2016). This study also mentioned the need to uncover the vector for GRBV to aid in mitigation strategies and policies (Bahder et al. 2016). A study in 2021 sought to do just that (Flasco et al. 2021).

Flasco et al. developed multiple assays to determine the transmission of what they thought to be the best candidate for a GRBV vector, *Spissistilus festinus* (Flasco et al. 2021). *S. festinus*, commonly known as the three-cornered alfalfa hopper, was able to acquire and maintain GRBV infection after being placed on a nonhost plant for GRBV, alfalfa (Flasco et al. 2021). The researchers designed three assays for GRBV infection, the first placing *S. festinus* on infected *Phaseolus vulgaris* and transferring to alfalfa to perform gut clearing then transferring to healthy *P. vulgaris* (Flasco et al. 2021). The second used infected *P. vulgaris* again but after the

intermission on alfalfa, *S. festinus* was transferred to healthy *V. vinifera* 'Carbernet franc' (Flasco et al. 2021). In the third assay, *V. vinifera* 'Cabernet franc' was used as both the initial infected and the end healthy plant (Flasco et al. 2021). The study found that *S. festinus* was able to transfer GRBV in all three assays, indicating it is a vector for GRBV (Flasco et al. 2021). The study also found that GRBV is not seed transmitted, meaning that seeds from an infected plant did not sprout into seedlings that were infected with GRBV (Flasco et al. 2021). All detection in the study was performed with PCR (Flasco et al. 2021). A later study found that GRBV was transmissible by *S. festinus* between cultivated and feral *Vitis* species, further proving *S. festinus*' role as a vector between reservoirs and vineyards (Hoyle et al. 2022).

GRBV has been found in various regions across the continental United States (Krenz et al. 2014). GRBV has been estimated to cost vineyards up to $68,548/ha (Ricketts et al. 2017). This loss is due to the economically significant symptoms of GRBV (Ricketts et al. 2017). Symptoms start with the titular red blotching in late spring and eventually progresses to reddening of the entire leaf in late summer, some of these leaves will fall off early as well (Sudarshana et al. 2015). These reddening symptoms occur in red berried cultivars, but in white berried cultivars, the red blotches are replaced with chlorosis and eventually necrosis on leaves (Sudarshana et al. 2015). The leaves are not the only tissues of the vine affected (Sudarshana et al. 2015). More importantly for growers, GRBV causes delayed ripening and decreased chemicals in the berries that affect wine quality including total sugars and phenols (Sudarshana et al. 2015). The visible symptoms of GRBV are often hard to distinguish from other diseases such as leafroll viruses and some nutritional deficiencies (Sudarshana et al. 2015). Because of this, it is critically important for proper mitigation and management to have effective DNA based diagnostics (Sudarshana et al. 2015).

Recombinase Polymerase Amplification

Polymerase chain reaction (PCR) is a method of amplifying specific segments of DNA (Saiki et al. 1985). It accomplishes this by heating DNA to a temperature that causes double stranded DNA (dsDNA) to denature and then cooling the DNA to a temperature that causes the oligonucleotide "primers" to anneal to the target DNA (Saiki et al. 1985). If the template DNA and the primers are complimentary, some of the DNA will bind to the primers (Saiki et al. 1985). These complexes can then be amplified by a polymerase enzyme, creating copies of target DNAs (Saiki et al. 1985). Since its inception, this amplification method has become the standard for diagnostics testing in applications ranging from medicine to forensics (Kaunitz 2015). However, DNA amplification and diagnostics via PCR require expensive equipment inside of a lab setting, making point-of-use applications limited (Piepenburg et al. 2006). That is why Piepenburg et al. published a new method of DNA amplification built upon methods cells utilize to amplify their own DNA in 2006 (Piepenburg et al. 2006).

Piepenburg et al. designed their novel amplification method using a number of proteins and enzymes that had been characterized before, some decades old (Piepenburg et al. 2006). As with PCR, their method, titled 'recombinase polymerase amplification' (RPA), uses a polymerase enzyme to amplify DNA (Piepenburg et al. 2006). The polymerase used in RPA is *Bacillus subtilis* Pol I (Bsu), a strand displacing polymerase (Piepenburg et al. 2006). Bsu was first characterized in an article in 1964 that compared its exonucleolytic properties with that of *Escherichia coli* polymerase (Okazaki and Kornberg 1964). The researchers found that Bsu had very little nuclease activity occurring during its reaction compared to *E. coli* polymerase, and that it overcame more inhibitors of amplification (Okazaki and Kornberg 1964).

PCR uses changes in temperature to bind primers to template DNA, RPA uses a suite of proteins to accomplish this instead (Piepenburg et al. 2006). Prokaryotic recombinase proteins can bind single stranded DNA (ssDNA) to homologous double stranded DNA. *E. coli* recA is the most well-known of these prokaryotic recombinase proteins (Shibata, Cunningham, et al., 1979). RPA uses the recombinase protein of bacteriophage T4 uvsX to anneal primers to homologous double stranded template (Piepenburg et al. 2006). Both recA and uvsX form complexes with ssDNA that displace and bind with dsDNA, however recA has been shown to also form complexes with dsDNA that displace and bind with other dsDNA molecules (Formosa and Alberts 1986). Recombinase protein uvsX has also been shown to have a higher rate of homologous pairing than recA (Formosa and Alberts 1986).

In *E. coli* recombination models, recA is assisted and catalyzed by *E. coli* single-strand binding (SSB) protein (Harris and Griffith 1988). These proteins form complexes with ssDNA and interact with homologous dsDNA to form displacement loops (D loops) (Harris and Griffith 1988). Recombinase protein uvsX has been shown to form D loops without the aid of a SSB protein (Harris and Griffith 1988). However the rate of D loop formation is low and requires specific ratios of usvX to nucleotides (Harris and Griffith 1988). The SSB protein of T4 bacteriophage is known as gene 32 protein (gp32) (Harris and Griffith 1988). When compared to reactions in the absence of gp32, D loop formation is far greater and requires less specific ratios of uvsX to nucleotide when gp32 is present (Harris and Griffith 1988). *E. coli SSB* protein has also been tested as a catalyst for D loop formation with uvsX but showed no evidence of aiding in the reaction (Harris and Griffith 1988).

The RPA reaction combines the functions of Bsu, uvsX, and gp32 to perform amplification (Piepenburg et al. 2006). The uvsX protein forms complexes with the ssDNA

primers, these nucleoprotein complexes then seek out homologous dsDNA (Piepenburg et al. 2006). Once the nucleoprotein complex has found homologous dsDNA, it forms a D loop by binding the ssDNA primer to homologous dsDNA and displacing the original bond (Piepenburg et al. 2006). This disruption is locked in place by gp32 (Piepenburg et al. 2006). The Bsu strand displacing polymerase can then use the D loop as a starting point for amplification of DNA (Piepenburg et al. 2006). ATP hydrolysis causes the exchange of uvsX and gp32 (Piepenburg et al. 2006). To prevent this interaction from occurring outside of D loops, T4 protein uvsY and the crowding agent Carbowax20M are used to push the reaction equilibrium in the direction of uvsX binding (Piepenburg et al. 2006).

Originally, RPA was designed with a real-time PCR replacement in mind (Piepenburg et al. 2006). Real-time PCR is similar to standard PCR with the added use of florescent dyes that interact with the reaction (Kralik and Ricchi 2017). These interactions are monitored by fluorometers and can then be interpreted by programs to determine many things, but the primary interest of the reaction is studying gene expression (Kralik and Ricchi 2017). Piepenburg et al. found success in using RPA to interact with the florescent dyes, however there was an issue with no template reactions (Piepenburg et al. 2006). After an extended period of time, usually thirty to forty minutes, reactions with no template DNA began to form nonspecific interactions that triggered the florescent dyes (Piepenburg et al. 2006).

To solve this issue, they designed a nucleotide “probe” with a tetrahydrofuran abasic-site mimic (THF) (Piepenburg et al. 2006). The THF probe also contains a 3’ block to prevent polymerization (Piepenburg et al. 2006). The THF site is designed to simulate the damage that is caused in dsDNA by oxidative free radicals (Takeshita et al. 1987). These sites act as a gap in the dsDNA sequence that cannot be easily repaired (Takeshita et al. 1987). Many organisms have

ways of dealing with these abasic sites, including *E. coli* and its endonuclease IV (Nfo) (Levin et al. 1988). Nfo recognizes abasic sites preferentially in dsDNA (Levin et al. 1988). In the RPA reaction, the nucleotides flanking the THF site contain a fluorophore and a quencher (Piepenburg et al. 2006). When the probe binds to homologous DNA, the nfo enzyme recognizes the abasic site and cuts the section 3' of the site away (Piepenburg et al. 2006). This causes the fluorophore to fluoresce and the probe to then function as a forward primer (Piepenburg et al. 2006). This interaction can then be measured by a fluorometer (Piepenburg et al. 2006).

The RPA detection method can be simplified even further by using lateral flow (LF) (Piepenburg et al. 2006). LF is a widely used technique for diagnostics that works by binding antibodies to dyes and to a test strip (Carlberg 2005). For RPA, the reverse primer and either a forward primer or a probe are labeled with antibodies (Ivanov et al. 2021). When the DNA in question is present, amplicons will form (Piepenburg et al. 2006). These amplicons can then bind to both the dye and the test strip, causing a band of color to appear only when the analyte is present (Piepenburg et al. 2006).

Since the publication of the original paper on RPA, many things have been discovered about the method (Lobato and O'Sullivan 2018). RPA has been used to successfully amplify DNA from a number of sample types including urine, stool, blood, seeds, leaves, and meat tissue (Lobato and O'Sullivan 2018). Some experiments have achieved amplification from as few as 10 copies of template DNA (Lobato and O'Sullivan 2018). One of the uses that RPA has been developed for is field testing for pathogens in plants (Daher et al. 2016).

One such case involved the development of a test to detect the strawberry pathogen *Phytophthora cactorum* (Lu et al. 2021). In this study, the researchers developed a method of crude DNA extraction using a mixture of polyethylene glycol (PEG) 200 and sodium hydroxide

(NaOH) as a lysis and extraction buffer. Using this buffer and LF strip visualization, the researchers were able to perform an RPA diagnostic test completely in the field. Through comparative testing on serial dilutions of genomic DNA, the researchers found that their RPA method was 100 times more sensitive than conventional PCR at a concentration of 100 fg for RPA detection (Lu et al. 2021).

INTRODUCTION

Grapes, primarily *Vitis vinifera*, are the third most important horticultural crop globally and are the foundation of an industry valued at over $68 billion annually (Alston and Sambucci 2019). Due to the use of grafting as the primary method of propagating grapevines, grapevines are host to over 70 viruses (Martelli 2017). These viruses cause damage to grapevines and therefore big economic loss to the grape and wine industry to varying degrees. *Grapevine red botch virus* (GRBV) is a single stranded DNA virus of the family *Geminiviridae* that is found throughout the grape growing regions in the United States and globally (Krenz et al., 2014, Bertazzon et al., 2021). Symptoms of this virus include reddening of leaves in red berried cultivars and chlorosis in white berried cultivars, delayed fruiting, and decreased sugars and phenols (Sudarshana et al. 2015). The symptoms affecting berries are what make GRBV such an economically devastating virus, with an estimated annual loss of $68,548/ha to growers (Ricketts et al. 2017).

Grapevine vein clearing virus (GVCV), a double stranded DNA virus, belongs to the genus *Badnavirus* in the family *Caulimoviridae* (Zhang et al. 2011). GVCV is endemic to the Midwest region of the United States (Qiu and Schoelz 2017). The symptoms for this virus vary widely among cultivars but the most common symptom is its namesake: a chlorotic vein clearing of the leaves (Qiu and Schoelz 2017). Other symptoms include berry deformity, vine stunting, and sometimes vine death (Qiu and Schoelz 2017). While no economic studies have been published estimating the impact of GVCV, the virus has resulted in the removal of at least five vineyards in Missouri (Qiu and Schoelz 2017).

Polymerase chain reaction (PCR) is a method of DNA amplification that utilizes a fluctuation in temperatures to facilitate the binding of oligonucleotide primers and amplifying of target DNA. PCR is the diagnostic standard for detecting DNA in plant pathology (Kaunitz 2015). The reliance on precise temperature fluctuations over timed intervals leaves PCR dependent on expensive lab equipment. This limits onsite applications of PCR (Piepenburg et al. 2006). Recombinase polymerase amplification (RPA) provides an alternative. RPA is a method of DNA amplification that utilizes a number of proteins involved in bacterial recombination (Piepenburg et al. 2006). The use of these proteins makes RPA an isothermal reaction that can be performed under conditions far less stringent than PCR (Lobato and O'Sullivan 2018). The isothermal nature of RPA combined with its ability to overcome inhibitors to PCR makes it ideal for onsite applications with little to no laboratory equipment needed (Lobato and O'Sullivan 2018). When combined with a crude DNA extraction and lateral flow (LF) strips, RPA can be used to visualize results in as short as thirty minutes from time of sampling (Daher et al. 2016). An RPA diagnostics method was been designed for GRBV (Li et al. 2017), but it still needs equipment and can't be conveniently applied for onsite detection. In this study, we develop a crude DNA extraction protocol for grapevine leaves as well as onsite, equipment-free detection methods for GRBV and GVCV using RPA and LF strips.

MATERIALS AND METHODS

Grapevines for Crude DNA Extraction and RPA Testing

Vitis vinifera grape cultivars ‘Chardonel’ and ‘Vidal Blanc’ were used in this study. Clones of ‘Chardonel LBC0903’, the original grapevine found to be infected with GVCV, were used as GVCV positive control leaf tissue. A ‘Chardonel’ that tested positive for GRBV by conventional PCR was used as a GRBV positive control. ‘Chardonel 411’ is a GVCV and GRBV-free grapevine that was used as negative control leaf tissue. Tissue from these vines was used to refine and modify crude DNA extraction methods. Along with the aforementioned grapevines, ‘Chardonel’ and ‘Vidal blanc’ grapevines with unknown virus status were used in assessing the sensitivity, specificity, and repeatability of RPA for detecting GVCV and GRBV. All grapevines were grown under greenhouse conditions at the Missouri State Fruit Experiment Station in Mountain Grove, Missouri, USA.

Primer Design

Primers were designed for RPA reactions following the recommendations of TwistDx (TwistAmp DNA amplification kits assay design manual 2018). All primers were between 30 and 35 nucleotides long and amplicons were between 100 and 300 bp long. Reverse primers were labeled on their 5’ ends with biotin. Probes were each 49 nucleotides long. For both probes, the 5’ end was labeled with FAM, the 3’ end was labeled with a C-3 spacer, and the 31st nucleotide was replaced with a THF residue. Temperature compatibility was not taken into account due to the isothermal nature of RPA reactions. Using the sequencing software CodonCode Aligner, seven genomes of GRBV were aligned and highly conserved regions were

found. Primers and probes were then drafted from these regions and entered into ThermoFisher Multiple Primer Analyzer using the highest sensitivity setting available (Multiple primer analyzer - US 2023). Lack of dimers between the reverse primer and the probe were the chief factor in primer design due to the risk of false positives that these dimers can cause in lateral flow strips. Once the primers and probes were designed, they were entered into a NCBI Nucleotide BLAST search along with their amplicon to ensure specificity. This process was then repeated for GVCV. The sequences and features of these primers and probes are summarized in Table 1.

RPA Reaction

RPA reactions were initially performed using the recommended 50 µL reaction mix of TwistAmp Liquid Basic kit; that being 25 µL of 2x Reaction Buffer, 9.2 µL of 10 mM dNTPs, 5 µL of 10x Basic E-mix, 2.4 µL of 10 µM forward primer, 2.4 µL of 10 µM reverse primer, mixing and spinning the reagents, then 2.5 µL of 20x Core Reaction Mix was added, mixed and spun down. The above reaction mix was added to a 0.1 mL reaction tube and 2.5 µL of 280 mM MgOAc and 2.5 µL of template were added to the lid. After closing the lid the reagents are mixed well then spun down and heated to 37 °C for 40 minutes (TwistAmp® liquid DNA amplification kits combined instruction manual 2018).

The reaction volume was later decreased to a volume of 10 µL to decrease consumption of reagents. The new reaction volumes were as follows; 5 µL of 2x Reaction Buffer, 1.8 µL of 10 mM dNTPs, 1 µL of 10x Basic E-mix, 0.4 µL of 10 µM forward primer, 0.4 µL of 10 µM reverse primer, then mixing and spinning the reagents, 0.5 µL of 20x Core Reaction Mix, and then mixing and spinning down again. The master mix was then added to a 0.1 mL reaction tube and

0.5 µL of 280 mM MgOAc and 0.5 µL of template were added to the lid. After closing the lid, the reagents are mixed thoroughly, spun down, and then heated to 37 °C for 40 minutes.

When visualization on LF was introduced, the reaction volume was again changed. The new total reaction volume was 20 µL. Volumes and concentrations of individual products were scaled based on the reaction mix for the TwistAmp Liquid Exo kit. The reaction mix was as follows; 10 µL of 2x Reaction Buffer, 3.3 µL of 10 mM dNTPs, 2 µL of 10x Probe E-mix, 0.8 µL of 10 µM forward primer, 0.8 µL of 10 µM reverse primer, 0.2 µL of 10 µM probe, then mixing and spinning the reagents, 1 µL of 20x Core Reaction Mix, 0.4 µL of 50x Exo, and then mixing and spinning down again. The master mix was then added to a 0.1 mL reaction tube and 1 µL of 280 mM MgOAc and 1 µL of template were added to the lid. To help reduce nonspecific banding and false positives, the reaction was heated to 37 °C for a reduced 20 minutes.

Once the RPA reaction was completed, the product was visualized on Milenia Biotec HybriDetect strips (HybriDetect 2019). 10 µL of RPA product was mixed with 100 µL of HybriDetect Assay Buffer in a 1.5 mL tube and a HybriDetect Dipstick (LF strip) was placed in the mixture. The volume of RPA product was later reduced to 5 µL to help reduce false positives.

Gel Visualization of RPA products

RPA products had to be purified before a clear DNA band could be seen on an agarose gel. Multiple methods of purification were tested. Based on the recommendation of TwistDx, the first method attempted was a phenol/chloroform extraction (TwistAmp® liquid DNA amplification kits combined instruction manual 2018). The reaction was as follows; one volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the RPA product and mixed then centrifuged at room temperature for 5 minutes at 16,000 rcf, the top layer was transferred to a

new tube and placed at -20 °C overnight, then centrifuged at 4 °C for 30 minutes at 16,000 rcf. The supernatant was mixed with 150 µL of 70% ethanol and centrifuged at 4 °C for 2 minutes at 16,000 rcf, and then supernatant was removed. The tube was centrifuged again at 4 °C for 30 minutes at 16,000 rcf and residual supernatant was removed. The pellet (invisible) was air-dried for 10 minutes, then the DNA pellet was dissolved into 50 µL of sterile deionized water (How to use phenol / chloroform for DNA purification 2023).

High temperatures were also used in an attempt to denature the nucleoprotein complexes of RPA products. RPA products were treated with temperatures of 65 °C and 95 °C for 10 minutes. Lastly, the Zymo Research DNA Clean & Concentrator™ kit was used. The protocol was as follows; 5 volumes of DNA Binding Buffer was added to the RPA product and mixed, the mixture was transferred to a Zymo-Spin™ Column in a collection tube, centrifuged at room temperature for 30 seconds at 10,000 rcf, flow through was discarded, 200 µL of DNA Wash Buffer was added to the column and centrifuged at room temperature for 30 seconds at 10,000 rcf, flow-through was discarded, 200 µL of DNA Wash Buffer was added to the column and centrifuged at room temperature for 30 seconds at 10,000 rcf, flow-through was discarded, the column was centrifuged at room temperature for 1 minute at 15,000 rcf, flow through discarded, the column was dried for 1 minute, 10 µL of sterile deionized water was added and incubated at room temperature for 1 minute, and the column was centrifuged at room temperature for 30 seconds at 10,000 rcf. A comparative experiment was designed comparing the effects of treatments of 95 °C, the Zymo Research DNA Clean & Concentrator™ kit, and no treatment. Each treatment was performed on a 10 µL RPA reaction product and viewed on a 1% agarose gel. The 1% agarose gel was made by mixing 1 g of agarose with 100 mL of 1x Tris/Borate/EDTA (TBE) buffer and 3.5 µL of GelRed (Biotium). The Phenol/Chloroform

extraction was excluded from this comparative experiment. The Zymo kit was used for the rest of this study.

Crude DNA Extraction

Crude DNA extractions were performed in a series of increasingly rudimentary methods to achieve a method that could be performed in the field with minimal equipment. A crude extraction buffer of 6% polyethylene glycol (PEG) 200 and 0.08% NaOH in sterile deionized water was used in all of the extraction methods based on existing literature (Lu et al. 2021). The first method was as follows; 100 mg of leaf tissue was taken in the form of five discs punched with a single hole puncher and placed into a Homogenization Tube from the SYNERGY™ 2.0 Plant DNA Extraction Kit (OPS Diagnostics), 500 µL of extraction buffer was then added to the Homogenization Tube, the tube was placed in a homogenizer for two cycles of 1 minute each with a 30 second dwell period between them, the tube was then centrifuged at room temperature for 5 minutes at 16,000 rcf, supernatant was then collected and used as DNA template for RPA reactions. Next, the homogenizer was replaced as the mechanical lysis with a mortar and pestle; 100 mg of leaf tissue was collected in the same way as above and placed in a clean mortar, 500 µL of extraction buffer was then added to the mortar and the tissue was ground with a pestle, the solution was then pipetted into a 1.5 mL tube and centrifuged at room temperature for 5 minutes at 16,000 rcf, supernatant was then collected and used as DNA template for RPA reactions. Centrifugation was removed next; 100 mg of leaf tissue was collected in the same way as above and placed in a clean mortar, 500 µL of extraction buffer was then added to the mortar and the tissue was ground with a pestle, the solution was then pipetted into a 1.5 mL tube and solids were allowed to settle for 5 minutes, supernatant was then collected and used as DNA template for

RPA reactions. Finally, the need for a mortar was removed; 100 mg of leaf tissue was collected in the same way as above and placed in a 1.5 mL tube, 500 µL of extraction buffer was then added to the 1.5 mL tube, tissue was ground using a small plastic pestle, solids were allowed to settle for 5 minutes, supernatant was then collected and used as DNA template for RPA reactions. This series of extraction methods was tested on two Chardonel vines, one vine was virus free and the other vine was known to be infected with GRBV. Results were visualized on a 1% agarose gel after a Zymo clean-up was performed.

A second extraction buffer was compared to the PEG 200/NaOH solution to determine which was more effective. The second buffer consisted of 0.5 M NaOH in deionized water, this was based off of existing literature (Kishan et al. 2023). A series of four extractions was performed. Each extraction used 100 mg of leaf tissue and 500 µL of extraction buffer. The extractions were; 6% PEG 200 and 0.08% NaOH in a Homogenization Tube, 6% PEG 200 and 0.08% NaOH with a plastic pestle in a 1.5 mL tube, 0.5 M NaOH in a Homogenization Tube, and 0.5 M NaOH with a plastic pestle in a 1.5 mL tube. The PEG 200/NaOH buffer was used for the rest of this study.

Performing Field Trials

The methods of crude extraction and lateral flow visualization mentioned above were performed on the 29th of August, 2023, at the Missouri State Fruit Experiment Station in Mountain Grove, Missouri, USA. RPA reactions were prepared prior to the field test and were transported to the field on ice in a cooler. Each crude sample consisted of five discs punched using a hole puncher. The crude samples were each placed in 500 µL of PEG 200/NaOH extraction buffer in a 1.5 mL tube. The samples were then crushed using a plastic pestle for 5-8

minutes and allowed to settle. After another 5-10 minutes, three distinct layers appeared in the sample. The 1 μL of sample that was placed in the RPA reaction was taken from the middle layer. The RPA reaction was heated using ambient body temperature by clutching the 0.1 mL reaction tubes in hand for 20 minutes. All spin down steps were performed by placing the reaction tubes in a plastic shopping bag and spinning rapidly.

For GRBV, two suspected positives from an asymptomatic variety were selected as test positives. Leaves were collected in two categories: young and old. Young leaves were collected three leaves from the end of the shoot, old leaves were collected three leaves from the beginning of the shoot. As with GVCV, vines two spaces on either side of the positives were selected as test negatives, only young leaves were used for test negatives. A purified DNA positive was used as a control.

For GVCV, a visually symptomatic vine was selected as a test positive. Vines two spaces on either side of the positive vine were selected as test negatives. A known positive *Ampelopsis cordata* was also tested. A purified DNA positive was used as a control.

Testing the Limit of Detection

Multiple experiments were performed to determine the limitations of the RPA on crude extract. A temperature assay was designed to determine the reaction limits for RPA on crude with LF visualization. Four reactions were mixed and 1 μL of crude extract from a known positive plant was added to each. One reaction was then placed in each of the following temperatures for 20 minutes: 37 °C, 32 °C, 27 °C, and 22 °C.

An assay to determine the viability of composite sampling was also performed. Composite sampling consists of combining tissue samples from multiple plants to reduce the

number of tests needed to be performed. In the lab that this study was performed in these composite samples that were collected by using a hole puncher to punch discs of leaf tissue, 5 discs weighed about 100 mg. Crude extraction with a 1.5 mL tube and a plastic pestle was performed using 5 discs from known virus-infected leaves and no discs from virus free leaves, 4 discs from known virus-infected leaves and 1 punch from virus free leaves, 3 discs from known virus-infected leaves and 2 discs from virus free leaves, 2 discs from known virus-infected leaves and 3 discs from virus free leaves, 1 punch from known virus-infected leaves and 4 discs from virus free leaves, and no discs from known virus-infected leaves and 5 discs from virus free leaves. 1 μL of each of these crude extractions was then used as template for RPA reactions and visualized on LF strips. This assay was performed for both GRBV and GVCV.

A detection limit assay was performed using serial dilutions. For both GRBV and GVCV, RPA reactions were performed using the forward and reverse primers designed for this study. These RPA reactions were then cleaned using the Zymo kit protocol and visualized on a 1% agrose gel. The amplicons were then extracted from the gel using the Qiagen MinElute Gel Extraction Kit (MinElute gel extraction kit 2016). Once the amplicons were extracted, concentration in ng/μL was determined using a Thermo Scientific™ Nanodrop™ One. Molecular weight of the amplicons was determined using Molbiotools DNA Calculator (DNA calculator 2023). The concentration of the amplicons was then converted to number of molecules using stoichiometry. A series of dilutions for each virus was performed starting with 10^7 copies and continuing to 10^2 copies. The dilutions were placed in crude extract from a virus free plant to simulate the crude extraction conditions. An RPA reaction was performed on each of the dilutions and visualized on LF strips to determine a detection limit for both GRBV and GVCV. The LF strips were removed from the HybriDetect Assay Buffer after two minutes to help

eliminate false positives (Lu et al. 2021). The serial dilutions and the RPA reaction was performed three times independently for both viruses. After the first set of dilution reactions for each virus showed an ability to detect 10^2 copies, the second and third reaction for both GRBV and GVCV were diluted to 10^0 as the furthest dilution. PCR on gel was then used in a series of dilutions in water for comparison.

False Positive Elimination

Multiple experiments were performed in an attempt to eliminate the presence of false positives in the LF strip visualization method. The first of these attempts was to decrease the volume of the RPA product placed in the HybriDetect Assay Buffer. An RPA reaction assay composed of a crude extraction from a virus free plant, a crude extraction from a known virus-infected plant, a purified DNA positive control, and a no template negative control was created. The reaction products were then visualized on LF strips by mixing decreasing volumes of RPA product with the HybriDetect Assay Buffer. These volumes were 10 µL, 5 µL, and 1 µL. The volume of HybriDetect Assay Buffer was 100 µL for each visualization.

The next experiment was to dilute the RPA product prior to mixing it with the HybriDetect Assay Buffer. Another RPA reaction assay composed of a crude extraction from a virus free plant, a crude extraction from a known positive plant, a purified DNA positive control, and a no template negative control was created. In this experiment, the RPA products were diluted with water prior to mixing with the HybriDetect Assay Buffer. The dilutions were 5 µL RPA product in 5 µL H_2O, 2.5 µL of RPA product in 7.5 µL of H_2O, and 1 µL of RPA product in 9 µL of H_2O. A full-strength control of 10 µL RPA product mixed directly with the HybriDetect Assay Buffer was also used.

RESULTS

Primer Evaluation

Primers and probes fitting the recommendations for RPA were designed for GRBV and GVCV using the CodonCode Aligner software. The primers, probes, and amplicons were then cross referenced with NCBI Nucleotide BLAST (blastn) to ensure specificity. The Blastn results indicated that the primers and probes designed for GRBV and GVCV only amplified the DNA of their respective virus. These primers were evaluated to determine specificity or if any unexpected dimers existed. When visualized on a gel, only the DNA fragments with expected sizes were observed (Fig. 1). When analyzed with LF strips, RPA reaction using the designed primers generated purple bands as expected for both positive samples, and no band for negative control (Fig. 2). A faint band in the negative controls occasionally appeared after an extended period of time, usually between ten and twenty minutes after the strip was put in the reaction.

RPA Product Clean-Up

The RPA reaction forms nucleoprotein complexes that cause streaking and inaccurate banding when visualized on a gel (Piepenburg et al. 2006). Most methods used resulted in streaking on the gel and amplicons appearing higher than expected. To clearly visualize RPA reaction products on a 1% agarose gel, various methods of cleaning the RPA products were tested. When compared with an untreated sample, the heat treatment of 95 ºC showed less streaking but the amplicon remained slightly higher than expected. The phenol chloroform extraction yielded some improvement in the streakiness and band location, but the involved nature of the protocol made it impractical to use. The Zymo Research DNA Clean &

Concentrator™ kit resulted in a clean gel with amplicons appearing where they were expected (Fig. 3).

Eliminating False Positives on Lateral Flow Strips

False positive results can occur in lateral flow testing for various reasons. Dimers between oligonucleotides that are labeled with FAM and biotin are a common problem, however the use of a probe helps to eliminate this issue (Piepenburg et al. 2006). Another reason for false positives in lateral flow that specifically affects RPA is the crowding agent within the RPA reaction. The crowding agent along with some of the proteins within the RPA reaction can disrupt the normal function of the antibodies in the lateral flow test and cause a false positive (Frequently asked questions | Support | TwistDx 2023). To reduce incidences of the occasional false positives, a dilution test was performed. The amount of RPA product added to the HybriDetect Assay Buffer was reduced from 10 µL to 5 µL to 1 µL while maintaining 100 µL of the HybriDetect Assay Buffer. The false positive visually reduced in brightness as the volume of RPA product decreased and at 1 µL of RPA product, the false positive was undetectable. The brightness of the positive controls was not visually altered by the reduction in volume of the RPA product (Fig. 4). However, repeated use of this method showed an inconsistent disappearance of the false positive.

In another attempt to eliminate false positives, the RPA product was diluted in water prior to mixing with the HybriDetect Assay Buffer. The dilutions were 5 µL RPA product in 5 µL H_2O, 2.5 µL of RPA product in 7.5 µL of H_2O, and 1 µL of RPA product in 9 µL of H_2O. As with the volume reduction experiment, the brightness of the false positive reduced as the dilutions progressed. At a dilution of 1 µL of RPA product in 9 µL of H_2O, the false positive was

undetectable. Unlike the volume reduction experiment, band brightness of the true positives also reduced as the dilutions progressed. However, repeated use of this method also showed an inconsistent disappearance of the false positive.

During the detection limit experimentation, another method of reducing false positives was used. Based on literature, the LF strips were removed from the HybriDetect Assay Buffer after two minutes (Lu et al. 2021). This method resulted in the most consistent reduction of the false positive of the methods attempted in this study.

Developing Crude Leaf Extraction for RPA

The RPA reaction is less sensitive to inhibitors than PCR. Because of this, a crude DNA extraction method can be used with less equipment and time than would be required for PCR (Lu et al. 2021). The crude extraction was performed using a series of increasingly rudimentary conditions for mechanical lysis. The solution in which the plant tissue was lysed remained consistent throughout experiments; 6% PEG 200 and 0.08% NaOH in H_2O. The equipment simplifications were: a Homogenization Tube (OPS Diagnostics) in a homogenizer along with centrifugation; a mortar and pestle along with centrifugation; a mortar and pestle allowed to settle; and a plastic pestle in a 1.5 mL tube allowed to settle. Each of these methods resulted in detection of GRBV from virus-infected Chardonel grapevines (Fig. 5).

Along with the development of equipment-free crude extraction, a comparative test was performed between two crude extraction solutions present in previous literature to determine which was more effective. The two extraction solutions were; 6% PEG 200 and 0.08% NaOH in H_2O and 0.5 M NaOH in H_2O (Kishan et al., 2023). These two solutions were compared using the two extremes of mechanical lysis, the Homogenization Tube method and the plastic pestle

method. The PEG 200/NaOH solution resulted in detection in positive plants while the 0.5 M NaOH solution resulted in no detection (Fig. 6).

Performing Equipment-Free Testing

The goal of this study is to develop simple, equipment-free methods of detecting GVCV and GRBV that can be performed onsite. On the 29th of August, 2023, an onsite trial was performed to test GRBV and GVCV at the Missouri State Fruit Experiment Station in Mountain Grove, Missouri, USA. A visually symptomatic positive plant was selected for GVCV testing while asymptomatic grapevines were selected for GRBV. Using the equipment-free method developed in this study, GVCV was successfully detected onsite within 35 minutes of sampling (Fig. 7). GRBV was not detected in the suspected positive plant, however the purified DNA positive control resulted in a positive LF strip under field conditions.

Testing the Limit of Detection

The temperature limitations of the equipment-free method were tested using a series of decreasing temperatures. The RPA reaction with LF strips performed under conditions as low as 32 °C. The optimal temperature for reactions run for 20 minutes was determined to be 37 °C.

The primary diagnostics method of PCR in the lab involves taking composite samples of five plants. To determine whether RPA could perform under these conditions, an assay was designed to find a detection limit to the crude extract. The assay involved replacing discs of virus-positive plant leaves with discs of virus-free plant leaves from five to one. The resulting RPA reactions were visualized on LF strips. Both viruses were detectable in a single disc of positive tissue combined with four discs of negative tissue (Fig. 8).

The limit of detecting both viruses in the crude extraction was also tested. This was done using a series of dilutions of the amplicon in a crude leaf extraction. This test was performed three times independently for both GVCV and GRBV. In the case of GVCV, the detection limit was determined to be 100 copies. In the case of GRBV, the detection limit was determined to be 1,000 copies (Fig. 9). The limit of detection by the standard PCR using the same primers was performed also. The limit of detection of GVCV was determined to be 10^4 copies.

DISCUSSION

False Positives

The appearance of false positives in LF visualization of RPA reaction products is well known (Lu et al. 2021; Ivanov et al. 2021; Lobato and O'Sullivan 2018). There are two main causes of these false positives. The first potential cause is a dimer formed from the FAM and biotin labeled oligonucleotides. In theory, the only scenario where both the FAM and biotin are attached to the same strand of DNA is when the target DNA is present for both to bind to. However, if no precaution is taken to ensure that no dimers form between the two labeled oligonucleotides, a false positive can occur (Ivanov et al. 2021). The second potential cause is interference from the RPA reagents themselves. The proteins and crowding agents in the RPA reaction can cause the antigens in the LF strip to bind without the presence of antigenically labeled amplicons, resulting in a false positive (Frequently asked questions | Support | TwistDx 2023). The false positives that appeared throughout this study are most likely due to the second cause for a number of reasons.

Dimer-related false positives were observed early in this study. Prior to designing primers and probes specifically for this study, RPA primers were created by extending previously designed primers to the desired 30-35 bp length of RPA. These primers showed successful amplification on agarose gel and were subsequently labeled with FAM on the forward and biotin on the reverse. When these labeled primers were used in an RPA reaction and visualized on LF strips, distinct positive lines appeared consistently in the negative controls. These RPA reactions were then verified with agarose gel visualization and no positive bands were seen in the negative controls, ruling out contamination in the reactions. When probes were designed for these primers

in the hope of eliminating the false positives, the same results were obtained. This was true for both viruses. These primers and probes were later analyzed using ThermoFisher Multiple Primer Analyzer using the highest sensitivity setting available and multiple dimers were found between the labeled primers and probes (Multiple primer analyzer - US 2023). As a direct result of this, the highest priority for designing primers for this study was to avoid dimers between the labeled primers and probes.

The false positives that appeared throughout this study differed from the dimer-related false positives in multiple ways. First, the dimer related false positives appeared consistently. The positives in this study did not show up consistently and could not be predicted. The dimer related false positives also appeared brighter than the false positives in this study and were indistinguishable from true positives. The false positives in this study varied in brightness but were consistently less bright than true positives. The false positives in this study also took longer to appear than their dimer counterparts.

Parallel to this study, a matrix of primer and probe concentrations was created to attempt to eliminate the false positives if they were dimer related. These concentration changes did not affect the appearance of the false positives enough to make a claim that the false positives are dimer-related. To further prove that the false positives in this study were not dimer-related, an RPA reaction that excluded oligonucleotides was performed and visualized on a LF strip. The false positive still appeared in the oligo-free reaction. This result is the key factor in the claim that the false positives that appear in this study are not dimer-related.

With the false positives established to be caused by the RPA reaction itself, different solutions needed to be developed. TwistDx recommends that RPA products be diluted prior to visualizing on LF strips (Frequently asked questions | Support | TwistDx 2023). Within this

study, multiple attempts to dilute the RPA products were made (Fig. 4). Initially, results were promising. However, repeated use of both methods of dilution showed inconsistency with their ability to remove the false positives. While the appearance of false positives within RPA LF visualization is well documented, few studies provide specific methodology to address these false positives.

An observation made during this study showed that the false positives were time sensitive. The true positives consistently appeared earlier than the false positives, a difference of between 1-5 minutes was noted. Initially, it was suggested that the LF strips only be read before 5 minutes due to this observation, but occasionally some false positives appeared too close to this time frame to be a satisfactory solution. After this time sensitivity trait was observed, a review of literature revealed a potential solution. One LF visualization protocol suggested that LF strips be removed after only two minutes in the strip solution (Lu et al. 2021). The authors do not specify why the LF strips should be removed quickly, however, the observations within this study suggest that this was a solution to a false positive problem. When the LF strip removal after 2 minutes was repeated in this study, the false positives stopped appearing while having little effect on the brightness of bands in the true positives.

Field Detection

GVCV was detected in grapevines in a vineyard using the equipment-free method developed in this study. The method was simple, requiring no major equipment, and rapid, with results determined within 35 minutes. This diagnostic method could be used by growers to determine if a suspected vine is infected in under an hour while remaining onsite, compared to the lengthy process of DNA purification and PCR that conventional diagnostics requires. The

proposed method of diagnostics is primarily designed with small sample sizes in mind. The mechanical lysis step using a plastic pestle becomes a bottleneck with a large sample size. This is partially remedied by adopting a composite sampling of five vines into a single reaction. If the composite sample is negative, then the grower knows that five vines are negative for the virus in question. If the composite is positive, the grower will need to perform individual tests to determine which of the five vines is positive. Another practical use of the proposed diagnostic method is to screen vines prior to purchase. This test would allow growers to know if vines are infected prior to making financial investments in purchasing and planting the vines.

GRBV was not detected in a field vine during this study. There are multiple factors that could have caused this failure to detect GRBV in the field. The vines selected as the test positives for GRBV were of an asymptomatic grapevine variety and were suspected positive based on composite PCR screening of the vineyard from the prior year. These dubious positives were unable to be confirmed using RPA or PCR on agarose gel due to their removal from the vineyard soon after the initial field trial was performed. Another factor in the failure of this test was sunlight. The FAM that labels the probe is photosensitive (Lanfranco et al. 2010). This photosensitivity gives the RPA reaction's readability on LF strips a limited time. The longer the probe is exposed to sunlight, the dimmer the positive bands will be. This was observed during the field trial as the GRBV test was the second performed and the purified DNA positive control for GRBV was dimmer than the purified DNA positive control for GVCV. To help with this photosensitivity issue, opaque reaction tubes could be used to limit the exposure of the FAM labeled probes to sunlight.

RPA based diagnostics has been developed for GRBV (Li et al. 2017). However, this method requires the purchase of a starter kit that includes a heat block and means of mechanical

lysis. The method developed here requires no heating equipment and uses a small, disposable plastic pestle for mechanical lysis.

Sensitivity

The detection limit of the crude extraction method for GVCV was determined to be 100 copies of the amplicon. RPA on LF strip has been shown to be able to detect single copies of amplicons, however, most detection limits fall between 10 and 1,000 copies (Lobato and O'Sullivan 2018). This places the detection limit of the GVCV method in the middle of the range of sensitivity. The detection limit of the crude extraction method for GRBV was determined to be 1,000 copies of amplicon. This puts the GRBV method on the lower end of the range of sensitivity. This sensitivity is also lower than another GRBV detection method using RPA that found a detection limit of 11-14 copies of amplicon cloned in a recombinant plasmid (Li et al. 2017).

Table 1. A list of primers used in this study.

Name	Sequence (5' to 3')	Position (nucleotides)	Amplicon (bp)	Gene
GRBV				
CP f	5'-agcggaagcatgattgagacattgacg-3'	1,073-1,099		
CP r	5'-aacgtatgtccacttgcagaagccgc-3'	1,329-1,304	231	CP
Rep f	5'-caagtcgttgtagattgaggacgtattgg-3'	2,567-2,595		
Rep r	5'-agccacacctacacgccttgctcatc-3'	2,884-2,850	318	Rep
GRBV RPA 1553 F	5'-gcgcggagaggtgacaaagacttacaagcgaa-3'	1,553-1,584		
GRBV RPA 1590 Probe	5'-gcattgactgaacctgatcgtagtagaactHaagttgaagaattgagag-3'	1,590-1,638		
GRBV RPA 1849 R	5'-tcgtgcctgattgtctggtattgaagtgtga-3'	1,819-1,849	300	V3
GVCV				
1320 RPA f	5'-ctcaggtccagaccctcaacgatcgtattg-3'	1320-1349		
1411 RPA r	5'-ggagatttgttctaccacgttatcaggaag-3'	1382-1411	92	ORF II
389 RPA f	5'-gaaaacagcagtaggagaggacggacaact-3'	389-418		
563 RPA r	5'-aaccgtgtaggggtgtgcgttcagatctct-3'	534-563	175	IR/ORF I
GVCV RPA 5389 F	5'-atcctcgcagggaatgatttctacattcct-3'	5,389-5,418		
GVCV RPA 5537 Probe	5'-ccatcaccaggattcaaactaccctggaacHtcaaaagatagcatactt-3'	5,537-5,585		
GVCV RPA 5648 R	5'-ggctcaggcatacttgcagctgcgagctca-3'	5,619-5,648	260	ORF III

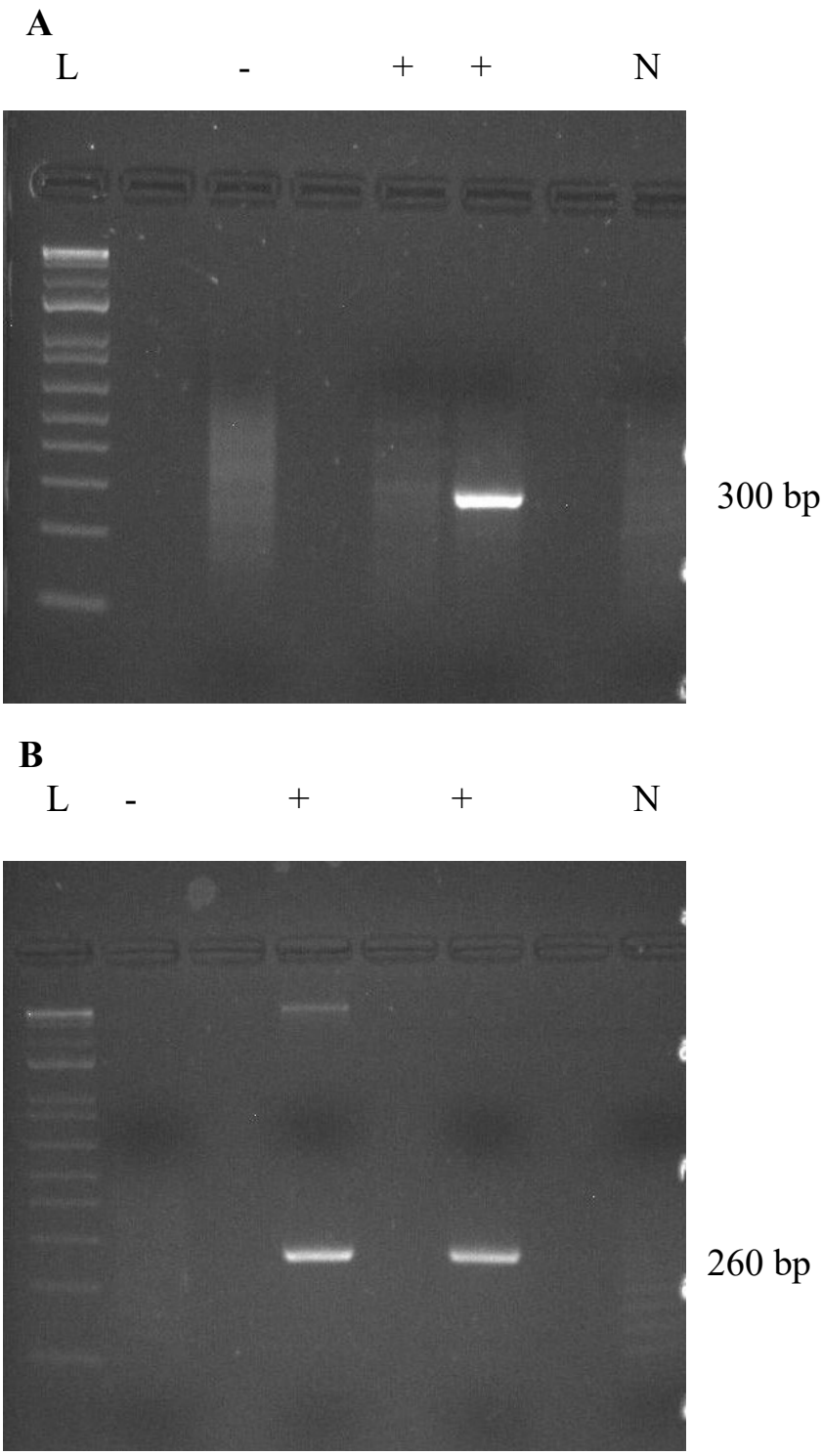

Fig. 1. Evaluation of GRBV and GVCV specific primers for RPA. **A**. GRBV primers located in the V3 gene that are used to amplify a DNA fragment of 300 bp. **B**. GVCV primers located in the ORF III that are used to amplify a DNA fragment of 260 bp. **L**: DNA ladder; **-** ; DNA that was extracted from virus-free grapevine leaf tissues; **+**: DNA that was extracted from virus-infected leaf tissues; **N**: negative control in which no DNA template was added.

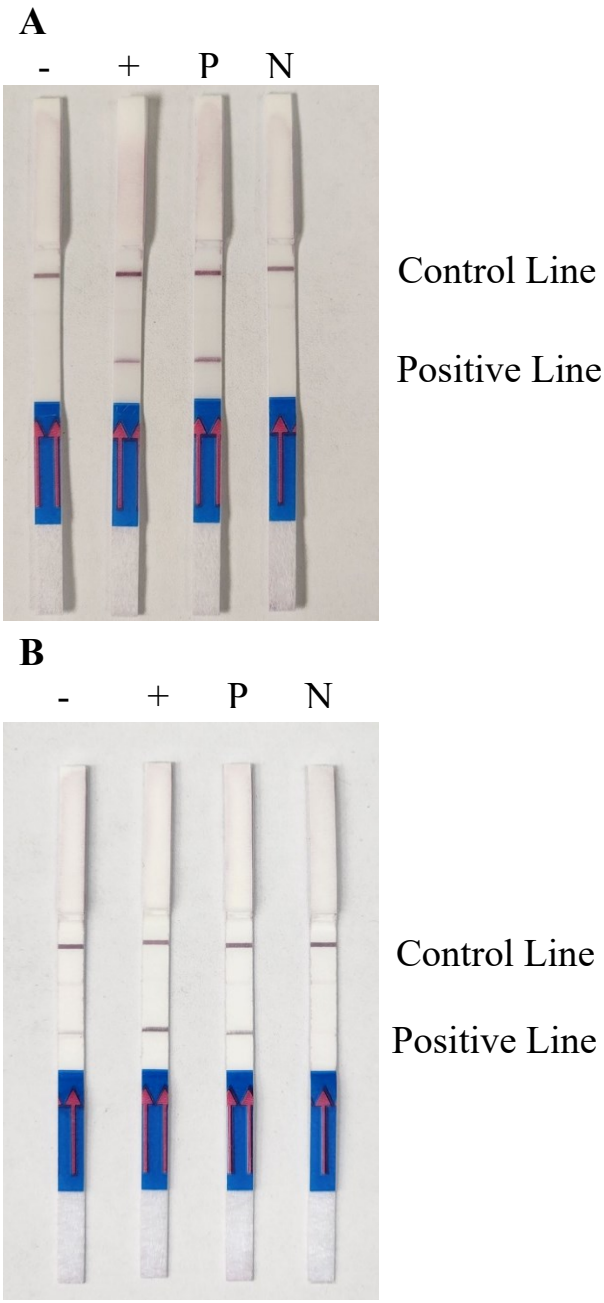

Fig. 2. Screening of GRBV and GVCV specific primers and probes for RPA on lateral flow assay. **A**. GRBV primers and probe located in the V3 gene that create a DNA fragment that has a biotin and FAM label. **B.** GVCV primers located in the ORF III that create a DNA fragment that has a biotin and FAM label. **- :** crude extract from a virus-free grapevine leaf tissue; +: crude extract from a virus-infected grapevine leaf tissue; **P**: grapevine DNA that was extracted from virus-infected leaf tissues; **N**: negative control in which no DNA template was added.

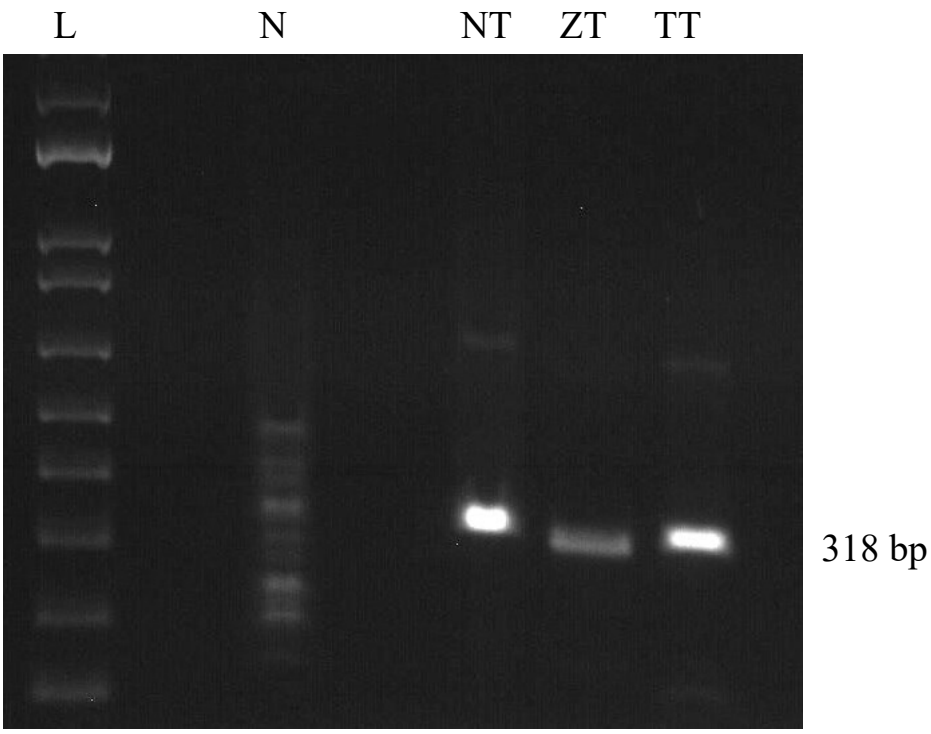

Fig. 3. Testing methods for cleaning up RPA-generated DNA fragments that were amplified using GRBV Rep primers with an expected amplicon of 318 bp. **L**: a DNA ladder; **N:** no template negative control; **NT** is no treatment; **ZT**: the Zymo^{TM} DNA Clean-Up Kit; **TT**: treatment at 95^{o}C.

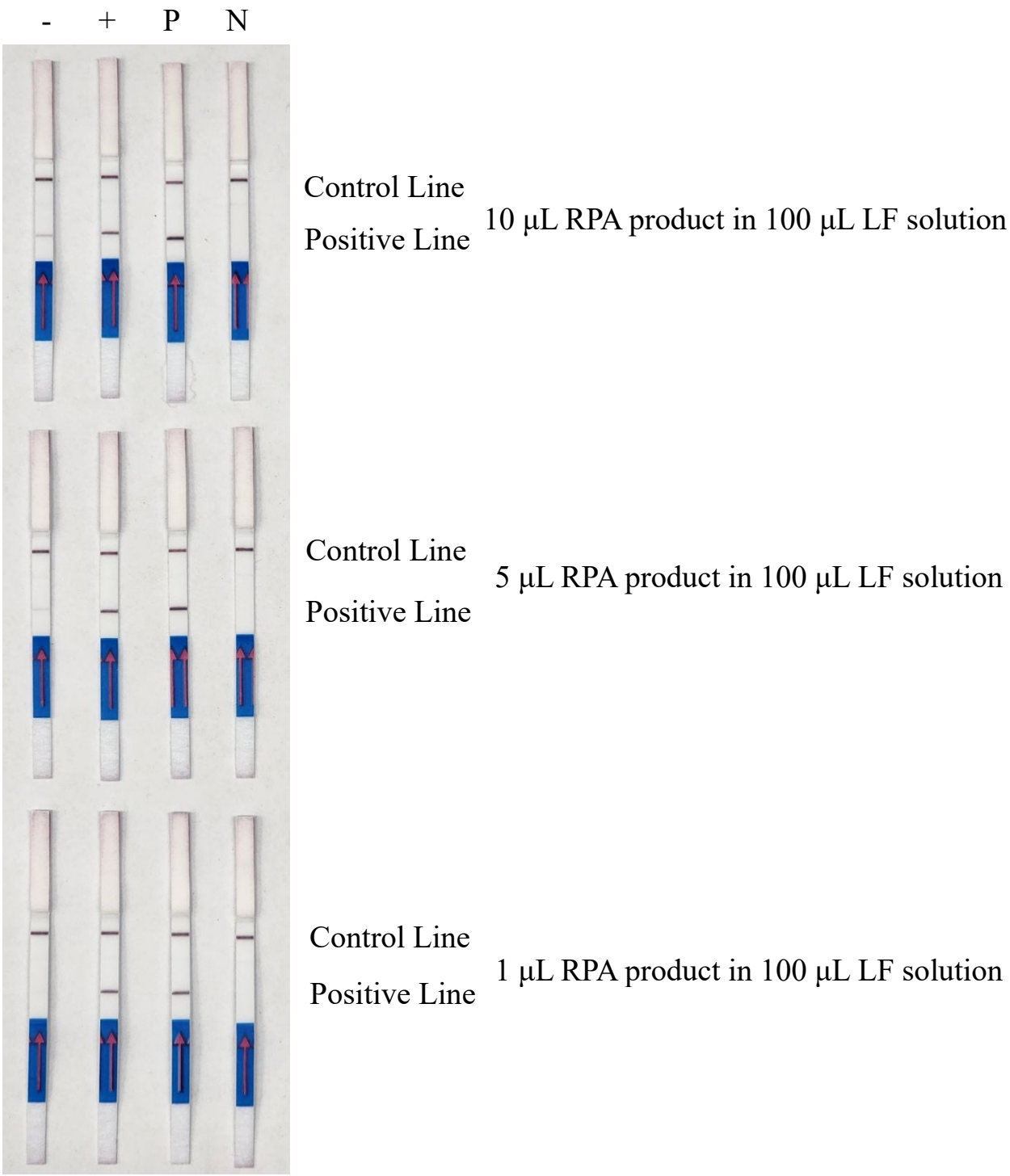

Fig. 4. Dilution of RPA product to eliminate false positives in lateral flow assay created by crowding agent interference. GVCV primers and probe located in the ORF III that create a DNA fragment that has a biotin and FAM label. **-** : crude extraction from virus-free grapevine leaves, **+:** crude extraction from virus-infected grapevine leaves; **P**: DNA extraction from virus-infected grapevine leaves; **N**: a no template negative control.

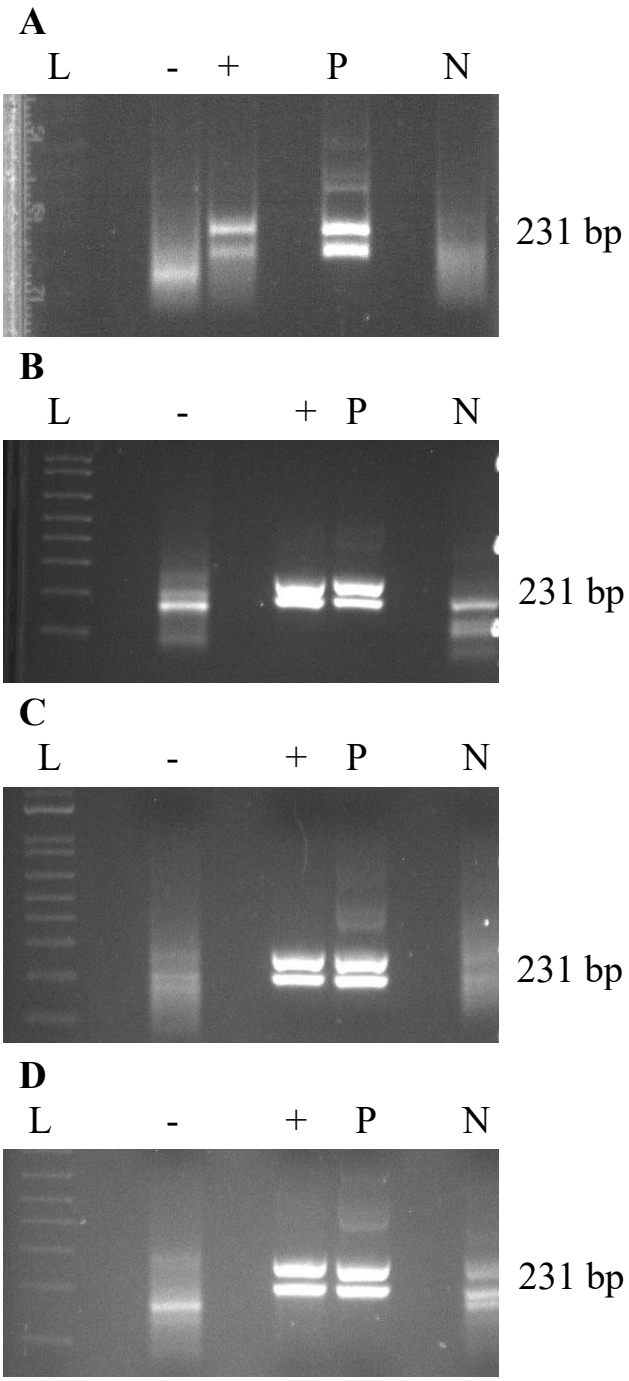

Fig. 5. Developing a mechanical lysis method of getting crude leaf extraction for RPA detection of GRBV using CP primers for an amplicon size of 231 bp. **A**. Lysis using a bead beater tube and centrifugation; **B**. Lysis using a mortar and pestle and centrifugation; **C**. Lysis using a mortar and pestle and self-settling; **D**. Lysis using a plastic pestle in a 1.5 ml plastic tube and self-settling. **L:** a DNA ladder, **-** : crude extraction from virus-free grapevine leaves, **+:** crude extraction from virus-infected grapevine leaves; **P**: DNA extraction from virus-infected grapevine leaves; **N**: negative control in which no DNA template was added.

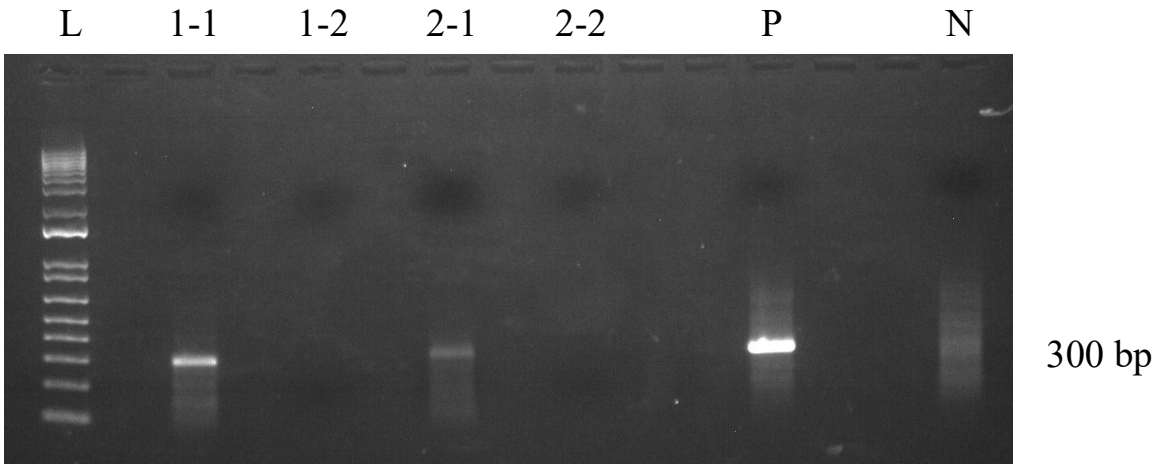

Fig. 6. Comparison of crude leaf extraction methods for RPA detection of GRBV using primers for an amplicon size of 300 bp. **1-1.** Crude leaf extraction using a plastic pestle in a 1.5 mL tube and a solution of 6% PEG 200 and 0.08% NaOH, **1-2**: crude leaf extraction using a plastic pestle in a 1.5 mL tube and a solution of 0.5M NaOH, **2-1**: crude leaf extraction using a bead beater tube with centrifugation and a solution of 6% PEG 200 and 0.08% NaOH; **2-2** crude extraction using a bead beater tube with centrifugation and a solution of 0.5 M NaOH. **L** is a DNA ladder, **P** is a purified DNA positive control, and **N** is a no template negative control.

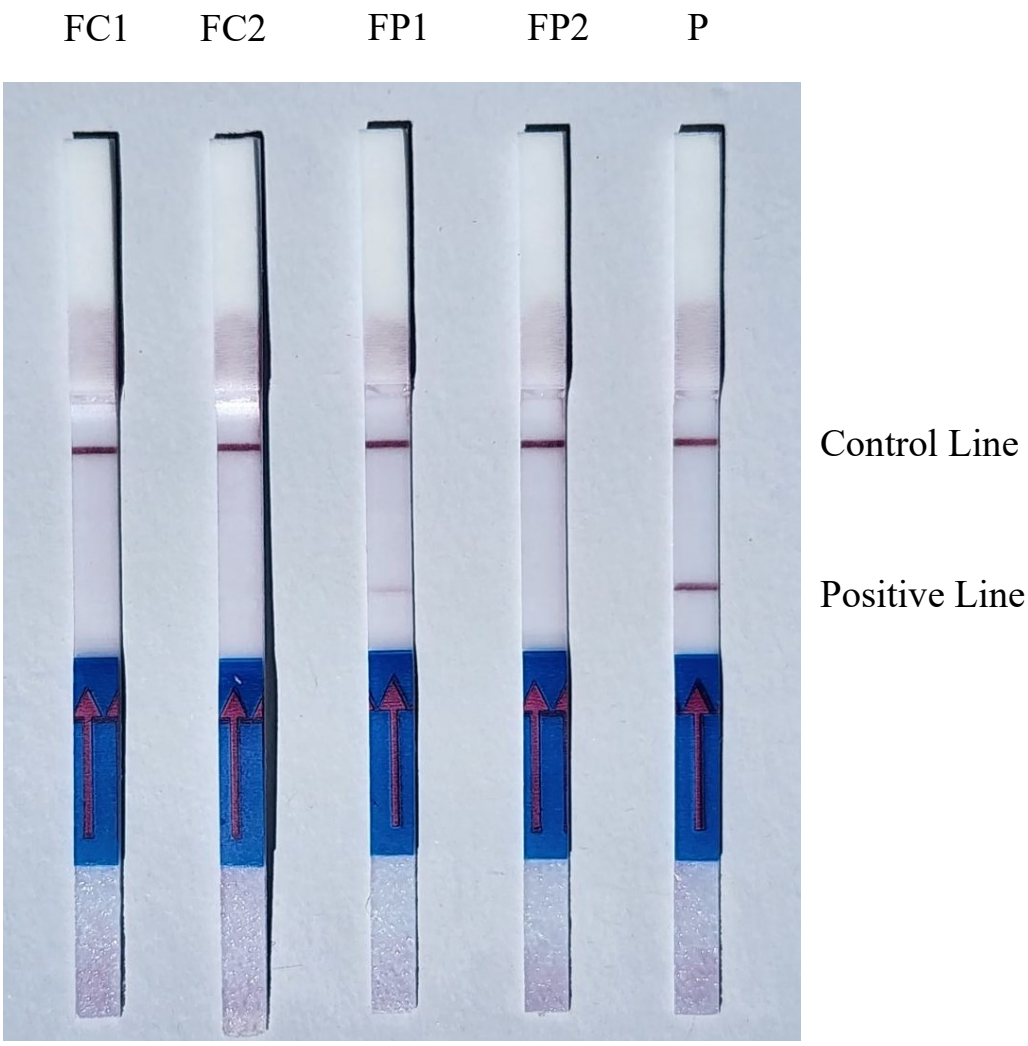

Fig. 7. Field trial of the equipment-free RPA method for detecting GVCV. GVCV primers and probe located in the ORF III that create a DNA fragment that has a biotin and FAM label. **FC1**: Crude extract from a grapevine two vines away from GVCV-infected grapevine in the row. **FC2**: Crude extract from a grapevine two vines away from GVCV-infected grapevine in the row in the opposite direction. **FP1**: Crude extract from the GVCV-infected grapevine that shows symptoms. **FP2**: Crude extract from a GVCV-infected *Ampelopsis cordata* grown in greenhouse conditions. **P**: Purified DNA positive control.

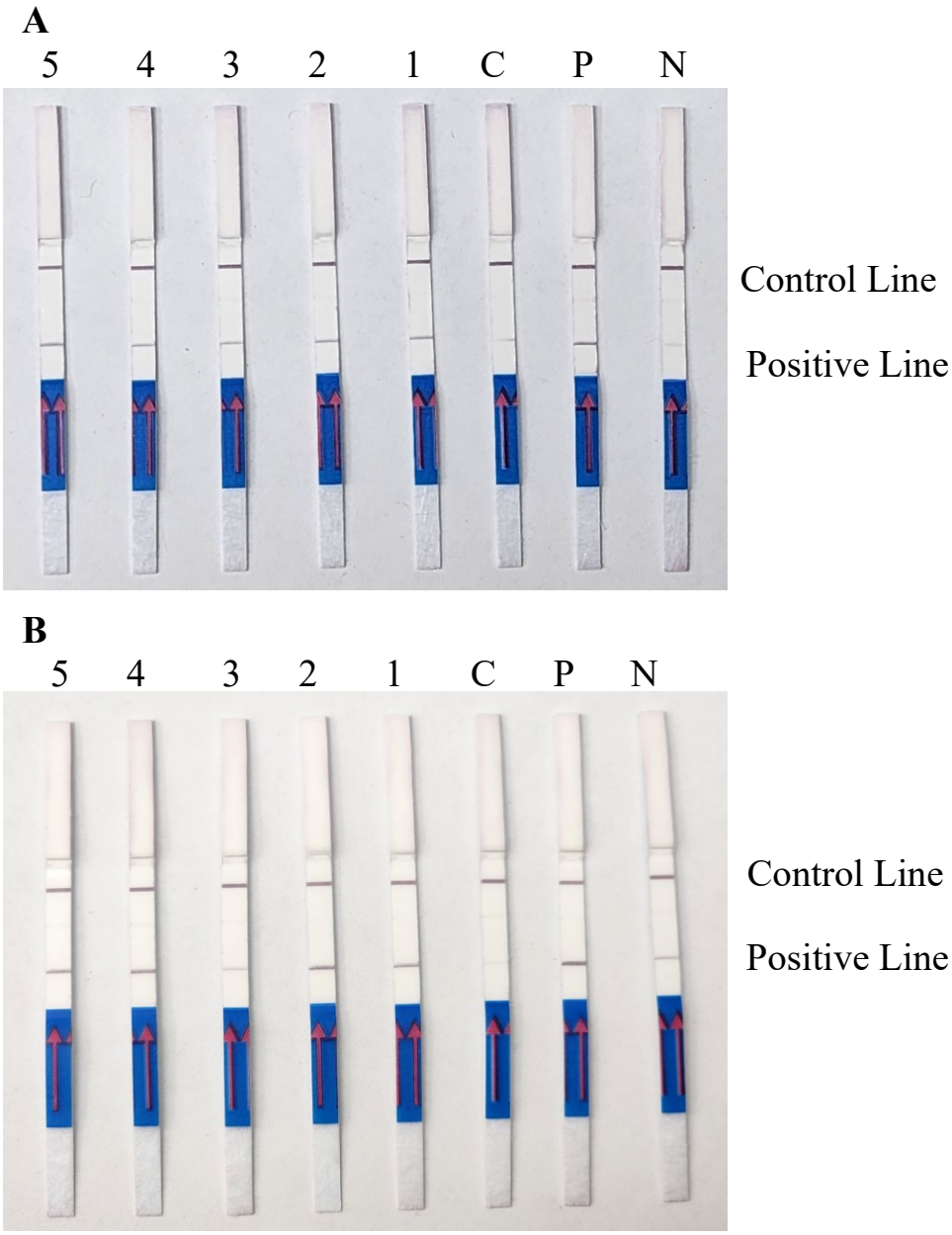

Fig. 8. Composite sampling of grapevines using crude extraction methods and RPA on lateral flow assay. **A**. GRBV primers and probe located in the V3 gene that create a DNA fragment that has a biotin and FAM label. **B.** GVCV primers and probe located in the ORF III that create a DNA fragment that has a biotin and FAM label. **5**: crude extract from five discs of leaf tissue from a virus-infected grapevine and no discs from a virus-free grapevine; **4**: crude extract from four discs of leaf tissue from a virus-infected grapevine and one disc from a virus-free grapevine; **3**: crude extract from three discs of leaf tissue from a virus-infected grapevine and two discs from a virus-free grapevine; **2**: crude extract from two discs of leaf tissue from a virus-infected grapevine and three discs from a virus-free grapevine; **1**: crude extract from one disc of leaf tissue from a virus-infected grapevine and four discs from a virus-free grapevine; **C**: crude extract from a virus-free grapevine; **P**: grapevine DNA that was extracted from virus-infected leaf tissues; **N**: negative control in which no DNA template was added.

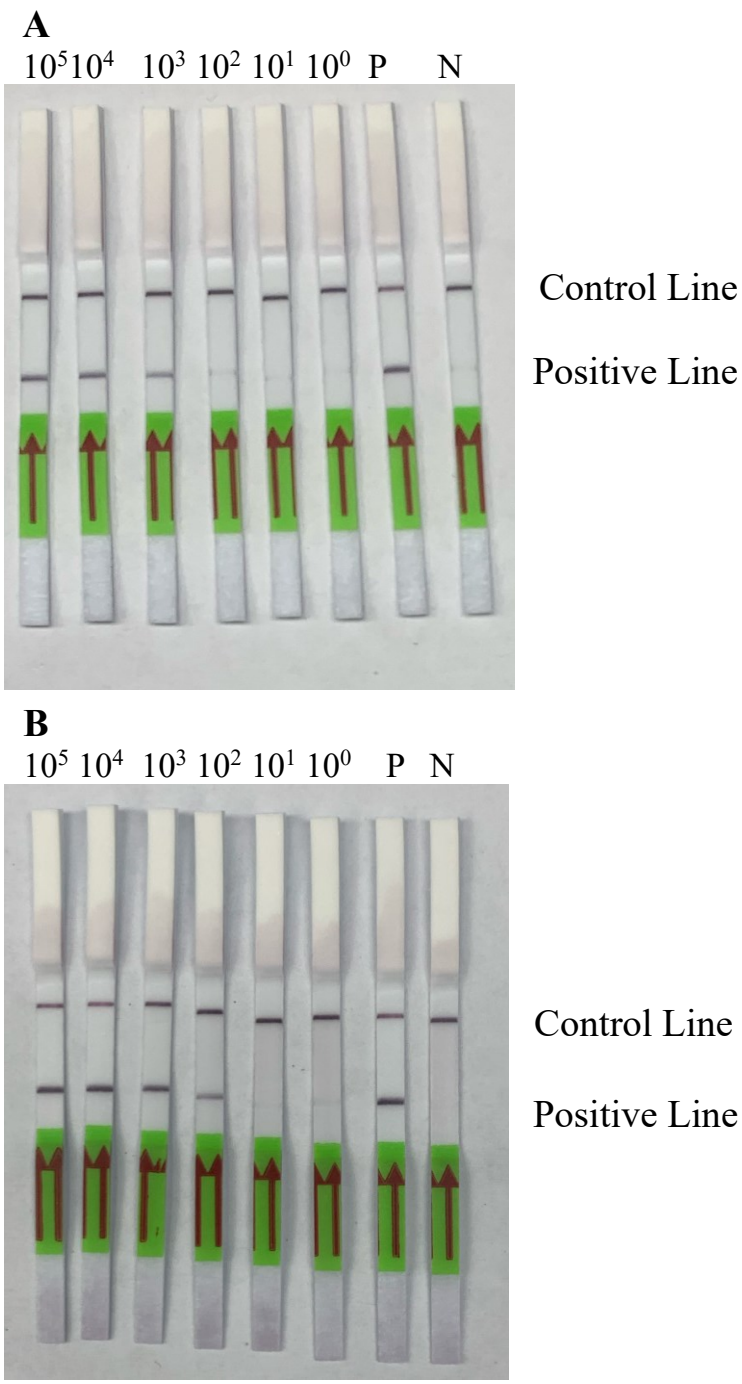

Fig. 9. Testing the limit of detection of crude extraction methods with RPA on lateral flow assay. **A**. GRBV primers and probe located in the V3 gene that create a DNA fragment that has a biotin and FAM label. **B.** GVCV primers and probe located in the ORF III that create a DNA fragment that has a biotin and FAM label. **10^5**: 10^5 copies of amplicon in crude extract; **10^4**: 10^4 copies of amplicon in crude extract; **10^3**: 10^3 copies of amplicon in crude extract; **10^2**: 10^2 copies of amplicon in crude extract; **10^1**: 10^1 copies of amplicon in crude extract; **10^0**: 10^0 copies of amplicon in crude extract; **P**: plant DNA that was extracted from virus-infected leaf tissues; **N**: negative control in which no DNA template was added.

LITERATURE CITED

Alston, J. M., and Sambucci, O. 2019. Grapes in the World Economy. In *The Grape Genome*, Compendium of Plant Genomes, eds. Dario Cantu and M. Andrew Walker. Cham: Springer International Publishing, p. 1–24. Available at: https://doi.org/10.1007/978-3-030-18601-2_1 [Accessed October 2, 2023].

Ambers, C. P. 2013. A historical hypothesis on the origin of the Norton grape. Journal of Wine Research. 24:85–95.

Bahder, B. W., Zalom, F. G., and Sudarshana, M. R. 2016. An evaluation of the flora adjacent to wine grape vineyards for the presence of alternative host plants of grapevine red blotch-associated virus. Plant Disease. 100:1571–1574.

Beach, S., Kovens, M., Hubbert, L., Honesty, S., Guo, Q., Pap, D., et al. 2017. Genetic and phenotypic characterization of *Grapevine vein clearing virus* from wild *Vitis rupestris*. Phytopathology®. 107:138–144.

Bertazzon, N., Migliaro, D., Rossa, A., Filippin, L., Casarin, S., Giust, M., et al. 2021. Grapevine red blotch virus is sporadically present in a germplasm collection in northern Italy. Journal of Plant Diseases and Protection. 128:1115–1119.

Carlberg, D. 2005. Lateral-flow assays: assembly and automation. In *Drugs of Abuse*, eds. Raphael C. Wong and Harley Y. Tse. Totowa, NJ: Humana Press, p. 99–114. Available at: http://link.springer.com/10.1007/978-1-59259-951-6_6 [Accessed May 7, 2023].

Cieniewicz, E., Thompson, J. R., McLane, H., Perry, K. L., Dangl, G. S., Corbett, Q., et al. 2018. Prevalence and genetic diversity of grabloviruses in free-living *Vitis* spp. Plant Disease. 102:2308–2316.

Daher, R. K., Stewart, G., Boissinot, M., and Bergeron, M. G. 2016. Recombinase polymerase amplification for diagnostic applications. Clinical Chemistry. 62:947–958.

Ding, S.-W., and Voinnet, O. 2007. Antiviral immunity directed by small RNAs. Cell. 130:413–426.

DNA calculator. 2023. Available at: https://molbiotools.com/dnacalculator.php [Accessed October 5, 2023].

Flasco, M., Hoyle, V., Cieniewicz, E. J., Roy, B. G., McLane, H. L., Perry, K. L., et al. 2021. Grapevine red blotch virus is transmitted by the three-cornered alfalfa hopper in a circulative, nonpropagative mode with unique attributes. Phytopathology®. 111:1851–1861.

Formosa, T., and Alberts, B. M. 1986. Purification and characterization of the T4 bacteriophage uvsX protein. Journal of Biological Chemistry. 261:6107–6118.

Frequently asked questions | Support | TwistDx. 2023. TwistDx limited. Available at: https://www.twistdx.co.uk/support/faqs/ [Accessed September 18, 2023].

Fung, R. W. M., Gonzalo, M., Fekete, C., Kovacs, L. G., He, Y., Marsh, E., et al. 2008. Powdery mildew induces defense-oriented reprogramming of the transcriptome in a susceptible but not in a resistant grapevine. Plant Physiology. 146:236–249.

Guo, Q., Honesty, S., Xu, M. L., Zhang, Y., Schoelz, J., and Qiu, W. 2014. Genetic diversity and tissue and host specificity of *Grapevine vein clearing virus*. Phytopathology®. 104:539–547.

Haible, D., Kober, S., and Jeske, H. 2006. Rolling circle amplification revolutionizes diagnosis and genomics of geminiviruses. Journal of Virological Methods. 135:9–16.

Harris, L. D., and Griffith, J. D. 1988. Formation of D-loops by the uvsx protein of T4 bacteriophage: a comparison of the reaction catalyzed in the presence or absence of gene 32 protein. Biochemistry. 27:6954–6959.
How to use phenol / chloroform for DNA purification. 2023. thermofisher.com. Available at: https://www.thermofisher.com/us/en/home/references/protocols/nucleic-acid-purification-and-analysis/dna-extraction-protocols/phenol-chloroform-extraction.html [Accessed October 4, 2022].
Hoyle, V., Flasco, M. T., Choi, J., Cieniewicz, E. J., McLane, H., Perry, K., et al. 2022. Transmission of grapevine red blotch virus by *Spissistilus festinus* [say, 1830] (hemiptera: membracidae) between free-living vines and *Vitis vinifera* 'Cabernet Franc.' Viruses. 14:1156.
HybriDetect. 2019. Available at: https://www.milenia-biotec.com/uploads/2019/07/MGHD_C.pdf.
Ivanov, A. V., Safenkova, I. V., Zherdev, A. V., and Dzantiev, B. B. 2021. Recombinase polymerase amplification assay with and without nuclease-dependent-labeled oligonucleotide probe. International Journal of Molecular Sciences. 22:11885.
Kaunitz, J. D. 2015. The discovery of PCR: ProCuRement of divine dower. Digestive Diseases and Sciences. 60:2230–2231.
Kishan, G., Kumar, R., Sharma, S. K., Srivastava, N., Gupta, N., Kumar, A., et al. 2023. Development and application of crude sap-based recombinase polymerase amplification assay for the detection and occurrence of grapevine geminivirus A in Indian grapevine cultivars. Frontiers in Plant Science. 14:1151471.
Kralik, P., and Ricchi, M. 2017. A basic guide to real time PCR in microbial diagnostics: definitions, parameters, and everything. Frontiers in Microbiology. 8 Available at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00108/full [Accessed May 6, 2023].
Krenz, B., Thompson, J. R., Fuchs, M., and Perry, K. L. 2012. Complete genome sequence of a new circular DNA virus from grapevine. Journal of Virology. 86:7715–7715.
Krenz, B., Thompson, J. R., McLane, H. L., Fuchs, M., and Perry, K. L. 2014. *Grapevine red blotch-associated virus* is widespread in the United States. Phytopathology®. 104:1232–1240.
Lanfranco, M. F., Anastasio, N. C., Seitz, P. K., and Cunningham, K. A. 2010. Chapter Eighteen - Quantification of RNA editing of the serotonin 2C receptor (5-HT2CR) ex vivo. In *Methods in Enzymology*, Constitutive Activity in Receptors and Other Proteins, Part B, ed. P. Michael Conn. Academic Press, p. 311–328. Available at: https://www.sciencedirect.com/science/article/pii/B978012381296400018X [Accessed October 22, 2023].
Levin, J. D., Johnson, A. W., and Demple, B. 1988. Homogeneous *Escherichia coli* endonuclease IV. Characterization of an enzyme that recognizes oxidative damage in DNA. Journal of Biological Chemistry. 263:8066–8071.
Li, R., Fuchs, M. F., Perry, K. L., Mekuria, T., and Zhang, S. 2017. Development of a fast AmplifyRP Acceler8 diagnostic assay for grapevine red blotch virus. Journal of Plant Pathology. 99 Available at: https://doi.org/10.4454/jpp.v99i3.3952 [Accessed October 22, 2023].
Lobato, I. M., and O'Sullivan, C. K. 2018. Recombinase polymerase amplification: Basics, applications and recent advances. Trends in Analytical Chemistry. 98:19–35.
Lu, X., Xu, H., Song, W., Yang, Z., Yu, J., Tian, Y., et al. 2021. Rapid and simple detection of *Phytophthora cactorum* in strawberry using a coupled recombinase polymerase amplification–lateral flow strip assay. Phytopathology Research. 3:12.

Martelli, G. P. 2017. An Overview on Grapevine Viruses, Viroids, and the Diseases They Cause. In *Grapevine Viruses: Molecular Biology, Diagnostics and Management*, eds. Baozhong Meng, Giovanni P. Martelli, Deborah A. Golino, and Marc Fuchs. Cham: Springer International Publishing, p. 31–46. Available at: https://doi.org/10.1007/978-3-319-57706-7_2 [Accessed October 22, 2023].
MinElute gel extraction kit. 2016. Available at: https://www.qiagen.com/us/resources/resourcedetail?id=0d0d73a7-e906-4a74-8f11-3b70311d7d66&lang=en [Accessed October 13, 2023].
Multiple primer analyzer - US. 2023. thermofisher.com. Available at: https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html [Accessed October 20, 2023].
Okazaki, T., and Kornberg, A. 1964. Enzymatic synthesis of deoxyribonucleic acid. Journal of Biological Chemistry. 239:259–268.
Perry, K. L., McLane, H., Hyder, M. Z., Dangl, G. S., Thompson, J. R., and Fuchs, M. F. 2016. Grapevine red blotch-associated virus is present in free-living *Vitis* spp. proximal to cultivated grapevines. Phytopathology®. 106:663–670.
Petersen, S. M., Keith, C., Austin, K., Howard, S., Su, L., and Qiu, W. 2019. A natural reservoir and transmission vector of grapevine vein clearing virus. Plant Disease. 103:571–577.
Piepenburg, O., Williams, C. H., Stemple, D. L., and Armes, N. A. 2006. DNA detection using recombination proteins ed. James Haber. PLOS Biology. 4:e204.
Qiu, W., Avery, J. D., and Lunden, S. 2007. Characterization of a severe virus-like disease in Chardonnay grapevines in Missouri. Plant Health Progress. 8:39.
Qiu, W., Petersen, S. M., and Howard, S. 2020. North American grape 'Norton' is resistant to grapevine vein clearing virus. Plant Disease. 104:2051–2053.
Qiu, W., and Schoelz, J. 2017. Grapevine vein clearing virus: diagnostics, genome, genetic diversity, and management. In *Grapevine Viruses: Molecular Biology, Diagnostics and Management*, eds. Baozhong Meng, Giovanni P. Martelli, Deborah A. Golino, and Marc Fuchs. Cham: Springer International Publishing, p. 315–330. Available at: http://link.springer.com/10.1007/978-3-319-57706-7_15 [Accessed May 1, 2023].
Ricketts, K. D., Gómez, M. I., Fuchs, M. F., Martinson, T. E., Smith, R. J., Cooper, M. L., et al. 2017. Mitigating the economic impact of grapevine red blotch: optimizing disease management strategies in U.S. vineyards. The American Journal of Enology and Viticulture. 68:127–135.
Rwahnih, M. A., Dave, A., Anderson, M. M., Rowhani, A., Uyemoto, J. K., and Sudarshana, M. R. 2013. Association of a DNA virus with grapevines affected by red blotch disease in California. Phytopathology®. 103:1069–1076.
Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., et al. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 230:1350–1354.
Sapkota, S., Chen, L.-L., Yang, S., Hyma, K. E., Cadle-Davidson, L., and Hwang, C.-F. 2019. Construction of a high-density linkage map and QTL detection of downy mildew resistance in *Vitis aestivalis*-derived 'Norton.' Theoretical and Applied Genetics. 132:137–147.
Shibata, T., Cunningham, R. P., DasGupta, C., and Radding, C. M. 1979a. Homologous pairing in genetic recombination: complexes of recA protein and DNA. Proceedings of the National Academy of Sciences. 76:5100–5104.

Shibata, T., DasGupta, C., Cunningham, R. P., and Radding, C. M. 1979b. Purified *Escherichia coli* recA protein catalyzes homologous pairing of superhelical DNA and single-stranded fragments. Proceedings of the National Academy of Sciences. 76:1638–1642.
Sudarshana, M. R., Perry, K. L., and Fuchs, M. F. 2015. Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. Phytopathology®. 105:1026–1032.
Takeshita, M., Chang, C. N., Johnson, F., Will, S., and Grollman, A. P. 1987. Oligodeoxynucleotides containing synthetic abasic sites. Model substrates for DNA polymerases and apurinic/apyrimidinic endonucleases. Journal of Biological Chemistry. 262:10171–10179.
TwistAmp® liquid DNA amplification kits combined instruction manual. 2018. Available at: https://www.twistdx.co.uk/wp-content/uploads/2021/04/twistamp-liquid-combined-manual_revb_v2-web.pdf [Accessed October 3, 2022].
TwistAmp DNA amplification kits assay design manual. 2018. Available at: https://www.twistdx.co.uk/wp-content/uploads/2021/04/twistamp-assay-design-manual-v2-5.pdf [Accessed April 10, 2023].
Uhls, A., Petersen, S., Keith, C., Howard, S., Bao, X., and Qiu, W. 2021. *Grapevine vein clearing virus* is prevalent and genetically variable in grape aphid (*Aphis illinoisensis* shimer) populations. Plant Disease. 105:1531–1538.
Zhang, Y., Angel, C. A., Valdes, S., Qiu, W., and Schoelz, J. E. 2015. Characterization of the promoter of grapevine vein clearing virus. Journal of General Virology. 96:165–169.
Zhang, Y., Singh, K., Kaur, R., and Qiu, W. 2011. Association of a novel DNA virus with the grapevine vein-clearing and vine decline syndrome. Phytopathology®. 101:1081–1090.