RING TEST FOR RECOMBINASE POLYMERASE AMPLIFICATION (RPA) OF *PHYTOPHTHORA RAMORUM*

A Project Paper

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Master of Professional Studies in Integrative Plant Sciences

By

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ABSTRACT

Viability of a new recombinase polymerase reaction (RPA) test developed by Agdia, Inc. for detection of *Phytophthora ramorum* was assessed through a ring test developed by the USDA's Agricultural Research Service (ARS). Four diagnostic laboratories in the National Plant Diagnostic Network (NPDN) carried out this ring test, testing 17 materials using RPA in parallel with an established enzyme-linked immunosorbent assay (ELISA). The purpose of this ring test was to determine the accuracy, repeatability, and reproducibility of RPA using different instruments for testing of *P. ramorum*, and assess its diagnostic potential in comparison to the existing ELISA protocol. The goals of this capstone were to carry out the RPA ring test on behalf of the Cornell University Plant Disease Diagnostic Clinic (CU-PDDC) to provide ARS with feedback on this RPA protocol and the resulting data, and to develop work instructions under the accreditation standard of the NPDN's and CU-PDDC's quality management system for this novel RPA test.

BIOGRAPHICAL SKETCH

Melissa Martens is a Master of Professional Studies student in the School of Integrative Plant Science at Cornell University. She was raised in Southern California, where she grew to love sunshine, the outdoors, and plants.

Melissa graduated from the University of California, Irvine with a degree in Biological Sciences, where she researched microbial community dynamics. While in her undergrad and after graduation, Melissa spent years working at a retail nursery. In this time, Melissa realized her professional passion for plants. She came to Cornell to deepen her technical knowledge, explore new career opportunities, and broaden her horizons.

After graduation, Melissa is eager to embark on a career involved in plant pathology and integrated pest management.

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Thank you to my parents for being my best friends and confidants. Without you, I would not be who I am today.

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LIST OF ABBREVIATIONS

ARS: Agricultural Research Service

CU-PDDC: Cornell University Plant Disease Diagnostic Clinic

Ct: quantification cycle

ELISA: enzyme-linked immunosorbent assay

GEB: general extract buffer

NEPDN: Northeast Plant Diagnostic Network

NPDN: National Plant Diagnostic Network

PBST: phosphate-buffered saline with Tween 20

PCR: polymerase chain reaction

PNP: p-Nitrophenyl Phosphate

qPCR: quantitative PCR

RPA: recombinase polymerase amplification

R.O.: reverse osmosis

SOD: Sudden Oak Death

SSB: single-strand binding protein

USDA: United States Department of Agriculture

WI: work instructino

PREFACE

Overview of *Phytophthora ramorum*

Phytophthora ramorum is a pathogenic oomycete capable of infecting a wide range of trees and woody shrubs, and is the causative agent of Sudden Oak Death (SOD)/ramorum blight. This pathogen was first observed in a population of tanoaks in California, and was identified as *P. ramorum* in the late 1990's (COMF Partners, 2021). Since then, the pathogen has been identified in 15 counties throughout Northern California, one county in Oregon, and multiple regions in European, most notably the United Kingdom (COMF Partners, 2021). *P. ramorum* has over 100 identified hosts, including multiple oak and tanoak species, along with a variety of common nursery plants such as *Camellia* spp., *Rhododendron* spp., *Viburnum* spp., and *Syringa vulgaris* (Shaw, 2007).

P. ramorum has had a devastating effect on coastal forests in California, and threatens timber and nursery industries across the country (Image 1, COMF Partners, 2021; APHIS USDA, 2022). Endemic California oaks are an iconic and ecologically essential aspect of the California landscape (Zavalet et al., 2007). Oak woodlands and savannahs comprise nearly a quarter of the natural habitat in California, and various endemic oak species provide food and habitat for animals and other understory plants (Zavalet et al., 2007). Oak populations also provide important ecosystem services, such as management of the watershed, and are an important cultural facet in their aesthetic appeal and common use in outdoor recreational settings (Zavalet et al., 2007). For the past several decades, there has been a concerning decline in oak populations in California, largely due to the introduction and spread of Sudden Oak Death (SOD) (Bolsinger, 1988; Zavalet et al., 2007).

Image 1. Dead oaks on hillside, Joseph O'Brien, USDA Forest Service, Bugwood.org

Although *P. ramorum*'s established infection areas are currently contained in California and Oregon, there is growing concern that it may be able to establish in other regions of the country. California is the top state in horticulture sales and among the top three states in horticulture production, followed closely by Oregon (USDA NASS, 2019). Because of this, there is great concern that horticulture products from the West coast may facilitate the spread of *P. ramorum* to other regions of the country. Surveying in 2001-2011 detected *P. ramorum* in 464 nurseries across 27 states, demonstrating the risk (USDA, 2011). This poses both an ecological and economic threat of national concern, as *P. ramorum* could devastate populations of native trees and shrubs, and significantly impact the country's multi-billion dollar timber industry (Cave et al., 2008).

Based on *P. ramorum*'s climate suitability range outlined in Meentemeyer et al. (2006), a national climate suitability map was created using climate data averaged across 2019 - 2022 (Figure 1, Meentemeyer et al., 2006). It was found that there was a significant range of suitable conditions across the Northeast, Midwest, and Southeast due to optimal climate conditions and

high host availability (Figure 1). This indicates the need to continue or strengthen widespread surveys and trace forward events in these areas to ensure *P. ramorum* infections are detected and controlled. Considering *P. ramorum* has already been detected in nursery stock throughout the eastern United States, there is great concern that this pathogen may be capable of establishing in these regions. Recognizing the increasing risk *P. ramorum* poses in the eastern United States is essential for directing the regulatory and management efforts needed to protect these areas.

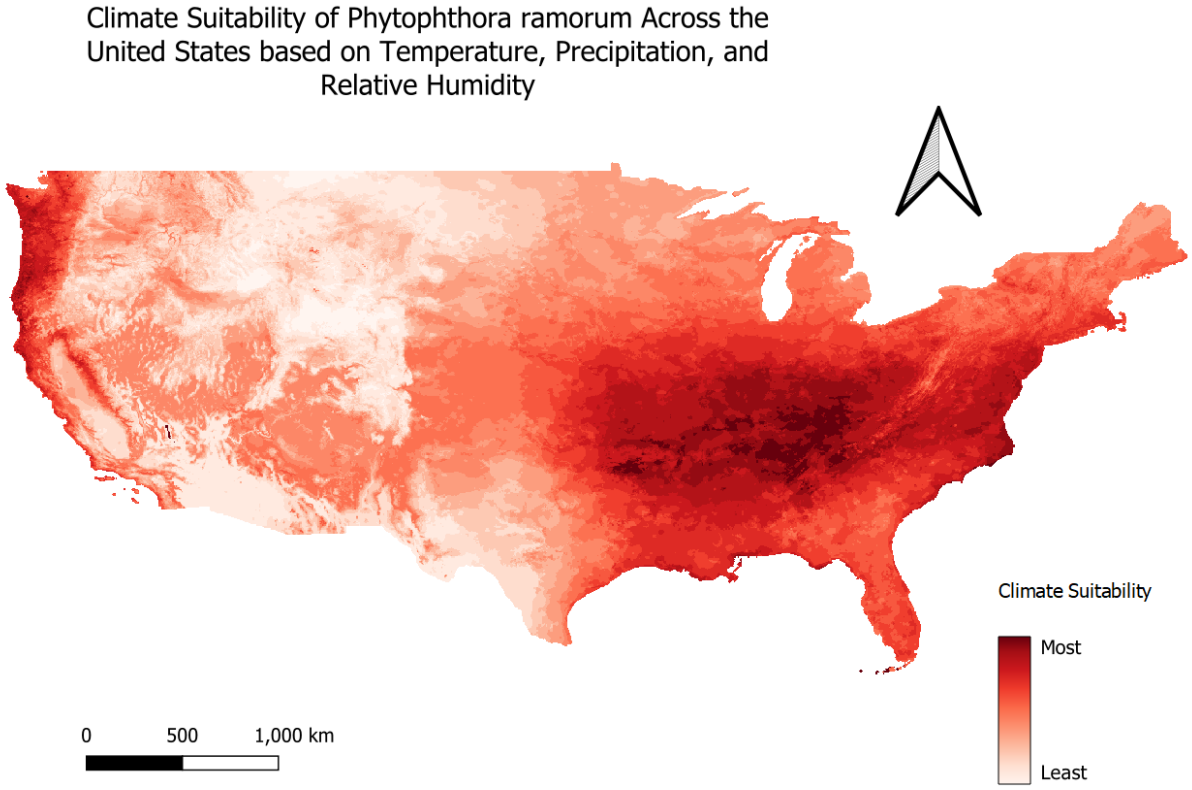


Figure 1. Climate suitability map of *Phytophthora ramorum* based on minimum temperature tolerance, maximum temperature tolerance, precipitation requirements, and relative humidity requirements (Meentemeyer et al., 2006).

Since there is a high risk that *P. ramorum* may spread to other regions of the country, it is imperative that the most effective testing methods are used to monitor the spread and movement of this disease. There are detailed procedures set up for regular surveying of interstate-shipping nurseries in high-risk areas when conditions are conducive to spread of the pathogen (USDA, 2022). Samples taken during these surveys are sent to diagnostic clinics for testing (USDA,

2022). Nurseries that are confirmed positive must destroy all host plants within a specific radii of the confirmed positive, monitor all host plants within a wider quarantine radii for symptoms for a 90-day quarantine period, and undergo further testing in following years (USDA, 2022). Positive detection may also result in subsequent widespread trace forward surveys to track recent shipments of potentially infected plant material out of the state (USDA, 2022). These regular surveys and potential trace forward events result in large volumes of samples being sent to diagnostic clinics across the country. Efficient and accurate diagnostic protocols are needed to ensure samples can be processed effectively, so that regulatory agencies can quickly take the necessary action to contain this pathogen.

Plant Disease Diagnostics

The Cornell University Plant Disease Diagnostic Clinic (CU-PDDC) is a member of the National Plant Diagnostic Network (NPDN), and is one of 12 member states that make up the Northeast Plant Diagnostic Network (NEPDN). The NPDN is a consortium of plant diagnostic laboratories formed by USDA National Institute of Food and Agriculture (NIFA) and Office of Homeland Security (NPDN, 2023). The missions of the NPDN is to serve as a "premier diagnostic system with the ability to quickly detect and accurately identify plant pests and pathogens and to communicate timely and accurate information" (NPDN, 2023). The NPDN sets high quality standards in diagnostic services for all labs in the network, and serves as a critical resource in monitoring the presence and spread of high-consequence plant pests and pathogens (NPDN, 2023). Accurate testing procedures and secure communication protocols within the network allows for rapid detection and response against notable threats (NPDN, 2023).

To maintain these high-quality diagnostic standards, the NPDN developed their own accreditation standard. This standard includes maintenance of a diagnostic quality system made up of several management and technical categories, which is a customized lab standard for plant pest and disease diagnostics. A critical part of this quality system is the development of documents that carefully detail procedures, instructions, and programs that are in-use in the lab. Documentation must be understandable and available to all lab personnel, and consistently updated with the most recent information (NPDN, 2022). A work instruction (WI) is a document that provides highly detailed instructions with exact steps on how to carry out an activity in the lab (NPDN, 2022). This ensures that protocols for molecular diagnostic tests are reproducible by all lab members trained to perform the procedure and carried out in the same way, ensuring consistency and accuracy in test results regardless of the test operator.

A critical part of this capstone project is the development of a thorough work instruction-worksheet combination for a novel diagnostic protocol for RPA detection of *P. ramorum* (Supplementary material Appendix 1, Fig. A1). The RPA procedure was first developed by researchers at UC Davis and Michigan State, and has been adapted by USDA ARS and recently made commercially available by Agdia, Inc. (Miles et al., 2015). This work instruction-worksheet serves as the guiding document for the work carried out in the following sections, and was designed to be reproducible by all lab members in the CU-PDDC (Supplementary material Appendix 1, Fig. A1).

INTRODUCTION

A new recombinase polymerase amplification (RPA) test kit for detection of *Phytopthora ramorum* was recently made commercially available by Agdia, Inc. This RPA test kit could be used with or in place of the existing diagnostic protocols for *P. ramorum*, which are elicitin qPCR, ITS qPCR and ELISA. RPA utilizes three core proteins: recombinase, single-strand DNA binding protein (SSB), and polymerase (Daher et al., 2016). These proteins are responsible for DNA unwinding, primer annealing, and replication, respectively (Daher et al., 2016). Recombinase and SSB activity substitutes for the heat cycles used in PCR for DNA denaturation and re-annealing, enabling RPA to be carried out under isothermal conditions (Daher et al., 2016).

This isothermal amplification technology has the benefits of being relatively simple, fast, and highly sensitive (Daher et al., 2016). For instance, RPA for *P. ramorum* requires just 20 minutes in an isothermal fluorometer, as where elicitin and ITS qPCR take approximately two hours to set up and execute, and ELISA may take 1-2 days. RPA for *P. ramorum* has less preparatory steps than the existing diagnostic procedures, as it does not require DNA extraction before processing and reagents are pre-combined in a lyophilized pellet. Comparatively, for elicitin and ITS qPCR, the operator must measure and combine reagents to create their own "master mix" for the reaction. The speed and simplicity of RPA may enable diagnostic clinics to process samples more efficiently, making testing more affordable (Daher et al., 2016). Additionally, the finalized RPA for *P. ramorum* protocol is intended for use with the small, compact Amplifire Isothermal Flourimeter by Agdia, which has the potential to be used in-field for near immediate results.

Inter-laboratory comparisons, such as ring tests, are an important approach for developing and maintaining quality diagnostic techniques in plant disease diagnostics. Ring tests can be used to validate new diagnostic methods, continuously monitor current diagnostic protocols, and examine potential variability in assay performance (Johnson and Cabuang, 2021). They are administered formally by an external organization, which provides the same blind sample material to a number of diagnostic clinics, and this external organization reviews the data to make conclusions about the accuracy and methodology of a particular protocol (Johnson and Cabuang, 2021). These inter-laboratory comparisons can also serve as an important assessment of a diagnostic clinic's internal quality control standard, by gauging the competence of technicians and checking for proper equipment calibration (Johnson and Cabuang, 2021).

In order to validate the efficacy, accuracy, and feasibility of RPA for *P. ramorum*, the USDA Agriculture Research Service (ARS), in collaboration with Agdia, Inc., organized a ring test that included four accredited diagnostic clinics in the National Plant Diagnostic Network (NPDN). This ring test consisted of 17 blind sample materials that were tested with Agdia's RPA test kit in parallel with Agdia's enzyme-linked immunosorbent assay (ELISA). The objective of this capstone project was to complete this ring test on behalf of Cornell University's Plant Disease Diagnostic Clinic (CU PDDC). Participating in the ring test will inform the ARS on the feasibility of this protocol under the realistic conditions of different diagnostic clinics, and help the ARS determine if RPA is equally or more effective than the ELISA assay for *P. ramorum*. This project had two main goals: 1) Provide results from CU-PDDC to ARS and Agdia, to be used in combination with data from the other labs, to validate RPA for *P. ramorum*, or inform the ARS of how it should be altered 2) Prepare work instruction for the RPA for *P. ramorum* protocol that meets the NPDN quality management system requirements, so this procedure can

be used for diagnosis of *P. ramorum* in the CU PDDC in the future. If it is found that RPA is a suitable diagnostic test for *P. ramorum* detection, RPA will be a valuable technology for rapid, affordable, simple, and accurate testing, enabling an improved monitoring system to limit and contain the spread of *P. ramorum*.

MATERIALS AND METHODS

Recombinant Polymerase Amplification (RPA) Procedure

This procedure was initially developed by Miles et al. (2015), and was subsequently adapted by USDA ARS and Agdia, Inc. to be made commercially available. Refer to the CU-PDDC work instruction-worksheet for a more detailed outline of what was executed for this ring test (Supplementary material Appendix 1, Fig. A1). All kit components were stored at 2-8°C, sample materials (Materials 1-17) were frozen at -20°C, and lyophilized test components were sealed with desiccant when not in use. Before beginning the procedure, kit components were warmed to room temperature (18-30°C) for 20-30 minutes. Leaf disc samples (Materials 1-17) were transferred to mesh extraction bags containing GEB2 buffer and macerated with Agdia's ball-bearing drill attachment. There were two samples for Material 9, and the extraction for each sample was carried out separately (Table 1).

After leaving macerated samples to sit in mesh extraction bags for five minutes at room temperature, Materials 1-8 were pooled accordingly: Materials 1 and 2, Materials 3 and 4, Materials 5 and 6, and Materials 7 and 8 (Table 1). Additionally, the two extractions of Material 9 were combined (Table 1). Pool of Materials 1&2 and pool of Materials 3&4 were diluted to 100x, 1000x, and 10000x using a serial dilution with GEB2 buffer (Table 1). Pool of Materials 5&6 and pool of Materials 7&8 were diluted to 100x, 1000x, and 10000x using a serial dilution with Material 9 (Table 1). Operator 1 and Operator 2 carried out separate dilutions, to be used in

separate Round 1 and Round 2 assays, respectively. Once pooling and dilutions were complete, 5 µL of each sample extract was transferred to 100 µL PD1 tubes in triplicates. From the PD1 tubes, 25 µL was transferred to the reaction plate, which consisted of 100 µL wells containing the lyophilized reaction pellet. The reaction tubes were immediately vortexed and centrifuged as soon as the plate was loaded.

Table 1. Workflow for processing of the 17 sample materials. 'UN' indicated undiluted, 'L2' indicates 2log or 10x, 'L3' indicates 3log or 100x, 'L4' indicates 4log or 1000x.

Material	Extraction	Pool	Dilution	Sample ID	RPA	ELISA
1	NO		Yes - GEB2	1&2UN	YES	NO
				1&2L2	YES	NO
2	NO			1&2L3	YES	NO
				1&2L4	YES	NO
3	NO		Yes - GEB2	3&4UN	YES	YES
				3&4L2	YES	YES
4	NO			3&4L3	YES	YES
				3&4L4	YES	YES
5	Operator 1		Yes - M9	5&6UN	YES	YES
				5&6L2	YES	YES
6	Operator 2			5&6L3	YES	YES
				5&6L4	YES	YES
7	Operator 1		Yes - M9	7&8UN	YES	YES
				7&8L2	YES	YES
8	Operator 2			7&8L3	YES	YES
				7&8L4	YES	YES
9a	Operator 1		NO	Not used	NO	NO
9b	Operator 1		NO	Not used	NO	NO
10	1 or 2		NO	10UN	YES	YES
11	1 or 2		NO	11UN	YES	YES
12	1 or 2		NO	12UN	YES	YES
13	1 or 2		NO	13UN	YES	YES
14	1 or 2		NO	14UN	YES	YES
15	1 or 2		NO	15UN	YES	YES
16	1 or 2		NO	16UN	YES	YES
17	1 or 2		NO	17UN	YES	YES

The isothermal RPA reaction was carried out in QuantStudio3. The reaction first ran for six cycles, with a cycle time of 20 seconds. At this point, the reaction was paused, and the plate was removed to be vortexed and centrifuged again. The reaction plate was then placed back in QuantStudio3 machine and allowed to run for a total of 40 cycles under the same conditions, including the first six cycles that were run before pausing the reaction. Materials 1-8 and

Material 12 were processed twice in two rounds, Round 1 and Round 2, each round carried out by a different operator (Table 2). Materials 10-17 (repeating Material 12) were processed once in a separate round, Round 3 (Table 2).

Table 2. Plate layout for each round of the RPA procedure. 'UN' indicated undiluted, 'L2' indicates 2log or 10x, 'L3' indicates 3log or 100x, 'L4' indicates 4log or 1000x.

Round 1: Operator 1											
1&2UN Rxn #1	1&2UN Rxn #2	1&2UN Rxn #3	1&2L2 Rxn #1	1&2L2 Rxn #2	1&2L2 Rxn #3	1&2L3 Rxn #1	1&2L3 Rxn #2	1&2L3 Rxn #3	1&2L4 Rxn #1	1&2L4 Rxn #2	1&2L4 Rxn #3
3&4UN Rxn #1	3&4UN Rxn #2	3&4UN Rxn #3	3&4L2 Rxn #1	3&4L2 Rxn #2	3&4L2 Rxn #3	3&4L3 Rxn #1	3&4L3 Rxn #2	3&4L3 Rxn #3	3&4L4 Rxn #1	3&4L4 Rxn #2	3&4L4 Rxn #3
5&6UN Rxn #1	5&6UN Rxn #2	5&6UN Rxn #3	5&6L2 Rxn #1	5&6L2 Rxn #2	5&6L2 Rxn #3	5&6L3 Rxn #1	5&6L3 Rxn #2	5&6L3 Rxn #3	5&6L4 Rxn #1	5&6L4 Rxn #2	5&6L4 Rxn #3
7&8UN Rxn #1	7&8UN Rxn #2	7&8UN Rxn #3	7&8L2 Rxn #1	7&8L2 Rxn #2	7&8L2 Rxn #3	7&8L3 Rxn #1	7&8L3 Rxn #2	7&8L3 Rxn #3	7&8L4 Rxn #1	7&8L4 Rxn #2	7&8L4 Rxn #3
12 Rxn #1	12 Rxn #2	12 Rxn #3	12 Rxn #1	12 Rxn #2	12 Rxn #3	12 Rxn #1	12 Rxn #2	12 Rxn #3	12 Rxn #1	12 Rxn #2	12 Rxn #3
GEB2 (-)	GEB2 (-)	GEB2 (-)									

Round 2: Operator 2											
1&2UN Rxn #1	1&2UN Rxn #2	1&2UN Rxn #3	1&2L2 Rxn #1	1&2L2 Rxn #2	1&2L2 Rxn #3	1&2L3 Rxn #1	1&2L3 Rxn #2	1&2L3 Rxn #3	1&2L4 Rxn #1	1&2L4 Rxn #2	1&2L4 Rxn #3
3&4UN Rxn #1	3&4UN Rxn #2	3&4UN Rxn #3	3&4L2 Rxn #1	3&4L2 Rxn #2	3&4L2 Rxn #3	3&4L3 Rxn #1	3&4L3 Rxn #2	3&4L3 Rxn #3	3&4L4 Rxn #1	3&4L4 Rxn #2	3&4L4 Rxn #3
5&6UN Rxn #1	5&6UN Rxn #2	5&6UN Rxn #3	5&6L2 Rxn #1	5&6L2 Rxn #2	5&6L2 Rxn #3	5&6L3 Rxn #1	5&6L3 Rxn #2	5&6L3 Rxn #3	5&6L4 Rxn #1	5&6L4 Rxn #2	5&6L4 Rxn #3
7&8UN Rxn #1	7&8UN Rxn #2	7&8UN Rxn #3	7&8L2 Rxn #1	7&8L2 Rxn #2	7&8L2 Rxn #3	7&8L3 Rxn #1	7&8L3 Rxn #2	7&8L3 Rxn #3	7&8L4 Rxn #1	7&8L4 Rxn #2	7&8L4 Rxn #3
12 Rxn #1	12 Rxn #2	12 Rxn #3	12 Rxn #1	12 Rxn #2	12 Rxn #3	12 Rxn #1	12 Rxn #2	12 Rxn #3	12 Rxn #1	12 Rxn #2	12 Rxn #3
GEB2 (-)	GEB2 (-)	GEB2 (-)									

Round 3: Operator 1 or 2											
10 Rxn #1	10 Rxn #2	10 Rxn #3	11 Rxn #1	11 Rxn #2	11 Rxn #3	12 Rxn #1	12 Rxn #2	12 Rxn #3	13 Rxn #1	13 Rxn #2	13 Rxn #3
14 Rxn #1	14 Rxn #2	14 Rxn #3	15 Rxn #1	15 Rxn #2	15 Rxn #3	16 Rxn #1	16 Rxn #2	16 Rxn #3	17 Rxn #1	17 Rxn #2	17 Rxn #3
GEB2 (-)	GEB2 (-)	GEB2 (-)									

Any sample that produced a quantification cycle (Ct) reading for both the internal control and the *P. ramorum* target was considered positive. Any sample that produced a quantification cycle (Ct) reading for both the internal control but not the *P. ramorum* target was considered negative. A threshold of 1,000 ΔRxn based on the amplification curves observed was used to determine Ct for both the *P. ramorum* target and internal control target.

Enzyme-Linked Immunosorbent Assay (ELISA) Procedure

Operator 1 and Operator 2 each executed their own ELISA assay for all sample materials. All kits components were stored at 4°C before use, and sample extractions from the RPA were frozen at 20°C. To prepare, samples were allowed to thaw and then vortexed and centrifuged. Fresh GEB2 buffer was prepared 30 minutes in advance at a concentration of 0.279 g GEB2 powder/5 mL reverse osmosis water (R.O. H_2O). PBST wash buffer was prepared by combining

20X PBST concentrate with 1000 mL of R.O. H_2O. The positive control was rehydrated with GEB2 buffer according to kit instructions. Finally, a humid box was set up by placing a moist paper towel in an airtight container.

Before loading samples, 120 µL of PBST was added to each well for four minutes and then removed. Samples were loaded in triplicates, with 100 µL of sample material transferred to each well. R.O. H_2O was used for the negative control, GEB2 was used as the buffer control, and freshly rehydrated *P. ramorum* included with the kit was used as the positive control. The plate was incubated for two hours at room temperature, and then washed eight times with PBST buffer. After the wash, 100 µL of enzyme conjugate buffer was added to each well. The plate was incubated again for two hours at room temperature, and then washed eight times with PBST buffer. After the second wash, 100 µL of the PNP substrate solution was added to each well, taking care not to expose the PNP solution to light while handling. The plate was incubated in the dark for one hour, and final results were recorded using a BioTek plate reader. The threshold for positive diagnosis was set as 2x the GEB buffer ODU.

RESULTS

The ELISA assay of the sample materials yielded results that aligned with what was expected by the ARS (Table 3). Materials 5-13 tested positive, and Materials 14-17 tested negative (Table 3). Materials 1-4 were not tested in the ELISA because they are the intended positive DNA controls for the RPA, and therefore would not react in an ELISA assay. These diagnoses will be used as the true diagnoses for comparison with the RPA results.

Considerable variability between replicates resulted in many inconclusive results for Round 1 and Round 2 of the RPA procedure (Table 3). In these runs, the amplification curves were abnormal, and many of the wells did not have adequate amplification of the internal control (Supplementary material Appendix 1, Fig. A2). Additionally, many of the amplification curves for the *P. ramorum* target started at very high levels but ended below the threshold, resulting in

undetermined Ct values and negative diagnoses that did not align with that of the ELISA results (Table 3, Supplementary material Appendix 1, Fig. A2).

Round 3 was a significantly smaller assay, consisting of only 27 wells, in comparison to 63 wells included in Round 1 and 2. Materials 10-13 tested positive and Materials 14-17 tested negative, which is consistent with the results of the ELISA (Table 3). All wells showed typical amplification curves of the *P. ramorum* target and adequate amplification of the internal control (Table 3, Supplementary material Appendix 1, Fig. A2).

Detailed observations throughout various stages of the RPA procedure were also recorded (Table 4). Notes on the difficulties, challenges, and unexpected occurrences are included for reference when considering the potential causes of unexpected outcomes, and potential areas where the work instructions need refining to adequately guide the methodological steps of this protocol.

Table 3. Comparison of RPA and ELISA results, with average Ct and ODU values and subsequent diagnostic conclusion – positive (+), negative (-), or inconclusive (INC). Threshold for RPA was set as 1000 ΔRxn for all Ct calculations. Threshold for ELISA was set as 0.2 ODU for Operator 1 results, and 0.36 for Operator 2 results, based on 2x the GEB2 buffer control value. UND denotes samples for which inadequate amplification occurred. INC denotes samples where not all replicates yielded Ct values. For these samples, average Ct values were only calculated across the replicates that yielded Ct values. OVERFL denotes samples with fluorescence levels above the OD reading capacity of the ELISA machine, and were therefore positive. Green cells are those that align with the expected values based on the ELISA, red cells are those that do not align with the ELISA results.

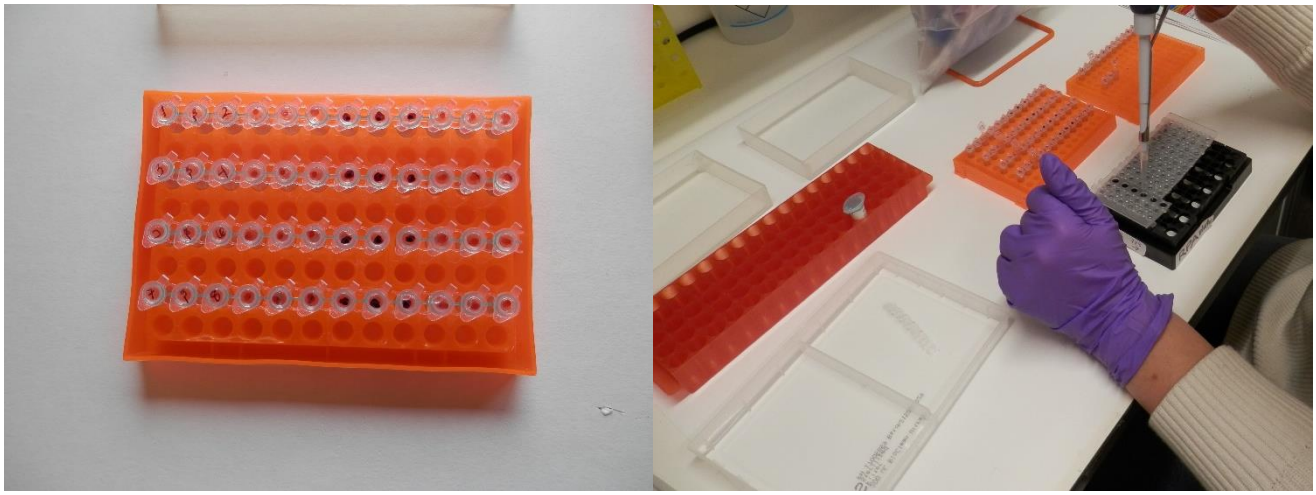
Material	RPA - Round 1 &3		RPA - Round 2		ELISA - Operator 1		ELISA - Operator 2	
	Avg Ct	Diagnosis	Avg Ct	Diagnosis	Avg ODU	Diagnosis	Avg ODU	Diagnosis
1&2 UN	14.38	INC	11.14	INC	Not tested with ELISA per Ring Test instructions			
1&2 L2	UND	(-)	UND	(-)				
1&2 L3	UND	(-)	UND	(-)				
1&2 L4	UND	(-)	UND	(-)				
3&4 UN	13.32	INC	12.20	INC				
3&4 L2	UND	(-)	UND	(-)				
3&4 L3	UND	(-)	UND	(-)				
3&4 L4	UND	(-)	UND	(-)				
5&6 UN	15.16	INC	UND	(-)	OVERFL	(+)	OVERFL	(+)
5&6 L2	UND	(-)	UND	(-)	OVERFL	(+)	OVERFL	(+)
5&6 L3	UND	(-)	UND	(-)	1.87	(+)	3.98	(+)
5&6 L4	UND	(-)	UND	(-)	0.52	(+)	0.36	(+)
7&8 UN	6.68	INC	UND	(-)	OVERFL	(+)	OVERFL	(+)
7&8 L2	UND	(-)	UND	(-)	3.47	(+)	1.23	(+)
7&8 L3	UND	(-)	UND	(-)	1.08	(+)	0.64	(+)
7&8 L4	22.70	INC	UND	(-)	0.72	(+)	0.25	(-)
10	14.67	(+)	Only tested by one operator		OVERFL	(+)	OVERFL	(+)
11	12.04	(+)			OVERFL	(+)	OVERFL	(+)
12 (Round 1 & 2)	6.71	INC	22.03	INC	OVERFL	(+)	OVERFL	(+)
12 (Round 3)	14.59	(+)						
13	16.6	(+)	Only tested by one operator per Ring Test Instructions		OVERFL	(+)	OVERFL	(+)
14	UND	(-)			0.10	(-)	0.24	(-)
15	UND	(-)			0.12	(-)	0.29	(-)
16	UND	(-)			0.17	(-)	0.22	(-)
17	UND	(-)			0.15	(-)	0.26	(-)
GEB2 Control (Round 1&2)	13.73	INC	UND	(-)	0.10	(-)	0.18	(-)
GEB2 Control (Round 3)	UND	(-)						
H2O Control					0.10	(-)	0.11	(-)
Positive Control					OVERFL	(+)	OVERFL	(+)

Table 4. Observations made during various steps of the RPA procedure.

Step	Observation
Test Kit Set-Up	**1.** In order to prepare for three separate rounds of assays, we had to cut the reaction plates. Cutting through the plate was very difficult, and required us to handle the base of the wells extensively. Some scratches were observed on the side of the wells from the scissors (Image 2).
Sample Preparation	**1.** There was a very small amount of Materials 1-4 provided, leaving little room for error during all subsequent steps. **2.** Samples were frozen between the mesh extraction and RPA reactions. Before Round 1 and 2, samples were removed from freezer and thawed, then added to PD1 tubes. Before Round 3, samples were thawed, vortexed, and centrifuged.
Dilution	**1.** We ran out of Material 9 when diluting Materials 5&6 and Materials 7&8. The ARS Research Lead had to send more Material 9 before we could proceed. Material 9 used in the Round 2 dilutions therefore consisted of samples sent to us days apart.
Transfer to PD1 Tubes	**1.** Even after tapping down the PD1 tubes, some PD1 solution remained stuck in the caps of some of the tubes. When opening the tube to transfer to sample, sometimes PD1 solution would flick off the top of the cap. **2.** The USDA/Agdia instructions said to "mix well" when adding sample to the PD1 tube. The first two rounds, we did this by pipetting up and down. The third round, we did this by pipetting up and down and thoroughly vortexing. **3.** Operator 1 changed pipette tips between transferring sample to each respective triplicate PD1 tube. Operator 2 did not. **4.** Having a detailed plan and organizing layout of PD1 tubes beforehand was critical to staying organized while transferring. Sample tubes, PD1 tubes, and reaction wells were all consistently organized with the same layout. The PD1 tube replicates were marked with different colors to help track where we were in the transfer process (Image 3). **5.** In Round 1 and 2, samples were mixed in PD1 tubes by pipetting up and down. In Round 3 samples were mixed in PD1 tubes by pipetting up and down, and then vortexing lightly.
Transfer to Reaction Plate	**1.** By the third assay round, both sides of the rows on the reaction plate had been cut. This made it difficult to get the caps off the wells, as there was no longer a pull tab for removal. We had to use a razor blade to loosen the caps before pulling them off. **2.** Removing the caps caused the pellets in the reaction wells to jostle around. We did not have any issues with a reaction pellet coming out of the well, but we were concerned this could occur while uncapping. **3.** When loading the reaction plate, we uncapped and loaded wells column by column, moving left to right. This systematic progression was helpful since the caps were attached in strips down the column, allowing us to progressively uncap, load, and recap wells moving from leftmost columns to rightmost columns (Image 3). **4.** Operator 1 opened and closed PD1 tubes one by one while transferring to reaction wells. Operator 2 opened PD1 tubes for entire reaction well column at once, and closed all PD1 tubes for that column before moving on to the next column. **5.** Round 3 involved less reactions, and the reaction plate was loaded much more quickly than the Round 1 and Round 2 plates.
Running Reaction in QuantStudio3	**1.** When the reaction plate was inserted into QuantStudio3, it took 2-3 minutes for the machine to warm up and initiate the protocol. **2.** When Operator 2 attempted Round 3, they accidentally placed the reaction plate one row down from the initial plate position when re-loading into the machine after the pause. The data from this run was invalid, and Round 3 had to be performed again. This mistake could easily be repeated by other operators, so it should be added to all work instructions to be cautious at the pause stage and ensure the plate is placed back in correct position.

Image 2. Process of cutting reaction plate.

Image 3. Parallel layout of PD1 tubes and reaction plate.



DISCUSSION

Before carrying out the RPA protocol, a detailed work instruction was developed to guide execution of the RPA assay (Supplementary material Appendix 1, Fig. A1). The work instruction in Figure A1 reflects extensive collaboration with ARS, Agdia, and the other diagnostic clinics over the course of multiple weeks. ARS provided an initial protocol to guide execution of the ring test, but it took this period of collaboration to develop a more detailed work instruction that was catered to guide the operators for the Cornell University Plant Disease Diagnostic Clinic. This highlights the complexity of designing ring tests and challenges in testing new diagnostic

procedures. Considerable questioning, trouble-shooting, and critical thinking is required on part of the external organization administering the ring test, the producer of the diagnostic test, and the operators of the diagnostic clinics to ensure all aspects of the ring test and protocol is uniformly understood. The final work instruction produced for the RPA assay adheres to NPDN standards to form a comprehensive guide so that the methodology is easily reproducible by other operators, and so that all parties involved can review and reflect on the methodology used by CU-PDDC to carry out their portion of the ring test.

Since this project is focused on assessing the feasibility of a molecular diagnostic test, and not necessarily the results of the test itself, detailed observations were recorded throughout every step of the procedure (Table 4). USDA ARS will be reviewing data from all labs involved in the ring test, and will be the organization making conclusions based on the data. It is the job of the clinics to carry out the test to the best of their ability with blind samples, and keep track of notable observations along the way that may be informative for the ARS. Notes on the difficulties, challenges, and unexpected occurrences while carrying out this test can help advise other diagnostic clinics on key technical details as they carry out their portion of the ring test, and may better inform future stages of this ring test. These types of observations are also critical in refining the diagnostic test to run as effectively and smoothly as possible throughout the wide range of diagnostic clinics that may utilize it once it is officially released. For this reason, a detailed observations table is provided, and reflections on how these observations may impact the accuracy of this RPA test will be discussed.

Round 1 and Round 2 of the RPA assay did not align with the ELISA results, as Materials 1-8 tested as either negative or were inconclusive, but these samples tested positive in the ELISA assays (Materials 1-4 assumed positive despite not being tested with ELISA). In Round 3, Materials 10-13 tested positive, and Materials 14-17 tested negative, aligning with the ELISA results. Round 1 and 2 both included 63 wells, as where Round 3 only involved 27 wells, therefore the Round 3 plate took significantly less time to load (Table 4). Additionally, Round 1 and 2 were each operators' first times executing this RPA assay. During Round 3, the operators

had discussed and reflected on the first two rounds, and the operator took more care to thoroughly vortex and centrifuge the samples at all stages of the procedure, although this was not explicitly outlined for all steps in the original protocol.

The CU-PDDC operators and ARS felt that the differences in reliable results between Round 1 and 2 versus Round 3 was attributable to the fact that the plates for Round 1 and 2 took significantly longer to load. The RPA reaction begins as soon as the PD1 diluted sample material is added to the lyophilized reagent pellet, and therefore amplification had already begun in the earliest loaded wells during the time it took to load the rest of the wells. It's likely that the extensive time it took to load the plates for Round 1 and 2 used up much of the reagents, resulting in the inconsistent levels of amplification recorded by the QuantStudio3. Additionally, having experience with the RPA procedure enabled the operator to carry out Round 3 more consistently and efficiently, likely contributing to the higher degree of consistency between replicates. Loading time will need to be taken into greater consideration for this procedure when working with reaction plates, or it may be determined that this test is only fit for use with the AmpliFire by Agdia, which is limited to only eight samples per run.

These results were informative to ARS and Agdia, Inc., and in response ARS coordinated with CU-PDDC to attempt the Round 1 step of the RPA again. This time, separate assays will be made for Materials 1-4 and Materials 5-8, so that only 30 wells will be loaded for each assay. This will enable the operator to load the plate much more quickly, in a time frame comparable to that of Round 3. This will allow CU-PDDC and ARS to determine if the inconsistent results from Round 1 and 2 were due to the length of time it took to load the plates, or if another factor was involved (lack of experience running the assay, contamination between samples, incompatibility with QuantStudio3, etc.). This next step in the ring test has yet to be carried out by the CU-PDDC, but will provide important information on whether the efficacy of the RPA for *P. ramorum* protocol is affected by the time it takes to load the reaction plate.

The preliminary results from the ring test for RPA detection of *P. ramorum* indicates that this test may be an adequate diagnostic tool for testing and monitoring *P. ramorum*, and may be

a suitable replacement for the ELISA assay. The results demonstrate that RPA is highly sensitive, and relatively fast and simple to execute. With this being said, further testing is needed to determine the limitations of the test, such as how preparation time affects the amplification curves. Considering the potential risk *P. ramorum* poses to other regions in the country that are suitable to the pathogen, and the economic losses *P. ramorum* is currently causing the nursery industry, increasingly productive diagnostic tools are needed for its detection. Implementation of RPA for *P. ramorum* in the NPDN could enable rapid, affordable, and accurate testing of suspect samples, allowing USDA and its subsequent regulatory agencies to more efficiently manage this damaging pathogen.

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SUPPLEMENTAL FIGURES

Appendix 1

Figure A1. Work instructions written to guide execution of the RPA for *P. ramorum* ring test.

PRAM Recombinant Polymerase Amplification (RPA) Date:_____
RING TEST WORKSHEET for *Phytophthora ramorum* Technician: _____
for AGDIA Rapid DNA Amplification Test Kit
Cornell University-Plant Disease Diagnostic Clinic

Preparation:

____ Before beginning, check for all necessary materials: 2x Amplify XRT plates, 3x 8 pack mesh bags, 24x 8- strip PD1 tubes, 2x microfilm plate seals, 1x 50 pack 1.5mL microcentrifuge tubes, 18x Nunc vials.

Note: There are 18 Nunc vials because there are two vials for material 9 – 9a and 9b.

____ All kit components should be stored in a refrigerator at 2-8°C. All samples should be frozen at -80°C

(-20°C if -80°C not available)

____ Review experimental design, sample handling and assignments in the chart below.

Material	Extraction	Pool	Dilution	Sample ID	RPA	ELISA
1	NO	Operator:	Yes - GEB2	1&2UN	YES	**NO**
				1&2L2	YES	**NO**
2	NO			1&2L3	YES	**NO**
				1&2L4	YES	**NO**
3	NO	Operator:	Yes - GEB2	3&4UN	YES	**NO**
				3&4L2	YES	**NO**
4	NO			3&4L3	YES	**NO**
				3&4L4	YES	**NO**
5	Operator 1:	Operator:	Yes - M9	5&6UN	YES	YES
				5&6L2	YES	YES
6	Operator 2:			5&6L3	YES	YES
				5&6L4	YES	YES
7	Operator 1:	Operator:	Yes - M9	7&8UN	YES	YES
				7&8L2	YES	YES
8	Operator 2:			7&8L3	YES	YES
				7&8L4	YES	YES
9a	Operator 1:	Operator:	NO	Not used	**NO**	**NO**

9b			NO	Not used	**NO**	**NO**
10	1 or 2	NO	NO	10UN	YES	YES
11	1 or 2	NO	NO	11UN	YES	YES
12	1 or 2	NO	NO	12UN	YES	YES
13	1 or 2	NO	NO	13UN	YES	YES
14	1 or 2	NO	NO	14UN	YES	YES
15	1 or 2	NO	NO	15UN	YES	YES
16	1 or 2	NO	NO	16UN	YES	YES
17	1 or 2	NO	NO	17UN	YES	YES

Getting started:

____ Before use, allow all kit components to warm to room temperature (18-30°C) for 20-30 minutes.

____ Turn on QuantStudio 3 instrument, check program settings listed on last page.

Note: An AmpliFire or compatible isothermal instrument can be used to perform this procedure.

____ Label mesh extraction bags with material numbers

Bag #	Sample ID #	**Bag #**	Sample ID #	**Bag #**	Sample ID #
1	M1-pre-extracted	**7**	M7	**12**	M12
2	M2-pre-extracted	**8**	M8	**13**	M13
3	M3-pre-extracted	**9a**	M9a	**14**	M14
4	M4-pre-extracted	**9b**	M9b	**15**	M15
5	M5	**10**	M10	**16**	M16
6	M6	**11**	M11	**17**	M17

Organize your tubes:

____ One operator labels five-15mL or similar tubes for pooling of 1&2, 3&4, 5&6, 7&8 and 9a&9b (not shown).

____ Both operators label 1.5mL tubes and organize tubes in tube holder accordingly:

Sample Layout: Operator 1

FREEZE	1&2UN	3&4UN	5&6UN	7&8UN	9A & 9B
USE	1&2UN	3&4UN	5&6UN	7&8UN	9A & 9B

Sample Layout: Operator 2

FREEZE	1&2UN	3&4UN	5&6UN	7&8UN				
USE	1&2UN	3&4UN	5&6UN	7&8UN				
FREEZE	10	11	12	13	14	15	16	17
USE	10	11	12	13	14	15	16	17

Dilution: Operator 1 & 2

10000X	1&2L4	3&4L4		5&6L4	7&8L4	
1000X	1&2L3	3&4L3		5&6L3	7&8L3	
100X	1&2L2	3&4L2		5&6L2	7&8L2	
USE	1&2UN	3&4UN	GEB2	5&6UN	7&8UN	M9

Sample Preparation – Plant Tissue:

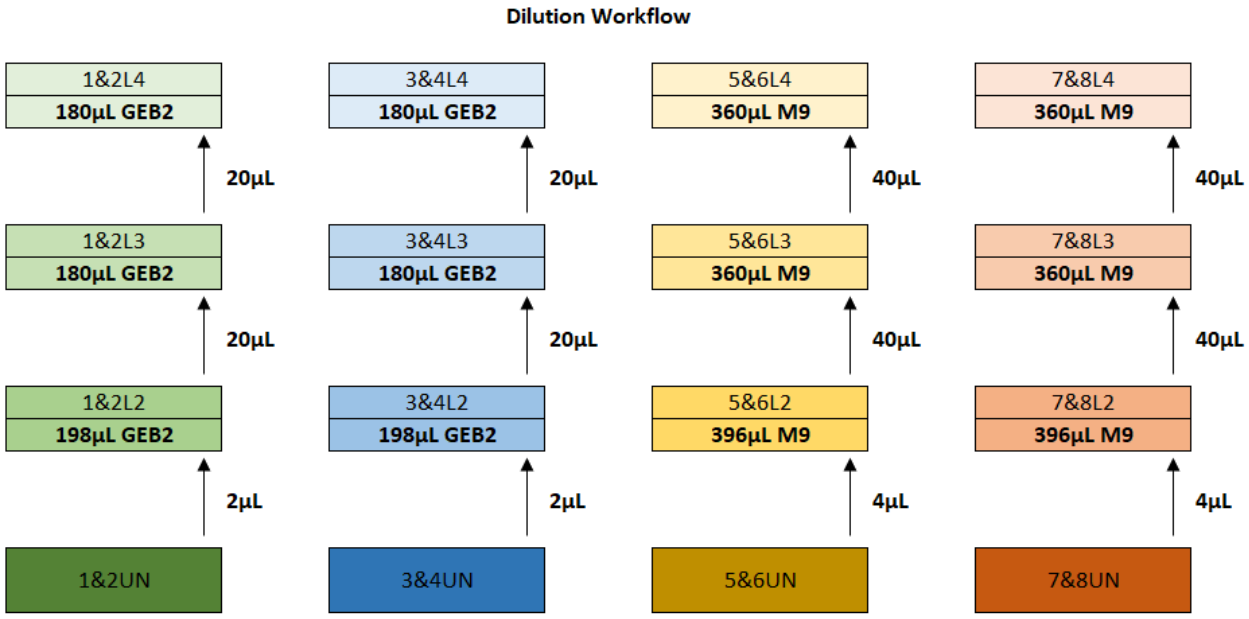
____ Refer to workflow diagram for operator assignment for each extraction.
____ Transfer leaf disc samples from Nunc vials into mesh extraction bag containing GEB2 buffer. You may use sterile toothpick or applicator stick if needed
Note: Materials 1-4 will not need extraction, all others (materials 5-17) will.
____ Macerate with ball-bearing drill attachment or blunt object (ex. pen)
____ Let rest for 5 minutes at room temperature.
____ While waiting, fill in table indicating which operator did each step (extraction/resuspension, pooling)

Pooling of Extracts:

____ One operator pools 1&2 and 3&4 by directly pipetting received amounts into the pooled tubes.
____ One operator pools ground 5&6, 7&8 and 9a&9b, into 15mL tubes using disposable pipettes.
____ Once 5-9 pooled in 15mL tube, transfer with disposable pipettes into four microcentrifuge tubes (one for use, one to store-freeze for each operator).
____ Materials 10-17 will not be pooled, but should be transferred from the extraction bag into one labelled microcentrifuge tube (only run by one operator).
Note: Transfer extra sample material into separate tubes to be frozen.

Dilution Preparation:

____ Follow the dilution workflow as shown in the chart below. Pooled 1&2 and 3&4 are diluted with GEB2 buffer and 5&6 and 7&8 are diluted with Material 9.



NOTE: REMEMBER TO USE GEB2 BUFFER FOR 1&2 and 3&4 and MATERIAL 9 FOR 5&6 and 7&8.

*The log2 yields 1:100 dilution, the log3 yields 1:1,000 dilution, the log4 yields 1:10,000 dilution

____ Mix all dilutions thoroughly by flicking or vortexing and centrifuging.

____ **At this point, you may pause in the workflow.** Resuspended, extracted, and diluted samples can be stored at -80°C (-20°C if -80°C not available) until resume workflow, and for ELISA assays done later.

Carrying Out Assays:

____ If stopped at previous step, remove samples from freezer, allow to thaw, vortex and centrifuge.

____ Remove **three** colored 100 µL Pellet Diluent Tubes (PD1) for each sample being tested, label caps with sample ID. Refer to the assay layout below. Note: Ensure liquid is at bottom of tube before use.

____ Transfer 5 µL of sample extract into the PD1 tube and mix well by pipetting up and down. Samples are now ready to be tested.

Test Protocol for Real-Time Detection in QuantStudio3:

____ Load program file "AmplifyRP CRT for Pramorum" and check conditions, see guide at end of sheet.

Note: In instrument settings, **ensure the cover temperature setting is turned off or 42°C**

____ Remove plate of reaction pellets from the white foil pouch. Secure plate in 200µL PCR tube rack and cut the number of pellets from the plate that are intended for use.

Note: Reaction pellets are light sensitive. Immediately place remaining plate back in the pouch.

____ Transfer 25 µL from the PD1 tube (colored) containing sample extract into the reaction pellet tube (clear). Refer to assay layout on the below. Only one operator will assay materials 10-17.

Note: Do not transfer more than 25 µL, DO NOT pipette up and down, reagents will stick to the tip.

REACTION HAS STARTED, do not pause mid-procedure.

Round 1: Operator 1											
1&2UN Rxn #1	1&2UN Rxn #2	1&2UN Rxn #3	1&2L2 Rxn #1	1&2L2 Rxn #2	1&2L2 Rxn #3	1&2L3 Rxn #1	1&2L3 Rxn #2	1&2L3 Rxn #3	1&2L4 Rxn #1	1&2L4 Rxn #2	1&2L4 Rxn #3
3&4UN Rxn #1	3&4UN Rxn #2	3&4UN Rxn #3	3&4L2 Rxn #1	3&4L2 Rxn #2	3&4L2 Rxn #3	3&4L3 Rxn #1	3&4L3 Rxn #2	3&4L3 Rxn #3	3&4L4 Rxn #1	3&4L4 Rxn #2	3&4L4 Rxn #3
5&6UN Rxn #1	5&6UN Rxn #2	5&6UN Rxn #3	5&6L2 Rxn #1	5&6L2 Rxn #2	5&6L2 Rxn #3	5&6L3 Rxn #1	5&6L3 Rxn #2	5&6L3 Rxn #3	5&6L4 Rxn #1	5&6L4 Rxn #2	5&6L4 Rxn #3
7&8UN Rxn #1	7&8UN Rxn #2	7&8UN Rxn #3	7&8L2 Rxn #1	7&8L2 Rxn #2	7&8L2 Rxn #3	7&8L3 Rxn #1	7&8L3 Rxn #2	7&8L3 Rxn #3	7&8L4 Rxn #1	7&8L4 Rxn #2	7&8L4 Rxn #3
12 Rxn #1	12 Rxn #2	12 Rxn #3	12 Rxn #1	12 Rxn #2	12 Rxn #3	12 Rxn #1	12 Rxn #2	12 Rxn #3	12 Rxn #1	12 Rxn #2	12 Rxn #3
GEB2 (-)	GEB2 (-)	GEB2 (-)									

Round 2: Operator 2											
1&2UN Rxn #1	1&2UN Rxn #2	1&2UN Rxn #3	1&2L2 Rxn #1	1&2L2 Rxn #2	1&2L2 Rxn #3	1&2L3 Rxn #1	1&2L3 Rxn #2	1&2L3 Rxn #3	1&2L4 Rxn #1	1&2L4 Rxn #2	1&2L4 Rxn #3
3&4UN Rxn #1	3&4UN Rxn #2	3&4UN Rxn #3	3&4L2 Rxn #1	3&4L2 Rxn #2	3&4L2 Rxn #3	3&4L3 Rxn #1	3&4L3 Rxn #2	3&4L3 Rxn #3	3&4L4 Rxn #1	3&4L4 Rxn #2	3&4L4 Rxn #3
5&6UN Rxn #1	5&6UN Rxn #2	5&6UN Rxn #3	5&6L2 Rxn #1	5&6L2 Rxn #2	5&6L2 Rxn #3	5&6L3 Rxn #1	5&6L3 Rxn #2	5&6L3 Rxn #3	5&6L4 Rxn #1	5&6L4 Rxn #2	5&6L4 Rxn #3
7&8UN Rxn #1	7&8UN Rxn #2	7&8UN Rxn #3	7&8L2 Rxn #1	7&8L2 Rxn #2	7&8L2 Rxn #3	7&8L3 Rxn #1	7&8L3 Rxn #2	7&8L3 Rxn #3	7&8L4 Rxn #1	7&8L4 Rxn #2	7&8L4 Rxn #3
12 Rxn #1	12 Rxn #2	12 Rxn #3	12 Rxn #1	12 Rxn #2	12 Rxn #3	12 Rxn #1	12 Rxn #2	12 Rxn #3	12 Rxn #1	12 Rxn #2	12 Rxn #3
GCD2 (-)	GCD2 (-)	GCD2 (-)									

Round 3: Operator 1 or 2											
10 Rxn #1	10 Rxn #2	10 Rxn #3	11 Rxn #1	11 Rxn #2	11 Rxn #3	12 Rxn #1	12 Rxn #2	12 Rxn #3	13 Rxn #1	13 Rxn #2	13 Rxn #3
14 Rxn #1	14 Rxn #2	14 Rxn #3	15 Rxn #1	15 Rxn #2	15 Rxn #3	16 Rxn #1	16 Rxn #2	16 Rxn #3	17 Rxn #1	17 Rxn #2	17 Rxn #3
GEB2 (-)	GEB2 (-)	GEB2 (-)									

____ Tightly cover plate with provided microfilm seal. Vortex to thoroughly mix the reaction and centrifuge to collect liquid to the bottom of the reaction tube.

Note: do not pipetting up and down, reagents will stick to the tip.

____ Place tubes into QuantStudio and initiate data collection

____ After 6 cycles (approximately 4 minutes), pause the ABI QuantStudio and remove the reactions. NOTE ORIENTATION OF PLATE BEFORE REMOVING. Quickly vortex, centrifuge, and reinsert the reactions into the QuantStudio.
Note: Ensure tubes are back in original position and orientation when you place back in QuantStudio

____ After reinserting, click the center of the display to go to control menu. Hit resume button in the bottom right, and cycle will begin again. Select the left arrow on the left side of the screen to return to the main display.

____ After all cycles have run, the instrument will beep indicating the test is complete.

____ Add Cq values to the chart below.

____ Interpret results as follows: Blue curve = FAM = *Pram* Red curve = CalRed = Internal control
Any sample that produces a Cq for both the internal control and P. ram targets is **POSITIVE**
Any sample that produces a Cq for the internal control but not the P. ram target is **NEGATIVE**
Any sample that fails to produce an internal control Cq is **INVALID** and should be retested

Post Results:

Round 1 Results:											
1&2UN:	1&2UN:	1&2UN:	1&2L2:	1&2L2:	1&2L2:	1&2L3:	1&2L3:	1&2L3:	1&2L4:	1&2L4:	1&2L4:
3&4UN:	3&4UN:	3&4UN:	3&4L2:	3&4L2:	3&4L2:	3&4L3:	3&4L3:	3&4L3:	3&4L4:	3&4L4:	3&4L4:
5&6UN:	5&6UN:	5&6UN:	5&6L2:	5&6L2:	5&6L2:	5&6L3:	5&6L3:	5&6L3:	5&6L4:	5&6L4:	5&6L4:
7&8UN:	7&8UN:	7&8UN:	7&8L2:	7&8L2:	7&8L2:	7&8L3:	7&8L3:	7&8L3:	7&8L4:	7&8L4:	7&8L4:
12:	12:	12:	12:	12:	12:	12:	12:	12:	12:	12:	12:
GEB2 (-)	GEB2 (-)	GEB2 (-)									

Round 2 Results:											
1&2UN:	1&2UN:	1&2UN:	1&2L2:	1&2L2:	1&2L2:	1&2L3:	1&2L3:	1&2L3:	1&2L4:	1&2L4:	1&2L4:
3&4UN:	3&4UN:	3&4UN:	3&4L2:	3&4L2:	3&4L2:	3&4L3:	3&4L3:	3&4L3:	3&4L4:	3&4L4:	3&4L4:
5&6UN:	5&6UN:	5&6UN:	5&6L2:	5&6L2:	5&6L2:	5&6L3:	5&6L3:	5&6L3:	5&6L4:	5&6L4:	5&6L4:
7&8UN:	7&8UN:	7&8UN:	7&8L2:	7&8L2:	7&8L2:	7&8L3:	7&8L3:	7&8L3:	7&8L4:	7&8L4:	7&8L4:
12:	12:	12:	12:	12:	12:	12:	12:	12:	12:	12:	12:
GEB2 (-)	GEB2 (-)	GEB2 (-)									

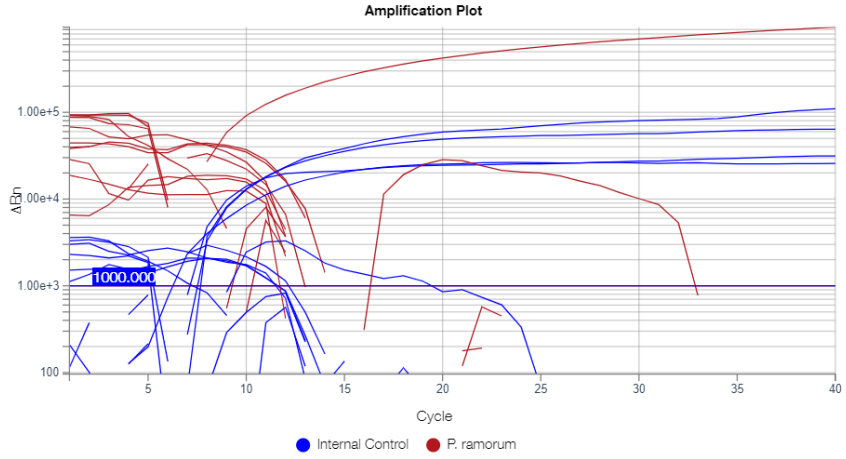
Round 3 Results:											
10	10	10	11	11	11	12	12	12	13	13	13
14	14	14	15	15	15	16	16	16	17	17	17
GEB2 (-)	GEB2 (-)	GEB2 (-)									

Experiment Properties in QuantStudio 3:

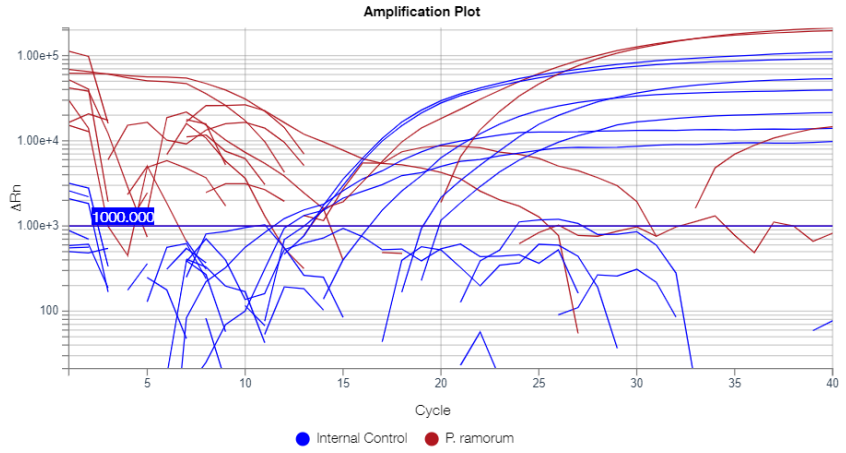
____ Open the experiment properties named “AmplifyRP CRT for Pramorum”.
____ Ensure reaction conditions are set to sample size of 25 µL, temperature of 42°C, cycle timing of 20 seconds, and 40 total cycles.
____ Make sure data collection is turned on by clicking the camera icon until it is highlighted
____ Ensure the pause feature box is checked, and set to pause after 6 cycles at 42°C
____ Ensure targets selected are “P. ramorum” and “Internal control”
____ Remember to use the template to run the procedure and save it named as you wish in the data folder.

NOTE: This is the end of part 1, proceed to the ELISA processing to complete the ring test.

A.



B.



C.

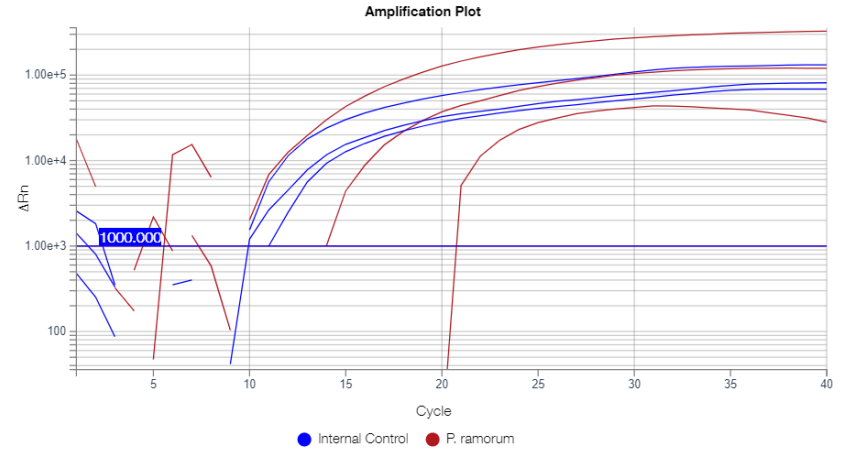


Figure A2. Sample of amplification curve results for Material 12 from Round 1, Round 2, and Round 3 of RPA.