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A RAPID DIAGNOSTIC TEST AND MOBILE "LAB IN A SUITCASE" PLATFORM FOR DETECTING *CERATOCYSTIS* SPP. RESPONSIBLE FOR RAPID `Ō HI`A DEATH

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

We describe a field compatible molecular diagnostic test for two new species of *Ceratocystis* that infect `ōhi`a (*Metrosideros polymorpha*) and cause the disease commonly known as Rapid `Ōhi`a Death. The diagnostic is based on amplification of a DNA locus within the internal transcribed spacer region that separates fungal 5.8S ribosomal genes. The assay uses forward and reverse primers, recombinase polymerase, and a fluorescent probe that allows isothermal (40°C) amplification and simultaneous quantification of a 115 base pair product with a battery operated fluorometer. DNA extractions are field compatible and can be done by heating wood drill shavings to 100°C in Instagene® solution containing Chelex® resin to bind potential amplification inhibitors. The initial heat treatment is followed by a short bead beating step with steel ball bearings and zirconium beads to release DNA. DNA is subsequently purified with a magnetic bead based extraction method that does not require silica columns or centrifugation. The assay is designed around a portable "lab-in-a-suitcase" platform that includes a portable fluorometer, miniature centrifuge, and heat block that operate off either 120V AC power sources or a 12 volt battery with a portable inverter, a magnetic rack designed for 1.5 ml tubes and magnetic bead DNA purification, pipettes and consumable reagents and tubes. The entire assay from DNA extraction to results can be performed in less than 90 minutes on up to six independent samples plus a positive and negative control. Sensitivity based on suspensions of *Ceratocystis* endoconidia (spores) that were added to wood shavings and processed under field conditions by Instagene® magnetic bead DNA extraction was up to 163 spores/mg wood for Species A and 55 spores/mg wood for Species B in 95% of replicates as determined by probit analysis. Sensitivity increased 5–10 fold to 19 spores/mg wood for Species A and 9 spores/mg wood for Species B when extractions were performed with a commercial, silica column based DNA purification kit. The test did not cross react with other common fungi that have been isolated from `ōhi`a.

INTRODUCTION

Rapid `Ōhi`a Death (ROD) is a suspected vascular wilt disease of the `ōhi`a lehua tree (*Metrosideros polymorpha*), a keystone species that is native to Hawaiian Islands. First confirmed in 2014 in the lower Puna District of Hawai`i Island (Keith et al. 2015), the disease is expanding throughout the Puna and South Hilo Districts of Hawai`i Island and along the Wailuku River watershed west of Hilo. More than 50,000 acres of private and state forest lands have been affected (http://cms.ctahr.hawaii.edu/rod/TheDisease.aspx). The disease is highly pathogenic in native `ōhi`a trees and can lead to significant mortality once symptoms become evident. This emerging disease is a threat to native forests because of its potential impacts in high value conservation areas and watersheds managed by Department of Interior Agencies, the State of Hawai`i, and State Watershed Partnerships. In response to this threat, the Hawai`i Department of Agriculture has placed an embargo on unrestricted movement of `ōhi`a, `ōhi`a products, and soil off of Hawai`i Island to stop spread of the infection.

The disease is caused by two new members of the *Ceratocystis fimbriata* species complex, a widespread group of closely related fungal pathogens that are incompletely characterized (Oliveira et al. 2015). One of these two new species is similar to *Ceratocystis platani* (Species A, ōhi`a wilt) while the second *Ceratocystis* (Species B, `ōhi`a decline) has affinities to isolates of *Ceratocystis* from taro (*Colocasia esculenta*) in Hawai`i and China and eucalyptus (*Eucalyptus sp.*) stumps in China (L. M. Keith, unpub. data, 2017). Infections caused by both species are

commonly referred to as ROD because of rapid progression of the disease once symptoms become evident. Both species of fungi have been detected alone and in combination in native `ōhi`a trees from Hawai`i Island. Close relatives of Species A are known pathogens of woody hosts, including sycamore (*Platanus occidentalis*), gum trees (*Eucalyptus spp.*), black wattle (*Acacia mangium*), pomegranate (*Punica granatum*), coffee (*Coffea spp.*), cacao (*Theobroma cacao*), and fig (*Ficus carica*) (Harrington 2013). Although relatives of Species B are typically associated with post-harvest disease on storage corms (Thorpe et al. 2005), isolates of Species B from `ōhi`a trees have proven to be as virulent as isolates of Species A on inoculated seedlings and saplings in laboratory tests (L. M. Keith, unpubl. data, 2017). Both species are considered to be significant threats to Hawai`i's native forests. Field-scale experiments and continued disease monitoring will reveal additional information about their potential long-term ecological impacts.

The primary mechanism of natural spread likely involves production and release of boring dust containing infective aleurioconidia by wood boring ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) with subsequent movement by wind and flowing water (Luchi et al. 2013, Grosclaude et la. 1991). Other hypothesized pathways for spread of the infection are currently under investigation and include movement of infected wood, soil and plant products, use of contaminated tools and equipment for landscaping, spread via contaminated footwear and vehicles, movement of infected soil by feral ungulates, and movement by birds through adhesion of sticky ascospores on plumage or through ingestion of beetle prey containing environmentally resilient aleurioconidia.

Given the need to more accurately identify the distribution of this pathogen and develop effective management actions, rapid and accurate field and laboratory diagnostic methods are needed to confirm infections in symptomatic trees. Methods that are currently being used are based on physical signs of infection (rapid browning of leaves, characteristic staining of the sapwood) and isolation of fungus with morphological characteristics consistent with *Ceratocystis* in tissue culture. A TaqMan® based qPCR assay has also been developed for rapid lab-based identification and differentiation of *Ceratocystis* Species A and B (W. B. Heller and L. M. Keith, unpubl. data, 2017), however the test reported here offers significant advantages in terms of portability and reduced instrumentation costs. Our objective was to develop a portable and rapid field diagnostic assay based on amplification of fungal DNA that can complement existing laboratory capabilities, provide diagnostic capabilities to organizations without dedicated laboratory facilities, and help to inform management actions in real time.

METHODS

Sample Collection and Field Extraction of DNA

Wood samples were collected from both healthy `ōhi`a trees and trees with symptoms of ROD by removing outer bark by hand and then drilling into the trunk with a battery operated hand drill and 3/8 inch drill bit. Drill shavings were collected directly into a sterile whirl-pak® bag (Thermo Fisher Scientific, Waltham, Massachusetts, 12.5 X 7.5 cm, catalog #08-512-5M). Approximately 100 mg of drill shavings were transferred with metal forceps into 2.0 ml screw cap microcentrifuge tubes (Thermo Fisher Scientific, Waltham, Massachusetts, catalog # 02-681-375) containing 0.3 g of 800 µm low binding zirconium beads (OPS Diagnostics LLC, Lebanon, New Jersey, catalog #BLBZ 800-250-34) and three, 3 mm stainless steel grinding balls (OPS Diagnostics LLC, Lebanon, New Jersey, catalog #GBSS 118-2500-09). Six hundred µl

of Instagene® Matrix (Bio-Rad Life Science Research, Hercules, California, catalog #7326030) was added with a pipette and disposable filter tips; tubes were vortexed briefly to wet the drill shavings (Scientific Industries Inc., Bohemia, New York, Vortex Genie 2, catalog #SI-0236). They were then centrifuged briefly (USA Scientific, Personal microcentrifuge, catalog #2631-0006) to pellet shavings at the bottom of the tubes. Grinding tubes were placed into a heating block (USA Scientific, Thermal-Lok Mini Dry Heat Bath, catalog #2520-1001, Ocala, Florida) that was preheated to 100°C (Appendix 1–3).

The wood/Instagene® mixture was heated for five minutes and removed from the block. Grinding tubes were transferred to a Vortex Genie 2 with a circular Vortex Adapter that holds 24 - 2 ml microcentrifuge tubes (Mo Bio Laboratories, Carlsbad, California, catalog #13000-V1-24). The grinding tubes were agitated at the maximum speed setting on the Vortex Genie 2 for 60 seconds to break up the wood sample and release fungal DNA. Following agitation, tubes were centrifuged for 30 seconds to pellet the drill shavings and all recoverable fluids were transferred with a pipette with disposable filter tips to 0.5 ml snap cap microcentrifuge tubes. The 0.5 ml microcentrifuge tubes were then centrifuged for 60 seconds to pellet fine particulates in the Instagene® wood extract and clarify the supernatant.

A magnetic bead based DNA purification protocol was used with reagents from a Bio-Nobile (Pargas, Finland) QuickPick™ SML Plant DNA kit that were purchased individually in bulk volumes (Sunrise Science Products, San Diego, CA, https://sunrisescience.com/). These included QuickPick™ XL Plant DNA Magnetic Particles (catalog #531000), QuickPick™ XL Plant DNA Binding Buffer (catalog #53300), QuickPick™ XL Plant DNA Wash Buffer (catalog #53500), and QuickPick™ XL Plant DNA Elution Buffer (catalog #53600). The extraction protocol followed the manufacturer instructions, skipping steps related to addition of lysis buffer and RNase, and starting with addition of up to 120 µl of clarified supernatant from the Instagene® wood extract to a 1.5 ml tube containing 190 µl of binding buffer and 7.5 µl of magnetic beads. The magnetic beads were allowed to bind DNA in the wood extract at room temperature for 5 minutes while the mixture was mixed every 60 seconds by gentle vortexing.

The mixture was placed into the magnetic rack (Invitrogen, ThermoFisher Scientific, Waltham, MA, MagnaRack, catalog #CS150000) to draw the magnetic beads to the side of the tube into a dark pellet. The remaining clear (but colored) extract was removed and discarded with a pipette and filter tip, leaving magnetic beads with bound DNA. The tube was removed from the magnetic position in the rack; 250 µl of QuickPick™ XL Plant DNA Wash Buffer was added; and the tube was gently vortexed to resuspend the pellet of magnetic beads. After a brief incubation lasting several minutes while other samples in the rack were processed, washes were repeated three more times.

After the last wash, the tube was removed from the magnetic position in the rack and 75 µl of QuickPick™ XL Plant DNA Elution Buffer was added to release DNA from the magnetic beads. The pellet was resuspended as described earlier, incubated for five minutes at room temperature, and then returned to the magnetic rack to collect the magnetic beads on the side of the tube. Elution buffer containing extracted DNA was then removed with a pipette and filter tip and transferred to a clean, labeled 0.5 ml tube for subsequent analysis.

Primer and Probe Design

Primers were designed to amplify a 193 bp fragment of 5S ribosomal DNA from the *Ceratocystis* 5S ribosomal internal transcribed spacer (ITS) region based on alignment of a 620 bp fragment of the ITS region from Species A (Genbank Accession KU043252) and Species B (Genbank

Accession KP203957). Conserved regions of ITS were targeted based on available sequence data for both probe and primer design to develop an assay that detects both Species A and Species B. Primer and probe designs were compatible with isothermal DNA amplification with recombinase polymerase (Piepenburg et al. 2006) and followed recommendations by the manufacturer (TwistDx, Cambridge, UK) to be compatible with the TwistAmp® exo kit. This DNA amplification chemistry allows isothermal real time detection of an amplification product with use of a proprietary 46–52 bp oligonucleotide probe containing a tetrahydrofuran (THF) residue flanked by a dT-fluorophore (a fluorophore attached to one of the non-base pairing positions on thymidine) and a corresponding dT-quencher group. These are placed at least 30 bp from the 5′ end of the probe so that the 5′ end of the probe can act as a forward primer when cut by Exonuclease III in the reaction mixture (Table 1). A block at the 3′-end by a suitable modification group prevents the entire 46–52 oligonucleotide probe from acting as an amplification primer. Our probe (LGC Biosearch Technologies, Novato, California), used a FAM dT-flurophore, Black Hole Quencher – 1 (BHQ-1) as a dT-quencher, and a standard C3 spacer, a short 3 carbon chain (C3) attached to the terminal 3′ hydroxyl group of the oligonucleotide, as a 3′ block. During the isothermal reaction, Exonuclease III in the proprietary reaction mixture cleaves the probe at the THF residue after it binds to the template DNA, separating the fluorophore and the quencher and generating a fluorescent signal (Piepenburg et al. 2006). As a result, the assay is similar to a TaqMan® quantitative PCR (qPCR), but proceeds exponentially at constant optimal temperatures ranging between 37°C–42°C.

Unlike standard PCR, there are no fixed rules for predicting how well amplification primers will perform, so we used an empirical "trial and error" approach to design and test different combinations of forward and reverse primers and probe. Both forward and reverse primers were 30–35 oligonucleotides in length as recommended by TwistDx, because recombination/priming with shorter oligonucleotides in the TwistAmp® reaction environment decreases sharply with size (TwistAmp® Combined Instruction Manual 2016). Locations of primer probe combinations on the ITS alignment were also chosen to minimize formation of hairpins and secondary structures. These were evaluated using functions available in Geneious R8.1.9 (Biomatters, LTD, Aukland, New Zealand). Hairpins longer than 5 bp that were stable at 40°C were avoided. For probe design, hairpins that included the tetrahydrofuran (THF) residue were also avoided, because exonuclease in the reaction mix could digest the probe before binding to target DNA and lead to false positive results.

Isothermal Assay Conditions and Primer Selection

All isothermal reactions were performed at 40°C in 50 µl volumes with a TwistAmp® exo kit (TwistDx, Cambridge, UK, catalog #TAEXO02KIT) in a T8 Isothermal Device (TwistDx, Cambridge, UK, catalog #T8Device). Individual tubes containing a proprietary mix of freeze dried components from the manufacturer were rehydrated with a master mix containing 29.5 µl rehydration buffer (supplied by the manufacturer), 5.5 µl of water, 3 µl of 10 µM forward primer, 3 µl of 10 µM reverse primer, 1 µl of 10 µM probe, 4 µl of DNA template (*Ceratocystis* Species A), 4 µl of magnesium acetate, and a single steel mixing ball dispensed with a Micro Ball Dispenser (TwistDx, Cambridge, UK, catalog #T1601balldispenser) (Appendix 4). The magnesium acetate was added to the caps of individual tubes rather than the reaction mix. After caps were carefully replaced, tubes were manually shaken simultaneously to introduce magnesium acetate to the reaction mix and initiate the isothermal reaction. Tubes were then briefly centrifuged to pellet the reaction mix and placed immediately into a T8 Isothermal Device (Appendix 5). This portable fluorometer measures fluorescence generated by the

isothermal reaction at 40°C over a 15-minute reaction after an initial magnetic mixing cycle with a steel ball bearing.

Table 1. Primer and Probe sequences.

Name	Direction	Sequence (5'-3')
Probe		
CF415P*	Forward	CGAAATGTATCGGCTGTTATACTTGCCAAC**F**C**H**CC**1**GTGTAGTATAAAATTT[C3]
		F = FAM
		H = THC
		1 = BHQ-1
		C3 = C3 Spacer
Primers		
268F	Forward	TGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
303F	Forward	TATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTC
325F	Forward	GCCAGGCATGCCTGTCCGAGCGTCATTTCACCACT
329F*	Forward	GGCATGCCTGTCCGAGCGTCATTTCACCACTCAAG
330F	Forward	GCATGCCTGTCCGAGCGTCATTTCACCACTCAAGG
354F	Forward	TTTGTTCTTGGCGTTGGAGGTCCTGTTCTCCCCTG
548R	Reverse	TCAACCTTGTGAAAGTTCAACAAAAGTTGAGGGGG
566R	Reverse	TTCCTACCTGATCCGAGGTCAACCTTGTGAAAGTT
503R	Reverse	GGCGTGTTACACAAGAACTTCAAAGTGTAAAAATT
509R	Reverse	TTTAGCGGCGTGTTACACAAGAACTTCAAAGTGTA
514R*	Reverse	GGGGGTTTAGCGGCGTGTTACACAAGAACTTCAAA
514BR*	Reverse	GAAGGTTTAGCGGCGGTGCTACACCAGAACTTCAAA

* Used in final assay

Six forward and five reverse primers were selected that flanked a 52 bp probe that targeted a conserved region in the alignment (Figure 1, Table 1). All possible forward and reverse primer combinations were evaluated based on slope of the curve as measured by changes in fluorescence (millivolts, mV) per unit time and the time needed for fluorescence to cross a threshold that was determined by averaging fluorescence measurements from control tubes that contained water rather than DNA. Tubes containing primer probe combinations that reached peak fluorescence with the steepest slopes and crossed the threshold in the shortest times were selected for further evaluation.

Assay Sensitivity

Endoconidia (spore) suspensions of both Species A and B were prepared by flooding colonized 2% malt extract agar (Becton Dickinson Company, Franklin Lakes, New Jersey, catalog #BD 21120) with sterile distilled water, scraping the surface with a rubber policeman, and filtering the agar washes through Miracloth (EMD Millipore, Billerica, Massachusetts, catalog #475855-1R) to remove hyphal fragments. Spores were enumerated with a hemocytometer to determine numbers per 100 µl of filtrate and diluted with sterile distilled water to prepare nine, two fold serial dilutions ranging in concentration from 100–25,600 spores per 100 µl of distilled water. A 100 µl aliquot of each dilution was combined with 100 mg of fresh sawdust from a healthy, uninfected `ōhi`a tree, yielding a series of wood samples spiked with 100–25,600 spores at concentrations ranging from 1 spore/mg of wood to 256 spores/mg of wood. Samples were extracted with NucleoSpin Plant II DNA extraction kit (Macherey-Nagel, Bethlehem, Pennsylvania, catalog #740770.250) following the manufacturer's protocol. Wood shavings were lysed with 300 µl of PL2 buffer and 10 µl of RNAse A (10 µg/µl) in 2.0 mL tubes with containing six, 3.0 mm zirconium beads (OPS Diagnositcs LLC, Lebanon, New Jersey, catalog # PFAW 3000-50-17). Samples were disrupted to release DNA with a FastPrep 24 homogenizer (MP Biomedicals, Santa Ana, California) for two, one minute intervals at 6.5 M/s with heating at 65˚C for 10 minutes between the two homogenization steps. Other extraction steps followed manufacturer recommendations for the NucleoSpin Plant II kit. DNA was eluted in a final volume of 100 µl.

A second series of serial two fold spore dilutions was prepared with uninfected `ōhi`a wood and extracted as described earlier using 500 rather than 600 µl of Instagene® Matrix and a magnetic bead cleanup and eluted in a final volume of 75 µl elution buffer. Extracted DNA from both the NucleoSpin Plant II Extractions and the Instagene® magnetic bead extractions were tested with a TwistAmp® exo kit in a T8 Isothermal Fluorometer as described previously.

Results from eight replicates of the NucleoSpin Plant II and Instagene® magnetic bead extractions were classified as either positive or negative based on 1) a fluorescence threshold (mean + 3.5 standard deviations) calculated from 90 measurements during the 900 seconds (15 minute) reaction period and 2) a 1st derivative (instantaneous slope) cutoff (mean + 3.5 standard deviations) determined from 60 fluorescence measurements from the exponential phase of the reaction between 150 and 750 seconds from the initiation of the assay. Threshold and 1st derivative cutoff values were calculated from 26 control reactions that were performed with water rather than template DNA. First derivative values were calculated for each fluorescence measurement with the T8 device software (T8 Desktop Application, version 2.4.3.0, Axxin, Fairfield, Australia). Samples were classified as positive if they exceeded both a fluorescence threshold (1497 mV) and 1st derivative cutoff value (1.75). The resulting positive and negative test results from the serial NucleoSpin Plant II extractions (72 replicates) and Instagene® magnetic bead spore extractions (72 replicates) were analyzed by probit regression

(Medcalc Software, Ostend, Belgium) to determine analytical sensitivity and confidence limits for detection of each spore dilution.

Assay Specificity

Specificity of the assay was determined by testing a panel of 24 samples of extracted DNA from trees with known infections of *Ceratocystis* Species A and B, uninfected trees, trees infected with other fungal pathogens, soil containing a diverse microbial community, and plants infected with other species/strains of *Ceratocystis*. All tests were run as described earlier. An assay was considered positive when it met both slope and fluorescence criteria described in the previous section.

T8 Program Parameters

We programmed the T8 isothermal fluorometer to detect the lowest spore dilutions that met criteria for distinguishing positive samples from controls that did not include template DNA. Initial average parameters were set so that unusually high levels of background fluorescence at the beginning of the reaction would invalidate a procedure as "indeterminate". Gradient parameters were set so that a positive sample required an average increase in fluorescence that exceeded 1.0 mV/second within a 180 second sliding window between 150 and 750 seconds after the start of a reaction. This was chosen based on slopes of serial spore dilutions so that mean 1st derivatives for fluorescence measurements during this period exceeded a value of 1.75. Amplitude parameters were set so that positive samples required a threshold value of at least 1,700 mV within a 30 second sliding window between 750 and 900 seconds at the end of the reaction. This setting was higher than the one used for this study to allow for operator and test variability under a wide range of conditions. Finally, we programmed the device so that samples had to pass criteria for both the gradient and amplitude tests (Figure 2) to be considered positive. Incorporation of these criteria into the fluorometer settings simplified reporting of results to the instrument operator and displayed final values as either "positive", "negative" or "indeterminate" at the end of a reaction, with the option to inspect individual amplification curves for verification.

Figure 1. Primer and Probe binding sites on a 320 bp region of the *Ceratocystis* Internal Transcribed Spacer (ITS). Primer and Probe sequences were based on an alignment of sequence data from *Ceratocystis* Species A (Genbank Accesion KP203957.1) and B (Genbank Accesion KU043252). Primers and probe were initially designed to amplify Species A.

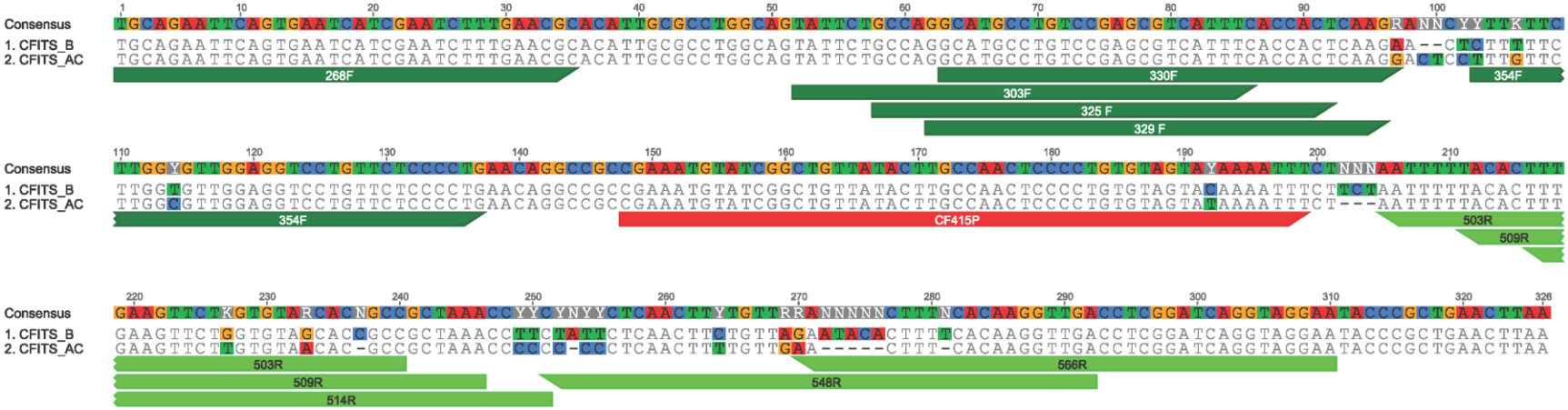
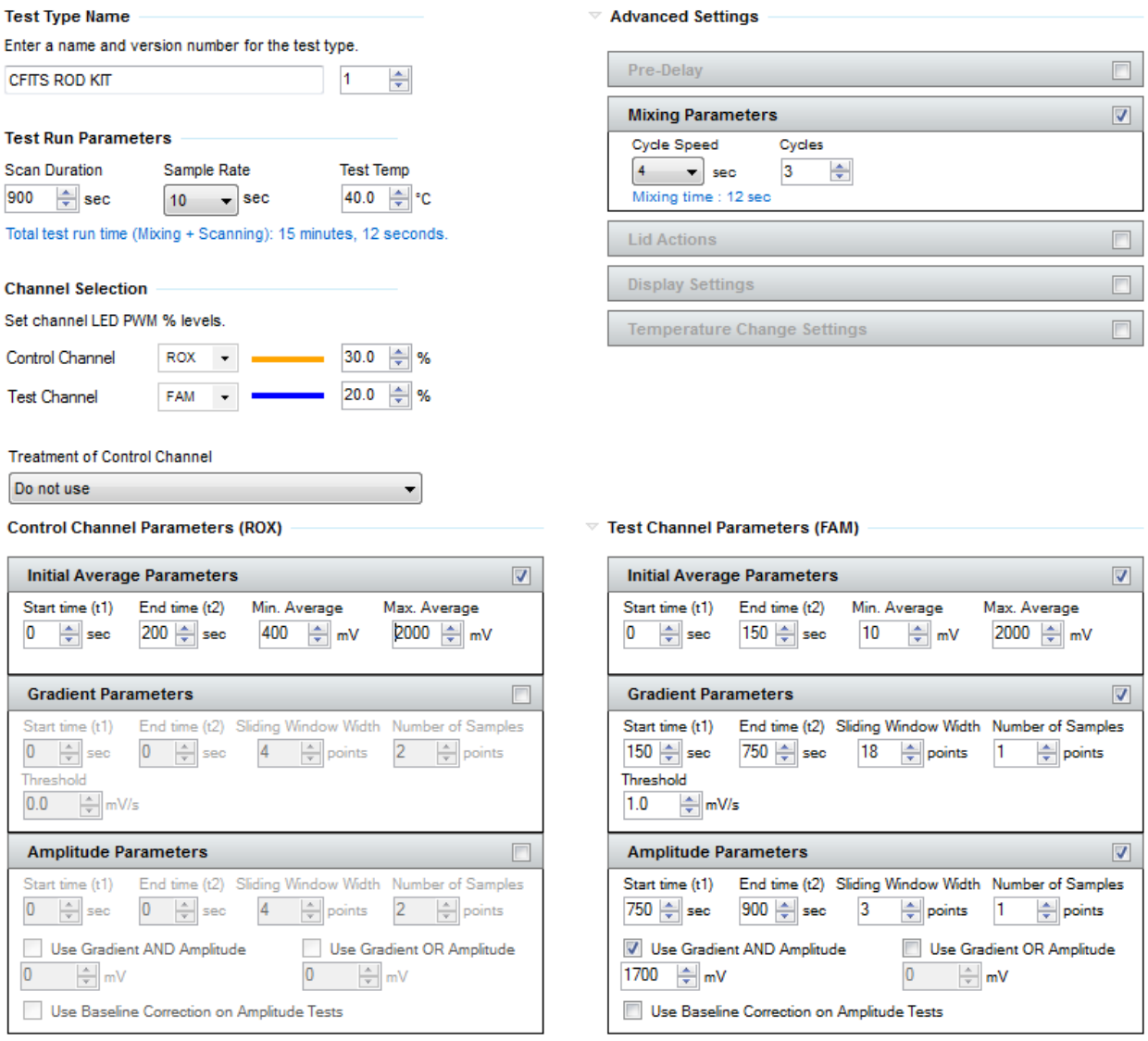


Figure 2: Program settings for the T8 isothermal fluorometer as viewed with the instrument software. The device was programed to distinguish positive and negative samples based on spore dilutions that were extracted with NucleoSpin Plant II Kit and the Instagene® magnetic bead extraction methods. All tests were run at 40°C for 900 seconds (15 minutes) with three, 4 second mixing cycles at the beginning of the incubation period. To be considered positive, a sample required both an average slope greater than 1.0 mV/second during a 180 second sliding window during the amplification phase of the test (150–750 seconds) and a final amplitude of at least 1,700 mV over a 30 second sliding window at the end of the test (750–900 seconds). These settings were chosen to maximize detection of low positive samples.

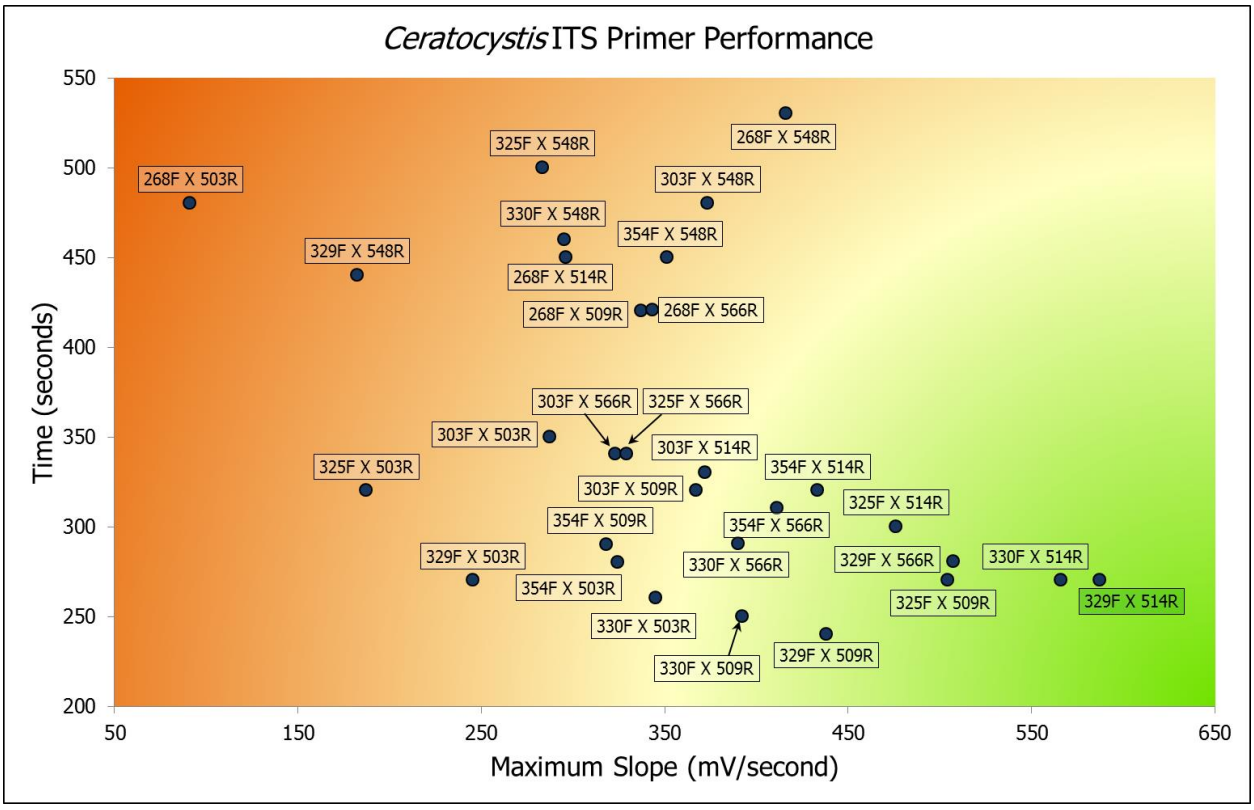


RESULTS

Primer Selection

Among primer pairs that we tested on DNA extracts from Species A, 329F X 514R was the best combination with Probe 415, crossing a 2000 mV fluorescence threshold within 4–5 minutes after the start of the reaction. While several other primer combinations had faster reaction times (e.g. 329F X 509R and 330F X 509R), curves from 329F X 514R had the steepest slopes (Figure 3).

Figure 3: Results of primer screens to determine reaction performance. Extracted DNA from an `ōhi`a infected with Species A was tested with 30 different primer pair combinations (5 reverse X 6 forward primers). Each primer pair was evaluated by amount of time in seconds (y-axis) to cross a threshold absorbance of 2000 mV and the rate of change in absorbance in mV/second (x-axis). Isothermal reactions with the best performing primer pairs crossed the threshold within the shortest time and had the steepest maximum slope during amplification (green shading). Primer pair 329F X 514R (dark green highlight) performed best.



Initial tests of this primer probe concentration on samples extracted from Species B were not as efficient in amplifying DNA, most likely because of five mismatches in DNA sequence between Species A and B in the region covered by 514R (Figure 1). To increase amplification efficiency for Species B and make sensitivity of the test more equal for both species, we designed a second reverse primer, 514BR (Table 1, Figure 1), that was complementary to the ITS sequence of Species B. We combined equimolar amounts of 514R and 514BR in reaction mixtures to make it more likely that both species would have an equal probability of being amplified.

Assay Sensitivity

Sensitivity of the assay was determined from extractions of spiked wood samples containing serial spore dilutions with a NucleoSpin Plant II DNA Kit and the Instagene® magnetic bead based purification protocol. Representative fluorescence curves and graphs of 1st derivative values are illustrated in Appendix 1.

Amplification was evident for dilutions as low as 1 to 2 spores/mg of wood shavings for samples spiked with Species B and extracted with both the Instagene® magnetic bead purification and Nucleospin Plant II Kits (Table 2). Positive detections using both threshold and instantaneous slope as criteria were inconsistent at lower spore concentrations, however. Detections did not exceed 50% of replicates until spore concentrations exceeded 8 spores/mg of wood shavings for Species B and 16–32 spores/mg of wood shavings for Species A.

Table 2. Number of test replicates out of a total of eight that were positive for Species A and Species B by different extraction methods. Replicates were not scored as positive unless they produced a reaction curve that exceeded both a minimum fluorescence threshold of 1497 mV and an instantaneous slope of 1.75 during the exponential phase of the reaction between 150 and 750 seconds after initiation.

	Instagene® Replicates Positive		NucleoSpin Plant II Replicates Positive	
Dilution	Species A	Species B	Species A	Species B
1	0	3	0	0
2	0	1	0	2
4	0	0	0	2
8	0	4	3	6
16	0	6	7	8
32	5	8	8	8
64	6	8	8	8
128	8	8	8	8
256	7	8	8	8

A probit analysis was used to determine confidence intervals for detecting Species A and Species B at different spore dilutions for each extraction method. Assay sensitivity was lower (curves were shifted to right) and confidence intervals were wider for spiked wood shavings extracted with Instagene® magnetic bead purification relative to the NucleoSpin Plant II Kit (Figures 4 and 5). Similarly, detection sensitivity was lower for Species A for both extraction methods. Based on probit analysis of 8 replicates, there was a 95% probability that 163 or more Species A spores/mg wood and 55 or more Species B spores/mg wood will be detected with Instagene® magnetic bead extractions (Table 3). By contrast, assays based on NucleoSpin Plant II Kit extractions were more sensitive, with a 95% probability that 19 or more Species A spores/mg wood and 9 or more Species B spores/mg wood will be detected (Table 4).

Figure 4. Probability curves and 95% confidence intervals for detecting Species A and Species B spore dilutions in wood shavings extracted with Instagene® Matrix and magnetic bead purification. Markers (○) identify each spore dilution.

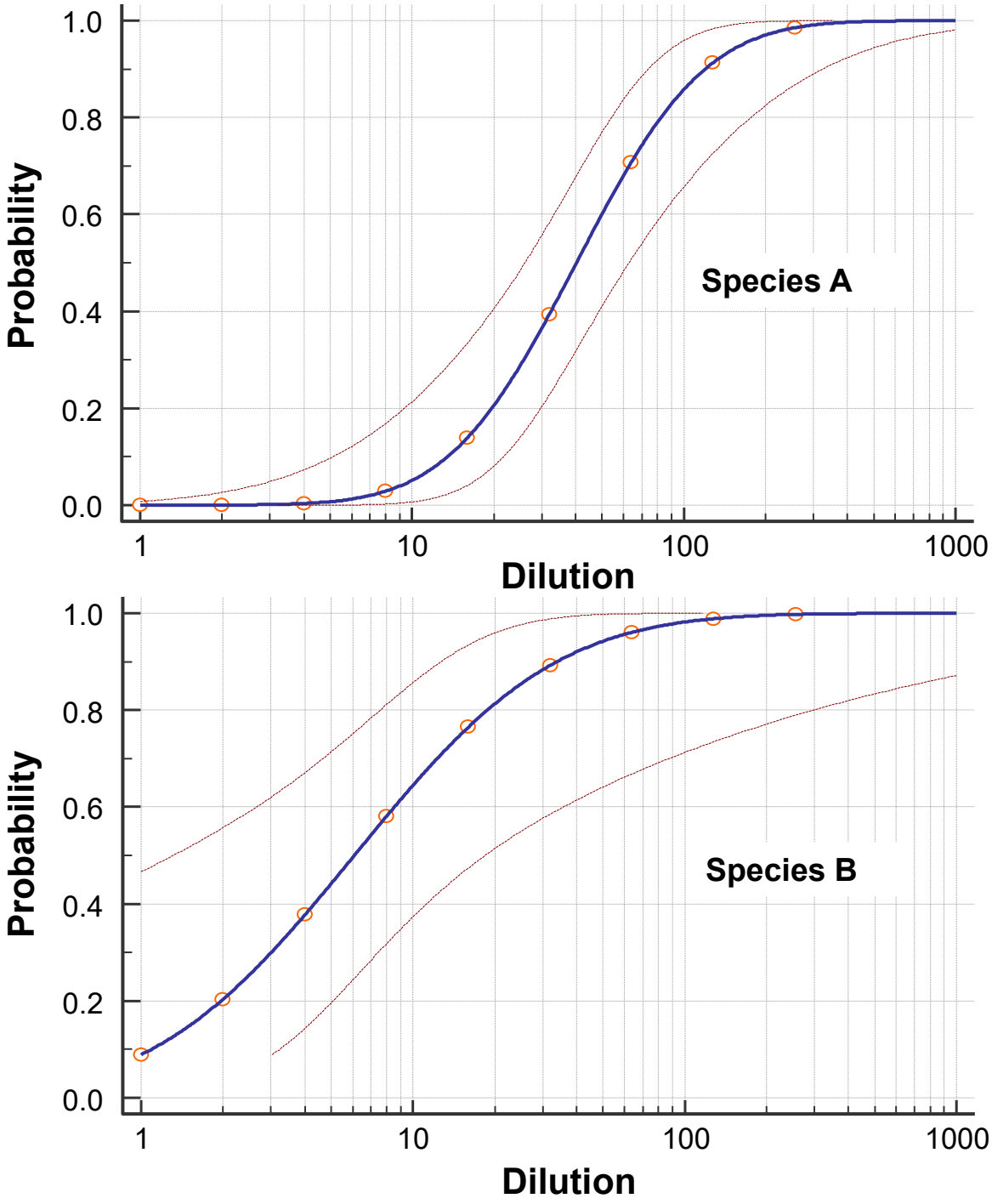


Figure 5. Probability curves and 95% confidence intervals for detecting Species A and Species B spore dilutions in wood shavings extracted with a NucleoSpin Plant II Kit. Markers (○) identify each spore dilution.

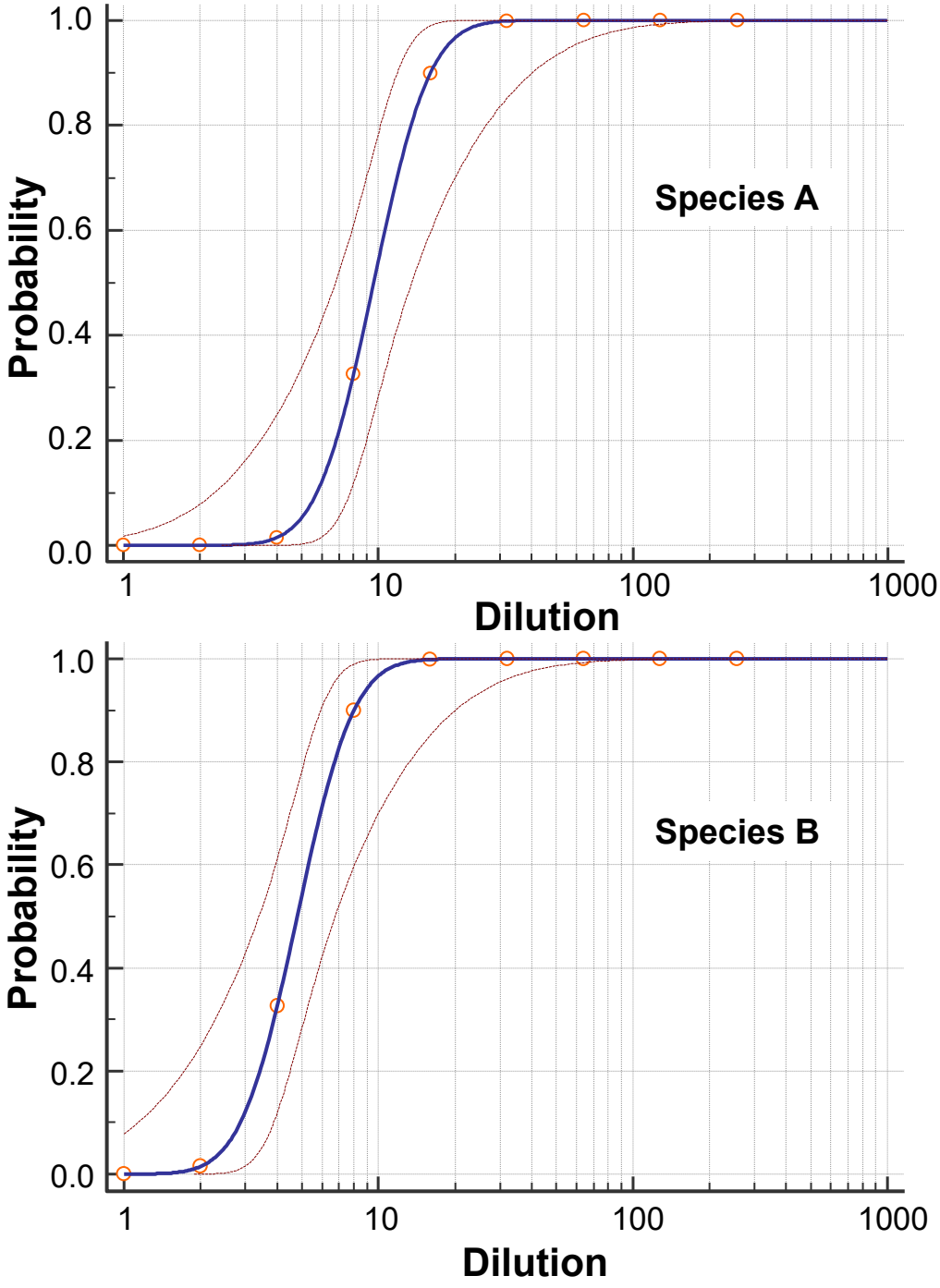


Table 3. Probability and 95% confidence intervals (indicated with dark shading) for detecting Species A and B in wood shavings extracted with Instagene® magnetic bead purification.

	Species A			Species B		
Probability	**Spores**	**95% Confidence interval**		**Spores**	**95% Confidence interval**	
0.010	6	1.1	11.0	0	0.0	1.3
0.020	7	1.7	13.1	0	0.0	1.6
0.025	8	1.9	13.9	0	0.0	1.7
0.050	10	3.1	17.0	1	0.0	2.3
0.100	14	5.1	21.7	1	0.0	3.2
0.200	20	9.4	29.8	2	0.1	5.1
0.250	23	11.7	33.9	2	0.1	6.2
0.500	40	26.0	62.9	6	1.3	18.4
0.750	72	47.7	141.2	15	6.0	153.7
0.800	82	54.2	176.5	19	7.6	298.3
0.900	120	74.1	324.6	34	12.7	1909.1
0.950	163	94.5	545.5	55	18.3	9417.4
0.975	214	115.8	861.2	84	24.5	38452.0
0.980	232	123.0	987.5	95	26.6	58601.3
0.990	292	146.1	1472.0	137	33.8	200411.1

Table 4. Probability and 95% confidence intervals (indicated with dark shading) for detecting Species A and B in wood shavings extracted with a NucleoSpin Plant II Kit.

	Species A			Species B		
Probability	**Spores**	**95% Confidence interval**		**Spores**	**95% Confidence interval**	
0.010	4	0.8	5.7	2	0.4	2.9
0.020	4	1.1	6.2	2	0.5	3.1
0.025	4	1.2	6.4	2	0.6	3.2
0.050	5	1.6	6.9	2	0.8	3.5
0.100	6	2.3	7.8	3	1.1	3.9
0.200	7	3.5	9.0	3	1.7	4.5
0.250	7	4.0	9.6	4	2.0	4.8
0.500	10	6.8	13.5	5	3.4	6.8
0.750	13	9.6	22.8	6	4.8	11.4
0.800	13	10.2	26.5	7	5.1	13.3
0.900	16	11.8	40.2	8	5.9	20.1
0.950	19	13.3	57.4	9	6.6	28.7
0.975	21	14.5	78.6	11	7.3	39.3
0.980	22	14.9	86.3	11	7.5	43.2
0.990	24	16.1	113.7	12	8.0	56.8

Assay Specificity

The recombinase polymerase assay correctly identified 23/24 known positive and negative samples in a blind test of extracted DNA from a variety of sources (Table 5). The assay did not detect Species B in one sample of wood shavings from an `ōhi`a tree, but was otherwise accurate in identifying all other Species A and Species B infections in the unknown samples. The assay detected closely related species of *Ceratocystis* that were isolated from taro (*Colocasia esculenta*), sweet potato (*Ipomoea batatas*) and ornamental *Syngonium* (arrowhead plant), but did not cross react with two common wood fungi, *Ophiostoma sp.* and *Leptographium bistatum* or with other fungal and microbial organisms in four soil samples.

Table 5. Results of a blind test to determine primer and probe specificity for the recombinase polymerase assay. Highlighted results indicate one case that did not agree.

Fungus	Host		DNA Source	Status	RPA
Ceratocystis sp. B	`Ōhi`a	*(M. polymorpha)*	Wood Shavings	+	+
uninfected	`Ōhi`a	*(M. polymorpha)*	Wood Shavings	-	-
Ceratocystis sp. B	`Ōhi`a	*(M. polymorpha)*	Wood Shavings	**+**	**-**
uninfected	`Ōhi`a	*(M. polymorpha)*	Wood Shavings	-	-
Ceratocystis sp. B	`Ōhi`a	*(M. polymorpha)*	Wood Shavings	+	+
Ceratocystis fimbriata	Taro	*(Colocasia esculenta)*	Cultured fungi	+	+
unknown			Soil	-	-
uninfected	`Ōhi`a	*(M. polymorpha)*	Wood Shavings	-	-
uninfected	`Ōhi`a	*(M. polymorpha)*	Wood Shavings	-	-
Ophiostoma sp.	Eucalyptus	*(Eucalyptus grandis)*	Cultured fungi	-	-
Ceratocystis sp. B	`Ōhi`a	*(M. polymorpha)*	Wood Shavings	+	+
Leptographium bistatum		*(Eucalyptus grandis)*	Cultured fungi	-	-
unknown			Soil	-	-
unknown			Soil	-	-
Ceratocystis fimbriata	Sweet potato	*(Ipomoea batatas)*	Cultured fungi	+	+
unknown			Soil	-	-
Ceratocystis fimbriata	Arrowhead plant	*(Syngonium sp.)*	plant tissue	+	+
Ceratocystis sp. A	`Ōhi`a	*(M. polymorpha)*	Wood Shavings	+	+
uninfected	`Ōhi`a	*(M. polymorpha)*	Wood Shavings	-	-
Ophiostoma sp.	`Ōhi`a	*(M. polymorpha)*	Cultured fungi	-	-
Ceratocystis sp. B	`Ōhi`a	*(M. polymorpha)*	Cultured fungi	+	+
Ceratocystis fimbriata	Arrowhead plant	*(Syngonium sp.)*	Cultured fungi	+	+
Ceratocystis sp. A	`Ōhi`a	*(M. polymorpha)*	Wood Shavings	+	+
Ceratocystis sp. A	`Ōhi`a	*(M. polymorpha)*	Wood Shavings	+	+

DISCUSSION

Isothermal DNA amplification is becoming increasingly popular for diagnostic assays because it can be readily adapted to office environments and field situations where expensive instrumentation and dedicated laboratory infrastructure are not available. A variety of amplification methods have been developed, including loop mediated isothermal amplification (LAMP), strand-displacement amplification, rolling circle amplification, helicase-dependent amplification, and recombinase polymerase amplification (RPA) (Yan et al. 2014). These methods amplify DNA in a constant temperature environment without the need for an expensive thermocycler. While LAMP has a longer history of use for diagnostic assays and has recently been adapted to other applications (Kundapur and Nema 2016), the popularity of RPA is growing rapidly. Primer and probe design for RPA is more similar to traditional PCR than LAMP and it can be used in a wide variety of formats that include amplification of a DNA template for cloning and sequencing, fluorescent probe based applications that are similar to quantitative TaqMan® PCR assays, and amplification of DNA products that can be detected with lateral flow test strips (Piepenburg et al. 2006). RPA has limitations, however, in that reagents and supplies are available from a single commercial source, reaction mixtures are still proprietary, and multiple primer and probe combinations often need to be tested empirically to optimize amplification of a desired target. Additionally, the RPA reaction environment can be tolerant of mismatches between target DNA and primers and probes (Daher et al. 2015), which has advantages when developing general assays, but can make assay design difficult if specificity is desired. However, numbers of publications using RPA technology in the past five years for diagnostic assays in the medical, veterinary and agricultural fields have increased exponentially (http://www.twistdx.co.uk/publications/).

We have developed an RPA assay that amplifies the two species of *Ceratocystis* (Species A and Species B) that cause ROD. We targeted multicopy ribosomal DNA loci rather than single copy nuclear genes to increase sensitivity, although qPCR assays have been successfully developed against both types of targets for these and other species of *Ceratocystis* (Heller and Keith, in preparation; Luchi et al. 2013). Assay development focused on available sequence data from the fungal 5.8s ribosomal ITS region for both species. Locating a suitable conserved region for primer and probe design was difficult because of the requirement for relatively long sequences for recombinase polymerase DNA amplification (30–35 nucleotides for primers and 46–52 nucleotides for probes). As a result, secondary structure in some promising conserved areas of the sequence caused formation of stable hairpins at the low temperature used for the reaction. The site we chose was highly conserved for the forward primer and had only a single mismatch for the probe sequence that was not present at critical 3' or 5' ends of the sequence (Daher et al. 2015). Our reverse primer, originally designed to match sequence from Species A, was replaced with equimolar mixtures of primers that matched both Species A and Species B, to improve performance in detecting both. The resulting assay was slightly more sensitive in detecting Species B with a limit of detection at half the concentration of Species A.

Given the tolerance of the recombinase polymerase for DNA mismatches, we were concerned about potential cross reactivity with other fungal species. However, we did not detect cross reactions in a panel of unknown samples that contained other species of fungi, soil samples, and samples from `ōhi`a trees that died of unknown (i.e. non-ROD) causes. We did, however, find that the assay detected closely related species of *Ceratocystis* in sweet potato, taro, and arrowhead plants (Table 2). This suggests that the assay may have wider applicability outside

of Hawai`i as a genus-specific test, but this may need to be established by a combination of both traditional alignment methods using ITS sequence data and trial and error when some sequence differences are present.

In addition to difficulties designing primers and a probe for *Ceratocystis*, we also faced challenges developing a rapid, field compatible method for extracting DNA from wood samples. DNA purification from wood is problematic under the best of circumstances given the abundance of natural compounds that can act as inhibitors. We based our method on reports that simple extractions from plant material with Chelex® resin can yield DNA of sufficient quality for PCR reactions (Hwangbo et al. 2010) and discovered that Instagene® extracts are compatible with reagents from magnetic bead based extraction kits for plants.

Some of our initial studies suggested that simply heating samples in Instagene® may be sufficient to recover DNA that can be used directly in the RPA reaction, but additional work revealed that these crude extracts could also lead to high initial background fluorescence that invalidated the test. We found that diluting the extracts 1:10 with elution buffer may be a suitable short cut and alternative to the magnetic bead extraction when results are needed quickly. However, comparisons of test sensitivity between the Nucleospin II Plant Kit and Instagene® magnetic bead extraction methods suggest that there is some carryover of contaminants after the magnetic bead clean up that can reduce test sensitivity. This was reflected in the wider confidence limits and lower detection limits for samples extracted with Instagene® (Tables 4, 5). Hence, additional purification steps are recommended if maximum test sensitivity is desired. Additional refinement of the extraction method by addition of more washing steps or increases in wash volume during each step may improve performance in the field.

In summary, we have developed a rapid, accurate, and sensitive field compatible test that detects the two *Ceratocystis sp.* that cause ROD. Several other closely related *Ceratocystis sp.* from sweet potato, taro, and arrowhead plants are also detected by the assay (Table 2), but they do not occur in `ōhi`a and false positive detections are unlikely when testing is limited to wood samples. Amplification of closely related species of *Ceratocystis* may make the assay desirable as a general screen for fungi in this genus and useful for detecting new introductions in horticultural material. Additional screening on a wider range of unrelated fungi will also help to verify specificity of this assay for *Ceratocystis*. Additional ITS sequence should be explored for primer and probe development to improve test sensitivity and specificity as genetic data becomes available. This might make it feasible to develop a multiplex assay that simultaneously detects and distinguishes Species A and Species B during the TwistAmp® exo reaction or with lateral flow test strips.

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LITERATURE CITED

Daher, R. K., G. Stewart, M. Boissinot, D. K. Boudreau, and M. G. Bergeron. 2015. Influence of sequence mismatches on the specificity of recombinase polymerase amplification technology. Molecular and Cellular Probes, 29:116–121.

Grosclaude, C., R. Olivier, J.-C. Pizzuto, and C. Romiti. 1991. Etude expérimentale du transport de l'inoculum de *Ceratocystis finbriata f. platani* par l'eau d'une riviére. European Journal of Forest Pathology, 21:168–171.

Harrington, T. C. 2013. *Ceratocystis* diseases. In: Infectious Forest Diseases, pp. 230–255, P. Gonthier and G. Nicolotti, eds. CABI, Centre for Agriculture and Biosciences International, Oxfordshire, UK.

Hwangbo, K., S. H. Son, J. S. Lee, S. R. Min, S. M. Ko, J. R. Liu, D. Choi, and W. J. Jeong. 2010. Rapid and simple method for DNA extraction from plant and algal species suitable for PCR amplification using a chelating resin Chelex 100. Plant Biotechnology Reports 4:49–52.

Keith, L. M. , R. F. Hughes, L. S. Sugiyama, W. P. Heller, B. C. Bushe and J. B. Friday. 2015. First report of Ceratocystis Wilt on Ōhi`a (*Metrosideros polymorpha*). Plant Disease 99: 1276.

Kundapur, R. R. and V. Nema. 2016. Loop-mediated isothermal amplification: beyond microbial identification. Cogent Biology 2:1137110.

Luchi, N., L. Ghelardini, L. Belbahri, M. Quartier, and A. Sanini. 2013. Rapid detection of *Ceratocystis platani* inoculum by quantitative real-time PCR assay. Applied and Environmental Microbiology 79:5394–5404.

Oliveira, L. S. S., T. C. Harrington, M. A. Ferreira, M. B. Damacena, A. M. Al-Sadi, I. H. S. Al-Mahmooli, and A. C. Alfenas. 2015. Species or genotypes? Reassessment of four recently described species of the *Ceratocystis* Wilt pathogen, *Ceratocystis fimbriata*, on *Mangifera indica*. Phytopathology 105:1229–1244.

Piepenburg, O., C. H. Williams, D. L. Stemple, and N. A. Armes NA. 2006. DNA detection using recombination proteins. PLoS Biology 4(7):e204.

Thorpe, D. J., T. C. Harrington, and J. Y. Uchida. 2005. Pathogenicity, internal transcribed spacer-rDNA variation, and human dispersal of *Ceratocystis fimbriata* on the family Araceae. Phytopathology 95:316–323.

TwistAmp® Combined Instruction Manual. 2016. http://www.twistdx.co.uk/support/twistamp_product_documentation/

Yan, L., J. Zhou, Y. Zheng, A. S. Gamson, B. T. Roembke, S. Nakayama, and H. O. Sintim. 2014. Isothermal amplified detection of DNA and RNA. Molecular Biosystems 10:970–1003.

APPENDIX 1: EQUIPMENT LIST

Equipment	Supplier	Catalog Number	Estimated Price*
T8 isothermal device	TwistDx[1]	T8Device	$5,500.00
Micro ball dispenser	TwistDx[1]	T1601balldispenser	$220.00
Mini heat block	USA Scientific[2]	#2520-1001	$220.00
Vortex Genie 2	Scientific Industries, Inc.[3]	#SI-0236	$400.00
Microcentrifuge	USA Scientific[2]	#2631-006	$300.00
Vortex adapter	Mo Bio Laboratories[4]	#13000-V1-24	$120.00
Magnetic MagnaRack™	ThermoFisher Scientific[5]	#CS150000	$400.00
800 watt inverter	Whistler Group[6]	XP800i	$60.00
P1000 LTS pipette	Rainin (refurbished)[7]	variable	$160.00
P200 LTS pipette	Rainin (refurbished)[7]	variable	$160.00
P10 LTS pipette	Rainin (refurbished)[7]	variable	$160.00
Pelican case	Pelican[8]	1560LFC	$250.00
Huffy 22 inch tool box	Local Hardware Store		$25.00
12 volt battery	Interstate Batteries[9]	DCM-0035	$120.00
		Total:	$8,095.00

* Substitute suppliers and equipment may be available at lower cost. Shipping costs not included in estimate.

[1] http://www.twistdx.co.uk/
[2] http://www.usascientific.com/
[3] https://www.scientificindustries.com/
[4] https://mobio.com/
[5] https://www.thermofisher.com/us/en/home.html
[6] https://whistlergroup.com/
[7] www.pipette.com
[8] http://www.pelican.com/
[9] http://www.interstatebatteries.com/m/cmp/natl/get-there

APPENDIX 2: CONSUMABLE SUPPLIES

Descriptions*	Supplier	Catalog Number
2 ml microcentrifuge tube with screw cap	Fisher Scientific[1]	02-681-375
3 mm steel grinding balls	OPS Diagnostics[2]	GBSS 118-2500-09
800 um zirconium beads	OPS Diagnostics[2]	BAWG 800-200-01
1.5 ml microcentrifuge tubes	Fisher Scientific[1]	13-698-791
0.5 ml microcentrifuge tubes	Fisher Scientific[1]	13-698-790
Plastic microtube rack	Fisher Scientific[1]	05-541
Plastic PCR rack	Fisher Scientific[1]	05-541-80
Liquid waste bottle (25 ml)	Fisher Scientific[1]	03-311-1J
Dry waste container	Local Store	
Pipette tips for P1000	Rainin[3]	17001864, 17002426
Pipette tips for P200	Rainin[3]	17001863, 17002429
Pipette tips for P10	Rainin[3]	17001865, 17002430
Gauze pads	Fisher Scientific[1]	13-761-52
10% bleach (100 ml bottle)	Local Store	
Sharpie fine tip pen	Local Store	
Disposable gloves	Fisher Scientific[1]	19-130-1597C
Styrofoam box and ice packs	Local Store	
TwistAmp® Exo Kit	TwistDx[4]	TAEXO02KIT
Instagene® Matrix	BioRad[5]	7326030
Forward Primer (10 µM)	Integrated DNA Technologies[6]	Custom synthesis[#]
Reverse Primers (10 µM)	Integrated DNA Technologies[6]	Custom synthesis
Exo Probe (10 µM)	LGC Biosearch Technologies[7]	Custom synthesis
PCR Grade Water	Fisher Scientific[1]	BP24701
Magnetic Beads (QuickPick XL Plant Kit)	Bio-Nobile[8]	#531000
Binding Buffer (QuickPick XL Plant Kit)	Bio-Nobile[8]	#53300
Wash Buffer (QuickPick XL Plant Kit)	Bio-Nobile[8]	#53500
Elution Buffer (QuickPick XL Plant Kit)	Bio-Nobile[8]	#53600

* Substitute products and suppliers may be available at lower cost

[#] Primers and probes are custom ordered based on DNA sequence (Table 1)

[1] https://www.fishersci.com/us/en/home.html

[2] http://www.opsdiagnostics.com/

[3] https://www.shoprainin.com/

[4] http://www.twistdx.co.uk/

[5] http://www.bio-rad.com/

[6] https://www.idtdna.com/site

[7] www.biosearchtech.com/

[8] https://sunrisescience.com/

APPENDIX 3: EXTRACTION PROTOCOL

Materials:

2 ml microcentrifuge tubes with screw caps (hereafter: grinding tubes) containing 0.3 g of 800 um Zirconium beads plus three, 3 mm steel beads

Extraction Protocol:

Preheat heating block to 100°C

Put on personal protective equipment, including safety glasses, lab coat, and disposable gloves. Swab work area with gauze pads moistened with 10% (v/v) bleach.

Measure approximately 100 mg fine drill shavings or sawdust into each 2 ml grinding tube and label with sample identification number.

Snap circular Vortex adapter onto Vortex Genie II

1. Mix Instagene® Matrix by shaking to resuspend resin beads before use. Using 1000 µl pipette, add 600 µl Instagene® Matrix to each grinding tube.
2. Touch grinding tube to Vortex mixer (touch setting) to agitate tubes and wet wood.
3. Briefly centrifuge to pellet shavings.
4. Heat tubes 5 minutes in heating block.
5. While tubes are heating, use the 1000 µl pipette to add 190 µl QuickPickTM binding buffer to an equal number of labeled 1.5 ml centrifuge tubes. Using the 20 µl pipette, add 7.5 µl QuickPickTM magnetic beads to each 1.5 ml centrifuge tube. Prepare and label an equal number of 0.5 ml centrifuge tubes.
6. Transfer grinding tubes to the Vortex adapter. Run at maximum setting for 60 seconds.
7. Centrifuge grinding tubes 60 seconds to pellet wood shavings.
8. Using a 1000 µl pipette, transfer as much liquid as possible from each grinding tube to empty 0.5 ml centrifuge tubes. Use a fresh tip between tubes.
9. Centrifuge 0.5 ml tubes containing wood extract for 60 seconds to pellet fine particles and clarify the extract.
10. Using a 200 µl pipette, Transfer 120 µl of clarified extract to 1.5 ml centrifuge tubes containing binding buffer and magnetic beads. Gently mix by flicking tube carefully with a finger or by touching the Vortex mixer (touch setting) set on low speed (setting 7–8).
11. Incubate 5 minutes at room temperature. Start timing after the last sample is prepared. Briefly touch to Vortex mixer every 60 seconds during incubation to mix.
12. Transfer tubes to magnetic rack, wait about 15 seconds for magnetic beads to move to sides of tubes next to magnet. Remove all liquid with a 1000 µl pipette from bottom of each tube, discard solution in waste container. Be sure to change pipette tips between each tube to prevent cross contamination.
13. Use the 1000 µl pipette to add 250 µl QuickPickTM wash buffer to each tube, remove from magnetic rack, and gently resuspend magnetic beads by carefully flicking tubes with a finger or by briefly touching to the Vortex mixer set on low speed (setting 7–8). Avoid creating droplets that stick to the upper walls of the tube.

14. Transfer tubes to magnetic rack and repeat steps 12 and 13 three more times for a total of 4 washes with 250 µl of wash buffer.
15. Using a 200 µl pipette, add 75 µl of QuickPick™ elution buffer, resuspend magnetic beads with finger or by briefly touching to Vortex mixer and incubate for 5 minutes to release bound DNA from magnetic beads. Briefly touch to Vortex mixer every 60 seconds during incubation.
16. Transfer tube to magnetic rack, allow beads to settle on tube wall, and use the 200 µl pipette to transfer all fluid to clean, labeled 0.5 ml centrifuge tubes.
17. Centrifuge the 0.5 ml tubes with extracted DNA 60 seconds before use, transfer to a new, clean and labeled 0.5 ml tube if a pellet is visible. DNA is ready for analysis

APPENDIX 4: PREPARATION OF STOCK MASTER MIX

1. With personal protective equipment on, swab the tabletop with 10% (v/v) bleach.
2. Remove PCR water, rehydration buffer, primers, and probe from freezer and allow to defrost.
3. Centrifuge all tubes briefly to pellet the solutions.
4. Label a 2 ml screw cap tube "Master Mix". Add reagents to the Master Mix tube in the following volumes (enough for one strip of 8 reaction tubes) with the 1000 µl pipette (rehydration buffer), 200 µl pipette (forward primers and water) and 20 µl pipette (probe and reverse primers). Vortex for a few seconds to mix, and then centrifuge to pellet the solution. Prepare enough master mix for the desired number of tubes (i.e. multiply volumes by number of tubes needed X 1.05 to allow for some extra volume). A stock master mix can be prepared ahead of time, frozen, and carried to the field for later use.

Master Mix	Units	Concentration	Volume per reaction (µl)	Volume per 8 reactions (µl)
Rehydration Buffer	X	1.0	29.5	247.8
Forward Primer (329F)	uM	10.0	3.0	25.2
Probe (P415)	uM	10.0	1.0	8.4
Reverse Primer (514BR)	uM	10.0	1.5	12.6
Reverse Primer (514R)	uM	10.0	1.5	12.6
Water	-	-	5.5	46.2
		Master Mix Total/reaction	42.00	352.8

APPENDIX 5: AMPLIFICATION PROTOCOL

1. Remove stock master mix, PCR water, magnesium acetate ($MgOAc_2$), and positive control DNA from the cooler, allow to defrost and centrifuge briefly to pellet the solutions. Remove foil packet containing reaction tubes from the cooler and allow it to reach room temperature.
2. Replace the centrifuge head that holds 6 tubes with the centrifuge head that holds two strips of 8 PCR tubes by loosening the set screw and then retightening after the exchange is made.
3. Power up the T-8, log in, and go to "Tests". Choose the test that you would like to run (CFITS ROD Kit) and enter a title for the procedure and labels for each of the reactions. Touch the "✔" on the bottom of the screen. The T-8 will then make an audible beep when the desired temperature is reached. Be sure that old reaction tubes have been removed from the machine before programming the T8.
4. Remove a strip of reaction tubes from the foil packaging, place it in the PCR rack with lid.
5. Using scissors, cut the desired number of tubes from the strip — allow one extra tube for a negative (PCR water) control and one extra tube for the positive control. Remaining extra tubes are stored in a snap-cap plastic container in the cooler.
6. Label detachable lids on the tubes with a Sharpie pen, including the positive and negative controls. Do not label the clear plastic lower portion of the tube as it needs to be transparent for the T-8 to measure fluorescence.
7. Remove the caps from the reaction tubes and place them in the PCR rack face up and behind the tubes.
8. Using the 200 µl pipette, add 42 µl of master mix to each reaction tube.
9. Place one steel ball bearing in each reaction tube with the micro ball bearing dispenser.
10. Using the 20 µl pipette, add 4 µl of extracted sample to the inner wall of each corresponding reaction tube, 4 µl of PCR water to the negative control tube, and 4 µl of positive control DNA to the positive control tube.
11. Using the 20 µl pipette, add 4 µL of $MgOAc_2$ to the caps of each of the tubes. This insures that the $MgOAc_2$ does not come in contact with the rest of the reaction mixture until ready to start the reaction. Gently replace caps on the tubes so that the $MgOAc_2$ remains in the roof of the caps.
12. Mix reaction tubes by inverting and shaking for several seconds by hand. This introduces the $MgOAc_2$ into the mixture and starts the isothermal reaction. Using a strip of empty PCR tubes as a balance, centrifuge the tubes briefly to pellet mixture. Immediately place the tubes into the T-8 in the same order that the machine wells are programmed. Close the lid of the T-8 - the lid will lock and test will begin. Results will be displayed as "+" for positive samples, "-" for negative samples, and "!" for undetermined. Undetermined results indicate that early stages of the reaction were above a pre-set baseline. This may be because of excess PCR inhibitors in the DNA temple and samples may need to be re-extracted with several additional magnetic bead washes or diluted 1:10 with elution buffer and retested. For a test to be valid, the negative control must be "-" and the positive control must be "+".

APPENDIX 6: REPRESENTATIVE T8 FLUORESCENCE AND 1ST DERIVATIVE CURVES

Figure A1: Assay sensitivity for detecting a serial dilution of Species A spores that were added to 100 mg of uninfected wood shavings and extracted with a Nucleospin II Plant Kit. Amplification above the threshold (**---**) was evident for dilutions as low as 4 (A4, ✦) spores/mg of wood shavings. Legend (right) refers to strain and number of spores/mg of wood shavings. NTC (no template control).

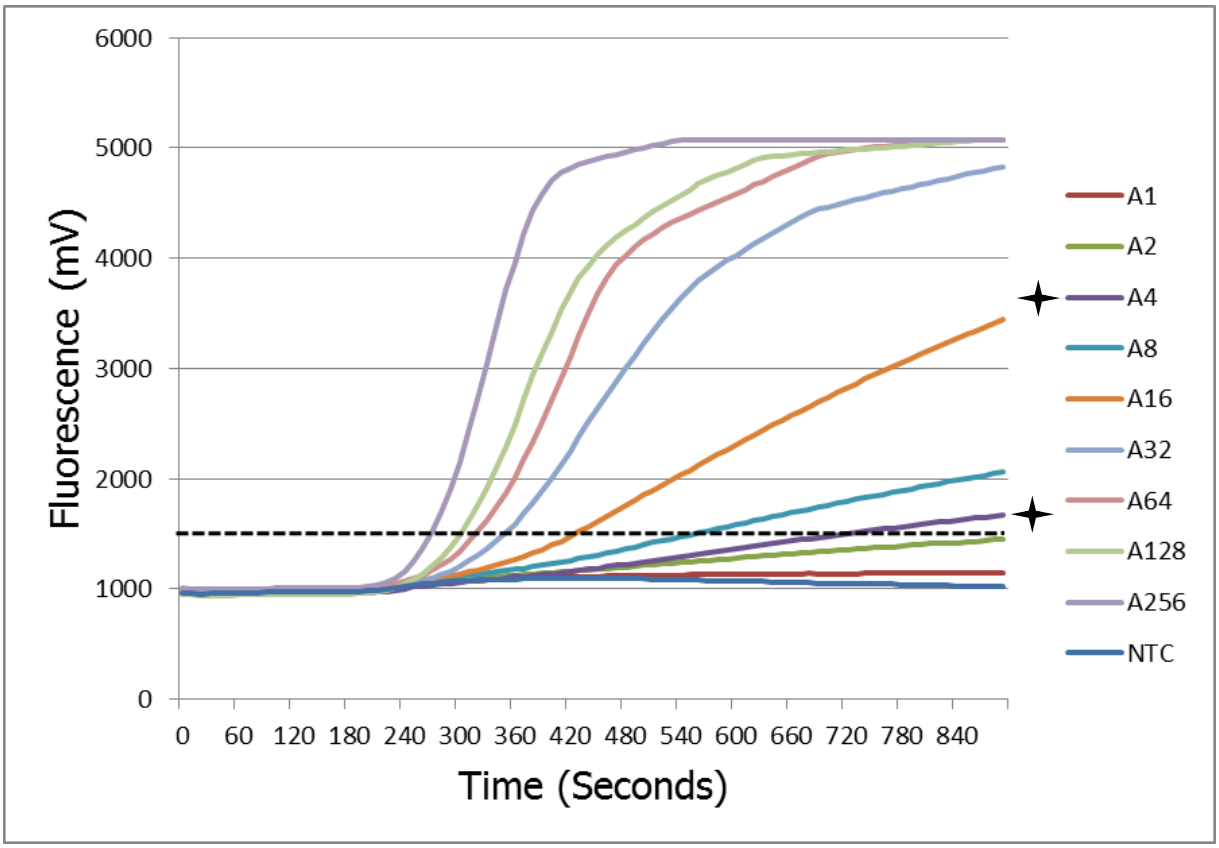

Figure A2: First Derivatives graph of data from Figure A1. For a replicate to be positive, it must cross a threshold of 1497 mV (Figure A1) and be above a derivative threshold of 1.75 (**---**). Note that spore dilution A8 (●) and A16 (★) both cross the threshold (Figure A1), but only A16 has a derivative curve higher than the derivative threshold (see below). Using these criteria, A16, but not A8 would be considered positive.

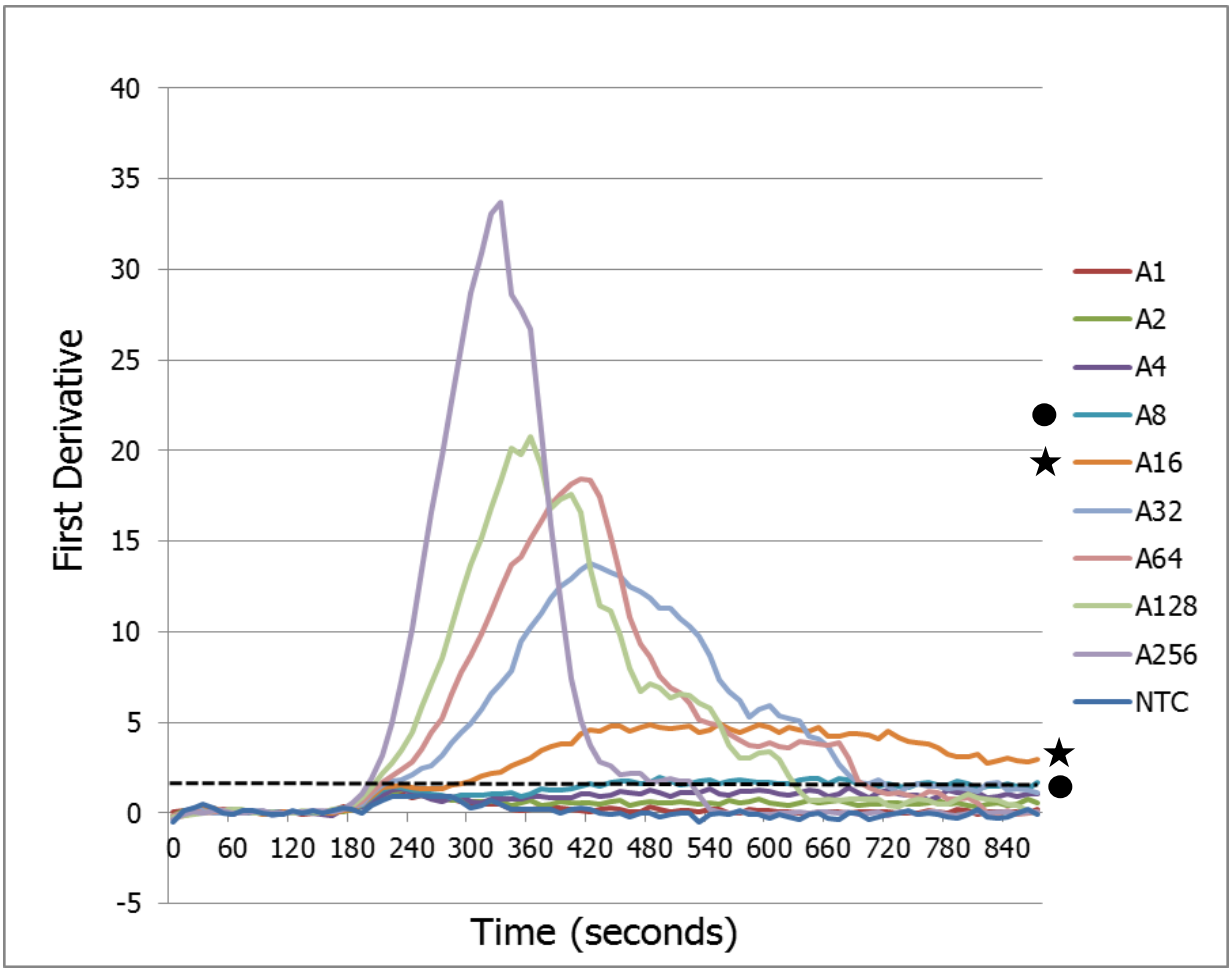

Figure A3. Essential equipment for the portable field kit. A. TwistDx T8 Isothermal Instrument. The fluorometer is powered by a 12-volt lithium ion battery and can test up to 8 samples at one. Data can be downloaded with a thumb drive (arrow). B. Adjustable heat block for initial sample processing with Instagene® Matrix. C. Portable centrifuge. Two heads are available for larger tubes (0.5 and 1.5 ml) and smaller reaction tubes (arrow). Heads are changed by loosening a set screw with a small screwdriver. D. Vortex Genie mixer with a 24 tube adapter for shaking samples with zirconium beads and steel balls to disrupt wood tissue and release DNA. E. Racks for holding 0.5 ml and 1.5 ml tubes (right) and 0.2 ml reaction tubes (left). During the isothermal reaction, samples are mixed magnetically with a steel ball bearing that is dispensed with a Micro Ball Dispenser (arrow). Containers of QuickPick™ magnetic beads, binding buffer, wash buffer and elution buffer are on right. F. Magnetic Rack for magnetic bead DNA purification and pipettes and tips for measuring and dispensing liquids. G. 800 Watt inverter for powering lab equipment from a 12-volt battery.

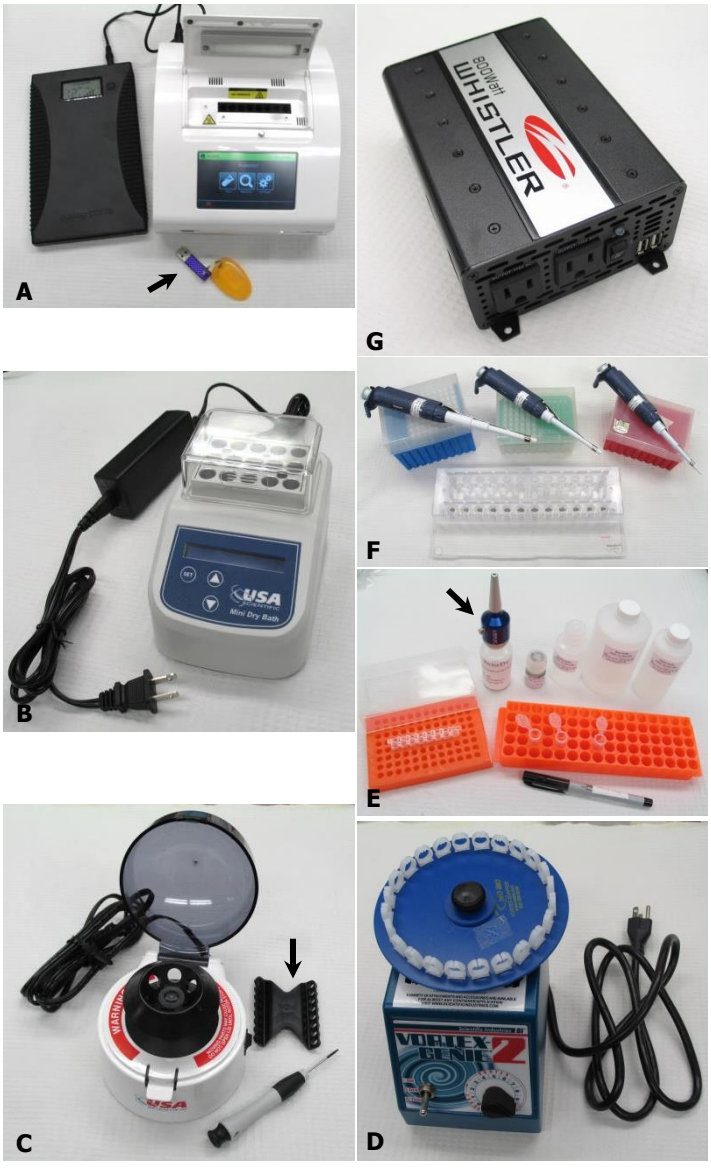

Figure A4: Padded pelican case for transporting equipment and consumable supplies (A). The pelican case has a padded lid with pouches for electrical cords, pipettes, and the lithium ion battery that powers the T8 isothermal instrument (A and B). The tool box for consumable reagents contains a small Styrofoam box and ice packs for temperature sensitive reagents, extraction solutions, pipette tips, 10% bleach, tube racks, gauze pads, gloves, and tubes (C).

Figure A5. Testing `Ōhi`a near Honoli`i Stream north of Hilo, Hawai`i. The test kit can be set up on the tailgate or cargo area of field vehicles or on a portable folding table under a collapsible tent or tarp for protection from rain.