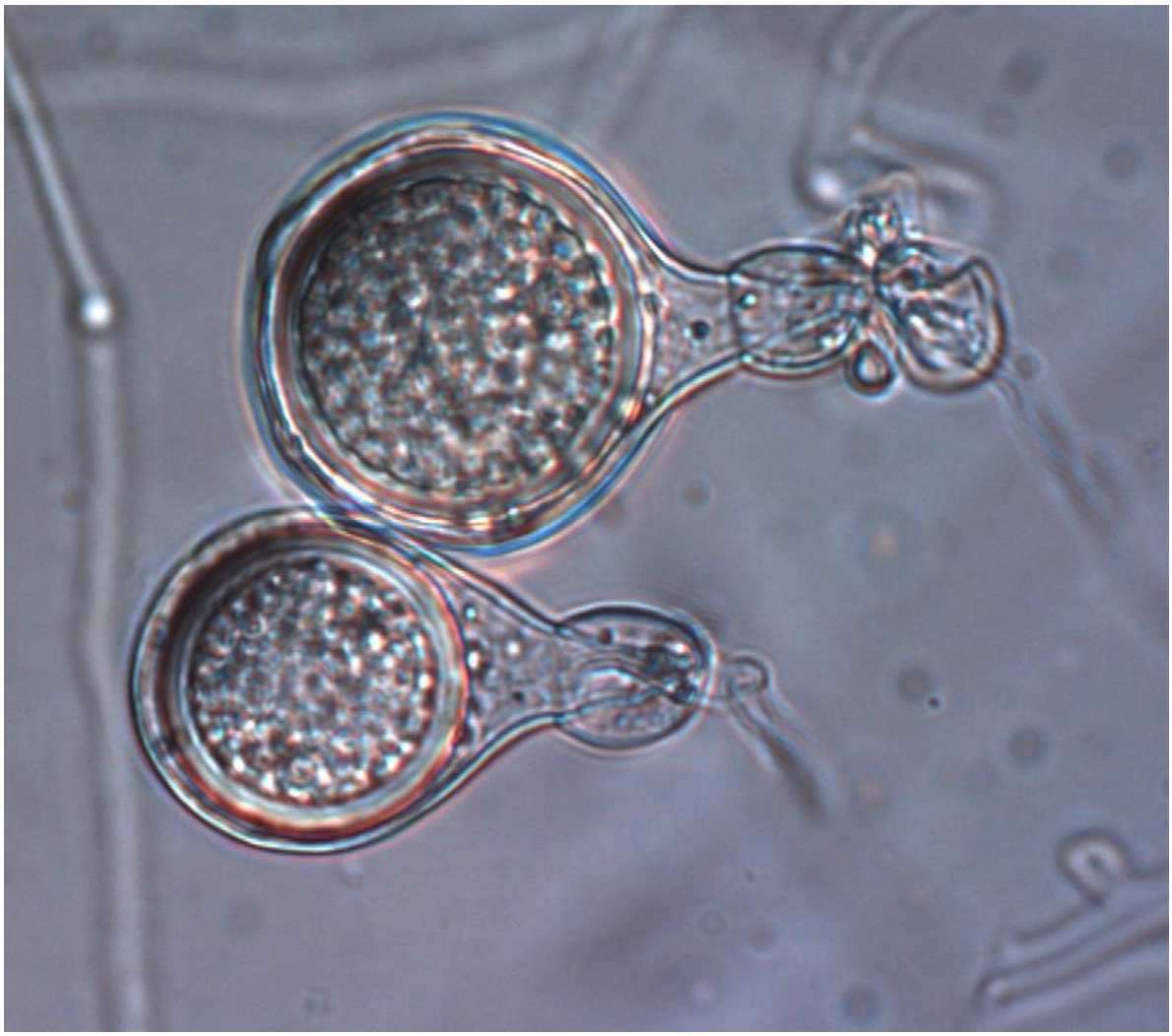
Comparison of a real-time PCR assay and a soil bioassay technique for detection of *Phytophthora* taxon Agathis from soil

Rebecca McDougal, Stan Bellgard, Peter Scott and Beccy Ganley



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REPORT INFORMATION SHEET

REPORT TITLE	COMPARISON OF A REAL-TIME PCR ASSAY AND A SOIL BIOASSAY TECHNIQUE FOR DETECTION OF *PHYTOPHTHORA* TAXON AGATHIS FROM SOIL
AUTHORS	REBECCA MCDOUGAL, PETER SCOTT AND BECCY GANLEY [SCION]; STAN BELLGARD [LANDCARE RESEARCH]
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EXECUTIVE SUMMARY

Comparison of a real-time PCR assay and a soil bioassay technique for detection of *Phytophthora* taxon Agathis from soil

Rebecca McDougal, Stan Bellgard, Peter Scott, and Rebecca Ganley

The problem

In order to slow the spread of *Phytophthora* taxon Agathis (PTA) and to undertake measures to limit its effect on individual trees and on ecosystems, specific and accurate detection of PTA is required for reliable delimitation and diagnostic purposes. The development of a real-time PCR assay for molecular detection of PTA from soil samples provides an opportunity to increase diagnostic throughput, accuracy and cost-effectiveness. However, a comparison of current PTA detection methods with the real-time PCR assay is needed to determine the advantages and disadvantages of the two individual methods.

This project

This project compares the efficacy of real-time PCR versus conventional soil bioassay for detection of PTA from soil samples. Samples collected from around trees known to be infected with PTA and a set of reference soil samples from Landcare Research were tested. The real-time PCR detection efficiencies were compared in a double-blind experiment between laboratories at Scion and Landcare Research. The results from the real-time PCR were then compared to conventional soil bioassay results to assess the robustness and reliability of each method.

Key Results

There were two deviations from the published real-time PCR assay: A reverse-complement of the published sequence was required and the probe was also shortened by two nucleotides; and DNA was ethanol precipitated and purified using an alternative kit as the automated DNA extraction X-tractor was not available to researchers at Scion.

The DNA extraction method was laborious with the number of samples tested. Issues identified with the method could bias the extraction procedure and influence the effectiveness of the DNA extraction when a large number of samples are required to be processed.

There was considerable variation in the detection or recovery of PTA both between organisations and between the detection methods used. Of the 26 soil samples tested 13 samples tested positive for PTA but only one soil sample, RT PCR 11, tested positive at both organisations using both detection methods.

Scion detected or recovered PTA from 13 of the soil samples tested and Landcare detected or recovered PTA from five samples, with four equivocal PCR results. The real-time PCR assay detected PTA in nine of the samples, with an additional three equivocal PCR results. The soil bioassay recovered PTA from eight of the soil samples. Of the 13 positive PTA detections, only four of these were detected or recovered by both the real-time PCR and the bioassay.

Implications of Results for Client

The overall rate of detection or recovery of PTA between real-time PCR and the bioassays was very similar and one technique over the other could not be recommended at this stage. Both methods are effective at detecting PTA in soil samples but changes to the current protocols could potentially improve detection rates. If real-time PCR was chosen as the preferred method of detection, then results that are inconclusive from the initial PCR reaction could be repeated with increased volumes of DNA or the bioassay could be subsequently performed.

Recommendations

Based on the results from this study we would recommend the following:

1. DNA extraction is trialled using a paint-shaker (or equivalent instrument) and results are compared to manual vigorous shaking.
2. The bulk method of DNA extraction used in this study (and changes recommended) are compared to DNA extraction using a kit (e.g. MO-BIO, 10g samples) with multiple sub-samples and pooling of extracts for PCR.
3. A DNA extraction internal control (e.g. spiked Phytophthora DNA or oospores from a different species) is used to assist in identifying soil sample extractions where DNA recovery is low.
4. A combination of both methods of detection are adopted for PTA detection from soil samples, until improvements, as discussed in this report, can be made to increase detection rates.

Comparison of a real-time PCR assay and a soil bioassay technique for detection of *Phytophthora* taxon Agathis from soil

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Introduction

Kauri dieback, caused by *Phytophthora* taxon Agathis (PTA), is a disease that poses a serious threat to our indigenous kauri. It was first discovered in 1972 on Great Barrier Island and since 2006, the organism has been found in the Auckland, Northland and Coromandel regions. The soil-based pathogen attacks the roots of kauri, destroying their ability to draw water and nutrients from the soil. PTA can kill kauri seedlings and trees of all ages and spreads easily through the transfer of organic matter.

The goal of the Kauri Dieback Management Programme (KDM) is to manage the disease which includes slowing the spread of PTA and to undertake measures to limit its effect on individual trees and on ecosystems. Specific and accurate detection of PTA is essential for reliable delimitation and diagnostic purposes. Until recently detection of PTA has been performed with traditional soil bioassay (baiting) techniques and plating of cork cambium tissue samples from the advancing edge of trunk lesions to *Phytophthora*-selective media. This diagnostic method is time-consuming as it is dependent on the pathogen growing out onto the selective media. The minimum time a sample could be completed is 15 days, although most samples take between 20 and 25 days due the time taken for the cultures to grow.

The development of a real-time PCR assay for molecular detection of PTA from soil samples provides an opportunity to increase diagnostic throughput, and potentially the accuracy and cost-effectiveness of sample numbers processed. Having an alternative, high-throughput system available is essential for the success of the programme's work. A preliminary investigation by (Bellgard, S., Landcare Research, pers. comm.) has shown that real-time PCR can be used to detect PTA in soil. In this study, 40 samples were tested from around a single infected tree. However, the real-time PCR assay has not been validated at any other sites, other than the Waitakere Ranges, and the variation between PTA detection using the real-time PCR assay with respect to the results obtained from conventional soil bioassay needs further parameterization. A comparison of current PTA detection methods with the real-time PCR assay will provide a clear understanding of the advantages and disadvantages of the two individual methods, with respect to accuracy, time and cost per sample. It is also expected to allow appropriate selection of, and improved confidence in, real-time PCR diagnostic approaches for field based diagnosis and future research projects.

This project aims to compare the efficacy of real-time PCR versus conventional soil bioassay for detection of PTA from soil samples. Samples will be collected from around trees known to be infected with PTA by KDM and an additional set of reference soil samples from Landcare Research will also be tested. The real-time PCR detection efficiencies will be compared in a double-blind experiment between two independent laboratories. This will include cycle threshold for PTA detection (Cq values), and quantification of intra- and inter-sample variation (including "true" and "technical/experimental" replicates). The results from the real-time PCR will then be compared to conventional soil bioassay results to assess the robustness and reliability of each method.

Materials and Methods

Soil samples

Twenty soil samples, approximately 2 kg each, from sites known to be infected with PTA were provided by KDM. Soil was collected from around trees and was sent to Scion without any location or sourcing data to allow for "blind" testing.

Five reference soil samples, 2 kg each, from Landcare Research were also used. These samples had been used for a previous study (Beever et al. 2010) where they had been collected from Twin Peaks Track Site (Huia) every month during 2009/10 and mixed in cement mixer. Each soil sample soil was a composite of three trees and had been stored at 10°C at Landcare Research since they were collected. These soil samples are tested yearly for PTA and are known to still contain infective propagules of PTA. Five samples from this collection were selected for analysis. A negative control, a soil sample taken from Landcare Research premises, was also included.

All soil samples were homogenised and dried for 2 days then divided into the appropriate amounts for the bioassay and real-time PCR assay. The samples were labelled Set A-D by an independent researcher and randomised within each set for "blind" testing. Duplicate 175g soil samples were analysed by standard bioassay procedure and duplicate 200g soil samples were analysed by DNA extraction and real-time PCR. For samples to be processed by real-time PCR, soils were sieved through a 2 mm sieve. Soils were not sieved for samples to be processed by bioassay as it is not part of the current Standard Operating Procedure (SOP; Appendix 1), as approved by the KDM.

Duplicate samples for the bioassay and real-time PCR assays were tested by researchers at Scion (Rotorua) and Landcare Research (Auckland). Samples were couriered to Landcare Research with the duplicate samples kept at ambient temperature until samples had been delivered to Landcare Research. Wherever possible, soil samples were treated the same, e.g. storage of samples upon receipt, work with soil samples to commence on the same day, to minimise sample variation. Soil sample temperatures were monitored in transit using iButtons.

Real-time PCR assay

Primers and probes for the specific detection of PTA as described by Than et al. 2013 were utilised for detection of PTA from soil samples.

Standard curves for between-laboratory comparison of the real-time PCR assay were produced using a genomic DNA preparation of the PTA type-strain ICMP 17027. The same DNA preparation was used in both laboratories. Standard curves were generated on real-time PCR instruments at both organisations using DNA concentrations ranging from 2 ng/µl to 2 fg/µl DNA as per Than et al. (2013). The specificity of the real-time PCR assay was also determined at both organisations by testing with against 2ng DNA from five *Phytophthora* species: *P. cinnamomi* (NZFS 102.17), *P. cryptogea* (NZFS 2558), *P. kernoviae* (NZFS 2646), *P. multivora* (NZFS 2750), and PTA (NZFS 3128 Scion; NZFS 3772 Landcare).

DNA extraction from soil samples were performed as described previously by Than et al. (2013). As Scion does not have an automated DNA extraction X-tractor machine, which are no longer commercially available, an alternative purification step was performed.

Instead, 1 ml of the DNA was concentrated by ethanol precipitation (final volume of 200ul) and 100µl of the concentrated DNA was purified using a MO BIO kit (Pro PowerClean® DNA Clean-Up Kit; #12997-50) was used to purify the DNA. The soil samples were extracted in three blocks, randomised as follows: Day1 - A, B, C, D, F, G, O, P; Day 2- E, I, K, N, Q, S, V, Z, X; Day 3 - H, J, L, M, R, T, U, W, Y.

Real-time PCR was performed on triplicate reactions for each soil sample as described previously by Than et al. (2013). The same PCR kit (TaqMan® Environmental Master Mix 2.0, Life Technologies) and Internal Positive control kit (TaqMan® Environmental Master Mix 2.0, Life Technologies) were also used as per the published method. At Scion, real-time PCR was performed on an Eco Real-time PCR instrument (Illumina) and at Landcare Research a Rotor-Gene 6000 instrument (Qiagen) was used.

Bioassay

Each soil sample was assayed as per the SOP (Appendix 1.) for bioassay detection of PTA.

Results and Discussion

Soil samples

The temperature of soil samples sent in chilly bins between organisations were recorded during transit. The average temperature for transit of soils was 15.1°C, with temperatures ranging from 12.5 °C to 20.5°C. The soil samples were at 20.5 °C (Scion's laboratory temperature) at the start of transit for approximately 20 mins and cooled down to 12.5 °C during transit. Although these temperature differences could influence bioassay recovery between labs this is considered unlikely as these variations have not impacted PTA bioassay results for other projects where soil samples have been sent and tested between multiple organisations (Scott, P., Scion, pers.comm.).

Deviations from published method

The PTA reverse primer and probe used in this study differed from the Than et al. (2013) published procedure (Table 1). In Than et al. (2013) the PTA reverse primer sequence was published in an incorrect orientation and resulted in no amplification product. Reverse-complement of the published sequence allowed successful amplification and detection of the amplicon. The probe was also shortened by two nucleotides to reduce the annealing temperature and increase sensitivity. The new probe was used for the duration of the project.

The final steps of the published PTA assay was unable to be performed at Scion, as Scion does not an automated DNA extraction X-tractor machine and they are no longer commercially available. A purification step was required as measurement of DNA (via Nanodrop) indicated the presence of contaminants in the DNA extracts. In addition to this, PCR on aliquots of selected extracts, spiked with genomic DNA and serially diluted, showed variable amplification and some PCR inhibition. To purify the DNA, 1 ml of the DNA was concentrated by ethanol precipitation (final volume of 100ul) and a MO-BIO kit (Pro PowerClean® DNA Clean-Up Kit) was used.

Table 1. Primer and probes used in Than et al. (2013) and this study

Primer or Probe name	Published sequence (Than et al. 2013)	Sequence used in this study	Change	Tm[1] of probes used in this study
ITS_PTA_F2	AACCAATAGTT GGGGGCGA	AACCAATAGTTG GGGGCGA	none	63.8
ITS_PTA_R3	GACGAGCTCTA TCATGGCGAG	CTCGCCATGATA GAGCTCGTC	Reverse/complement of published sequence[2]	64.2
ITS_PTA_ probe2	GGCGGCTGCTG GCTTTGGCT	AGCCAAAGCCAG CAGCCG	Reverse/complement of published sequence and removal of two C residues[3]	68.4

[1] As determined by Sequence Tm Utility (University of Utah, https://www.dna.utah.edu/utensils/RCC.php).
[2] The sequence of the published ITS_PTA_R3 primer was for the opposite DNA strand, and hence would not allow successful amplification when used in that orientation.
[3] As above for [1], but in addition, two C residues were removed to lower the Tm of the probe which makes it conform to primer and probe design rules for optimising sensitivity and specificity (Gundry, et al., 2011).

An internal positive control (IPC) kit (Life technologies) was used in the real-time PCR assays to detect PCR inhibition thereby preventing false-negative interpretation. No PCR inhibition was observed in any of the real-time PCR experimental runs. The DNA purification may not be necessary based on results comparing pre- and post-purified DNA (Appendix 2). Instead an additional 70% ethanol wash-step prior to elution from the silica could increase the purity of the DNA that is eluted, and the use of the IPC control kit would indicate if DNA is or is not suitable at this stage.

Difficulties encountered with the published method

The DNA extraction method was laborious with the number of samples tested. The main concern was that the vigorous, manual shaking step involved shaking a soil/buffer slurry in a 500 ml bottle with large steel ball bearings for 5 mins. Specifically it was noted that there was varying intensity in shaking of the bottles by different people; after two rounds, with breaks in between, arms tire from the shaking, and intensity of shaking decreases. These variations in this step are likely to introduce bias to the extraction procedure and influence the effectiveness of the DNA extraction. In addition to this the Nalgene bottle lids frequently broke during shaking from the ball bearings and this caused leakage of potentially contaminated soil slurry. Correct disposal of large volumes of soil slurry waste using this method was also difficult.

It has been previously shown that PCR-based detection of soil-borne pathogens is dependent on the soil sample size used for DNA extraction (Woodhall et al. 2012, Ophel-Keller et al. 2008). The method described by Woodhall et al. uses a paint-shaker to perform the lysis step, rather than manual shaking by hand. This could eliminate any potential bias in the method published by Than et al. 2013. Alternatively, it would be interesting to compare standard DNA extraction kits with multiple, replicate extractions for each soil sample rather than one or two bulk extractions. Pooling of DNA extracts from multiple smaller-scale extractions of the same soil sample could also overcome bias in DNA extractions (Feinstein et al 2009). In addition to changes in the DNA extraction method, it is also recommended that an internal control (i.e. Phytophthora DNA or oospores from a different species added to each sample at a standardised amount) is used for each extraction (Ophel-Keller et al. 2008). This control would allow identification of soils or extractions that have poor DNA recovery.

Real-time PCR assay

Standard curves were generated at both organisations, in triplicate. Representative standard curves for Scion (Figure1) and Landcare (Figure 2), show the high level of sensitivity and efficiency of the real-time PCR assay at both organisations. Specificity testing using DNA from four other *Phytophthora* species commonly found in soil in New Zealand, showed the assay is specific for PTA (Table 2). The lower the Cq value the higher the concentration of DNA present.

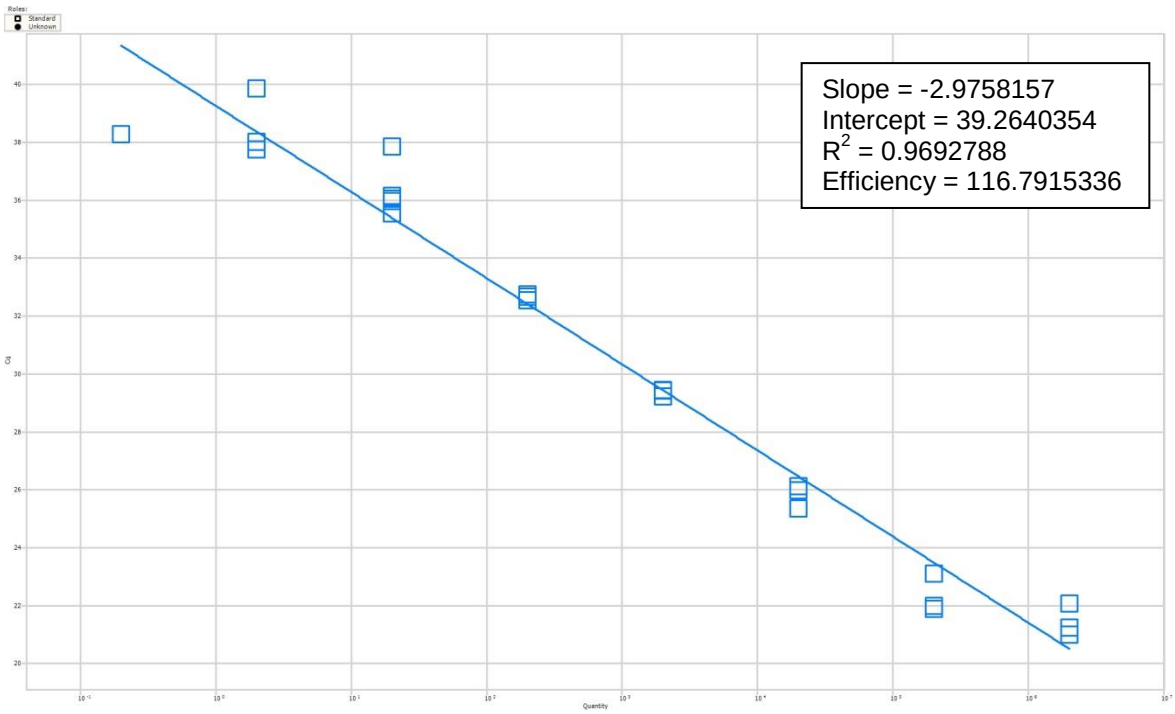

Figure 1. Scion real-time PCR standard curve of *Phytophthora* taxon Agathis DNA.

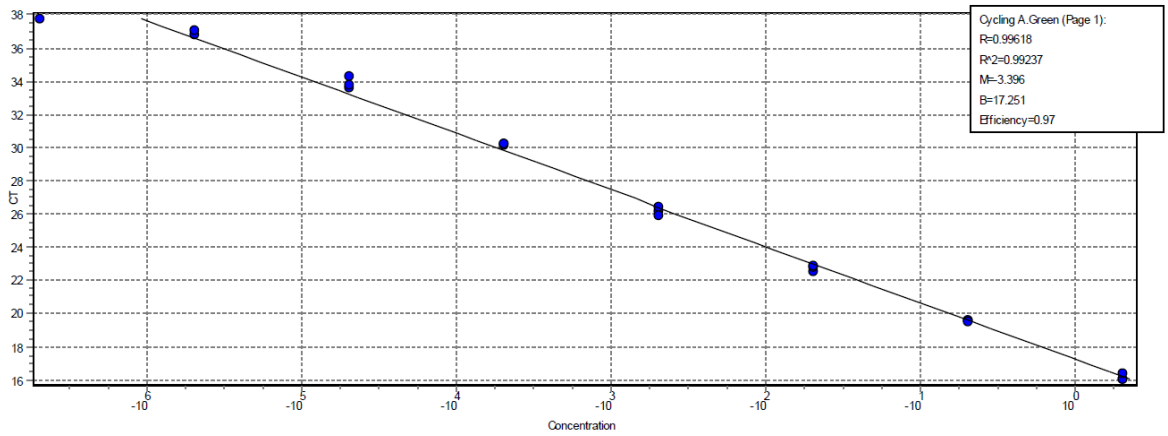

Figure 2. Landcare real-time PCR standard curve of *Phytophthora* taxon Agathis DNA.

Table 2. Specificity testing of ITS_PTA_probe2 with various *Phytophthora* species using the PTA real-time PCR assay

		Mean Cq value*	
Species	**NZFS number**	**Scion**	**Landcare**
P. cinnamomi	102.17	ND	ND
P. cryptogea	2558	ND	ND
P. kernoviae	2646	ND	ND
P. multivora	2750	ND	ND
PTA	3128	17.97	-
PTA	3772	-	16.90

* ND, not detected or amplification below limits of detection
- Not tested

The 20 soil samples provided by KDM, the five Landcare archive soil samples and the Landcare negative control were assayed at both organisations (Table 3; Appendix 3). Some soils samples contained less than required for the individual assays: samples LC1-6 and RT-PCR5 and RT-PCR6. For these samples, the available soils were divided into equal portions for testing.

Scion amplified PTA DNA in all three triplicate PCR reactions for both duplicate soils from six of the 26 samples and in one duplicate for three of the 26 samples. Landcare amplified PTA DNA in all three triplicate PCR reactions for both duplicate soils from three of the 26 samples, and in all three triplicate PCR reactions for one duplicate for one of the 26 samples, and in one or two of the triplicate assays for four of the 26 sample. Samples were only considered positive for PTA where all three reactions of the triplicate PCR reactions for each extract showed positive amplification. Of the 11 samples where PTA DNA was detected by the real-time PCR assay, only six were detected by both organisations. No amplification was detected in the negative soil sample (LCR 6; H5-8) by either organisation. All internal positive controls amplified, as did the control PTA DNA tested (Appendix 3).

For real-time PCR there can be difficulty in interpreting results when detecting a low concentration of DNA of interest in a sample (Hyatt et al 2007). Commonly thresholds for Cq values are used and if values exceed these thresholds then a samples is scored as 'not detected or amplification below limits of detection' (Hughes et al 2011). However, Cq value thresholds are not always recommended (Bustin et al. 2009). Based on the detection limits in the standard curves generated by Scion and Landcare, PTA could be reliably detected (i.e. Cq values were obtained for all replicates) at approximately Cq 36 and Cq 34 respectively (20 fg DNA). However, PTA was still detected at 2 fg DNA, and in some cases at 0.2 fg DNA, but just not in all replicates. The highest Cq values for this level of detection were just over 39 for Scion and just over 37 for Landcare. For this study we chose not to use a Cq threshold as the majority of Cq values fell below the highest standard curve Cq values obtained, meaning that Cq values (and therefore PTA DNA concentrations) fell within the range of detection for the assay (Appendix 3). For a sample to be scored positive, Cq values were required for all triplicate PCR reactions. Where Cq values were not obtained for all triplicate PCR reactions, samples were scored as an equivocal result (Hyatt et al 2007). PTA could possibly still be detected from such samples using the real-time PCR assay, by testing a larger volume of the extracted DNA.

Soil bioassays

The 20 soil samples provided by KDM, the five Landcare archive soil samples and the Landcare negative control were analysed at both organisations for the presence of PTA using the standard bioassay protocol (Table 3). Scion recovered PTA from a total of seven soil samples and for both duplicates for six of those samples and from only one of duplicates for one of the samples. Landcare recovered PTA from a total of three soil samples, and for all of those samples PTA was only recovered from one of the duplicates.

Comparison of real-time PCR and soil bioassay results

There was considerable variation in the detection or recovery of PTA both between organisations and between the detection methods used (Table 3). Of the 26 soil samples tested 13 samples tested positive for PTA but only one soil sample, RT PCR 11, tested positive at both organisations using both detection methods.

Scion detected or recovered PTA from 13 of the soil samples tested, whereas Landcare detected or recovered PTA from only five samples. For the real-time PCR a sample was considered positive for PTA when all triplicate PCR reactions for each extract showed positive amplification. In addition to Landcare's five positive samples they also had four equivocal PCR results, where PTA was detected in only one or two of the triplicate PCR reactions. Although, PTA was detected or recovered by Scion for these three samples, in one of the samples (RT PCR 9) PTA was not detected in Landcare's bioassay or by Scion. Although these equivocal PCR results could be positives, re-testing of samples like this would be recommended.

The real-time PCR assay detected PTA in nine of the samples, with an additional three equivocal PCR results. The soil bioassay recovered PTA from eight of the soil samples. However, of the 13 positive PTA detections, only four of these were detected or recovered by both the real-time PCR and the bioassay.

The overall rate of detection or recovery of PTA between real-time PCR and the bioassays was very similar and one technique over the other could not be recommended at this stage. However, the low number detected by both assay types suggests changes to the current protocols could be effective in improving detection of PTA. In addition to the recommended changes to the real-time PCR assays described previously, slight changes to soil preparation and the bioassay could also potentially increase recovery yields. Regardless of improvements that could be made, these results are better than results from a study where real-time PCR assays were compared to isolation from plant samples for *P. kernoviae* (Hughes et al. 2011). In this study, real-time PCR detected 19 positive samples out of 526 tested and *P. kernoviae* was isolated from 15 of these samples; isolation yielded four samples that were not detected by real-time PCR.

Table 3. PTA detection in soil samples from real-time PCR and soil bioassays

		Real-time PCR[b]		Bioassay[c]	
Sample name	**Sample ID[a]**	**Scion**	**Landcare**	**Scion**	**Landcare**
RT PCR 1	Q	-	-	-	-
RT PCR 2	Y	-	-	-	-
RT PCR 3	E	-	-	-	-
RT PCR 4	C	-	-	-	-
RT PCR 5	U	-	-	-	-
RT PCR 6	X	-	-	-	-
RT PCR 7	S	-	-	-	-
RT PCR 8	P	-	-	-	-
RT PCR 9	O	-	E	-	-
RT PCR 10	J	2	2	-	-
RT PCR 11	F	1	2	1	1
RT PCR 12	N	-	-	2	-
RT PCR 13	A	2	-	-	-
RT PCR 14	I	2	E	-	-
RT PCR 15	V	-	-	-	-
RT PCR 16	Z	2	E	2	-
RT PCR 17	K	2	2	-	1
RT PCR 18	G	1	-	-	-
RT PCR 19	D	-	-	-	-
RT PCR 20	L	2	1	-	-
LC 1	B	1	-	2	-
LC 2	T	-	-	2	-
LC 3	W	-	E	2	-
LC 4	R	-	E	2	1
LC 5	M	-	-	-	-
LC 6[d]	H	-	-	-	-

[a] Sample ID = sample identification code given to samples for blind testing and randomisation purposes

[b] 1 = PTA detected in all three triplicate PCR reactions for one of duplicate soil samples
2 = PTA detected in all three triplicate PCR reactions for both of the duplicate soil samples
E = equivocal PCR result, PTA detected in only 1 or 2 of triplicate PCR reactions (as described by Hyatt et al 2007).

[c] 1 = PTA detected in one of the duplicate soil samples
2 = PTA detected in both duplicate soil samples

[d] Landcare Research negative soil sample

- Not detected or recovered

Conclusions and Recommendations

The real-time PCR assay and the standard bioassay procedure for detecting PTA from soil samples are both effective methods. From the results obtained in this study it is very difficult to say which method is more effective, and at this stage, one method could not be recommended over the other. If real-time PCR was chosen as the primary method of detection, then samples that are inconclusive could be repeated using real-time PCR with increased volumes of DNA or the bioassay could be performed.

Soil analysis of any type is prone to variability. Sampling strategy and sample size can influence the outcomes of the analysis (Woodhall et al. 2012, Ophel-Keller et al. 2008). While large soil samples are most effective for detection, the published method is laborious and prone to bias, and therefore not suitable for large sample numbers. The use of a DNA extraction internal control would assist in identifying soil sample extractions where DNA recovery was low, allowing more confidence in negative detections.

Based on the results from this study we would recommend the following:

1. DNA extraction is performed using a paint-shaker (or equivalent instrument) as per Woodhall et al. 2012, and results compared to manual vigorous shaking.
2. The bulk method of DNA extraction used in this study (and changes recommended) is compared to DNA extraction using a kit (e.g. MO-BIO, 10g samples) with multiple sub-samples and pooling of extracts for PCR.
3. A DNA extraction internal control is used to assist in identifying soil sample extractions where DNA recovery is low.
4. A combination of both methods of detection are adopted for PTA detection from soil samples, until improvements, as discussed in this report, can be made to increase detection rates.

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Appendix

1. Soil baiting standard operating protocol (SOP) for *Phytophthora* taxon Agathis (PTA)

Drying of soil samples (pretreatment-phase)

- Thoroughly mix soil samples in their bag to remove jumps
- Label container
- Measure desired amount of soil Into bait container, e.g.
    - 175 g into 1-Ltake-away container
    - 90 g into 680-mi circular plastic pottle
    - 20 gin 300-mi plastic cup
- Air-dry on lab bench for 2 days
- Check soil each day and crumble clods with pop-sticks (use a new pop-stick for each sample)

NB. Alternatively, soils can be dried on paper towels on laboratory bench being aware of potential for aerial contamination of soil surface.

Moist Incubation (stimulating-phase):

- Using a spray-squirt bottle, moisten soil samples in containers with 80 water (using a fine mist)
- Spray enough moisture to make soil surface shine. Respray after 1h,targeting dry spots / clods of soil
- Apply lid loosely
- Incubate in light for 4 days at room temperature (20—22°C)

Bait tissue preparation

- One day after the commencement of moist incubation, prepare desired amount of lupin seed, allowing for five lupins per soil bioassay.
- Needles of Himalayan cedar (Cedrus deodara), harvested directly off tree (age of needles not considered important)
    - Pull off whorls
    - Pull needles off from leaf base
    - Five needles per soil bioassay

Bioassay

- Inundate the soil very slowly with RO or distilled water, e.g.:
    - 500 ml for l-L take-away container
    - 300 ml for 680-mi circular plastic pottle
    - 150 ml for 300-mi plastic cup

N.B. aiming to achieve a depth of RO water of 5-10 cm above soil surface

- Minimise soil disturbance and water turbulence
- The soil must not be mixed once flooded
- Sprinkle five 2-cm lengths of Himalayan cedar leaflets on water surface
- Add five lupin radicles to the water surface (suspended on polystyrene floats, or floated on the water surface)
- Incubate at 20°C in light for 2 days.

Bait processing

- After 2 days remove the bait tissues
- Wash in single rinse of sterile RO water
- Soak In 70% ethanol (ETOH) for 30s

- Remove from ETOH, rinse in sterile RO water
- Blot dry on paper towels
- Place leaflets/lupins onto P_5ARPH
- Label and seal plates and incubate in the dark at 18—20°C for two-days

Colony isolation

- Isolate *Phytophthora*-like cultures to V8 juice agar
- Check V8 juice agar plates after 4 days
- If cultures are free of contamination, sub-culture to PDA

Usual timeline

- Set up soils to dry on day 1
- Commence moist incubation on Day 3
- Commence lupin germination on Day 4 (p.m.)
- Flood and bait on Day 7
- Harvest and plate out baits on Day 9
- Check cultures on Day 11, sub-culturing to V8 juice agar where necessary
- Re-check cultures on Day 14 for new colonies, and sub-culture to V8 juice agar

N.B. the transparency of V8-juice agar can be improved through clarification

2. Comparison of DNA detection from pre- and post-purified DNA

			Pre-purification			Post-purification		
Sample Name	**Sample ID**	**Dilution**	**Cq**	**Cq Mean**	**Std. Dev. Cq**	**Cq**	**Cq Mean**	**Std. Dev. Cq**
RT PCR 9	O6	undiluted	22.38	20.22	1.87	18.40	20.43	1.77
RT PCR 9	O6	undiluted	19.07			21.37		
RT PCR 9	O6	undiluted	19.20			21.54		
RT PCR 7	S6	undiluted	19.11	18.98	0.14	21.09	19.51	1.37
RT PCR 7	S6	undiluted	18.83			18.74		
RT PCR 7	S6	undiluted	18.99			18.70		
RT PCR 9	O6	1:10	23.38	23.41	0.09	22.23	22.24	0.02
RT PCR 9	O6	1:10	23.35			22.24		
RT PCR 9	O6	1:10	23.51			22.27		
RT PCR 7	S6	1:10	22.25	22.47	0.22	22.09	22.28	0.18
RT PCR 7	S6	1:10	22.68			22.29		
RT PCR 7	S6	1:10	22.49			22.45		
RT PCR 9	O6	1:50	26.52	26.14	0.34	24.17	24.62	0.39
RT PCR 9	O6	1:50	25.92			24.80		
RT PCR 9	O6	1:50	25.96			24.88		
RT PCR 7	S6	1:50	24.50	24.51	0.04	24.25	24.41	0.28
RT PCR 7	S6	1:50	24.56			24.73		
RT PCR 7	S6	1:50	24.48			24.25		

3. Scion and Landcare real-time PCR assay results of soil samples

		Scion data*								Landcare data*						
		Soil samples			Internal positive control					Soil samples				Internal positive control		
Sample name	Sample ID	Cq	Cq Mean	Std. Dev. Cq	Cq	Cq Mean	Std. Dev. Cq	Sample name	Sample ID	Cq	Cq Mean	Std. Dev. Cq		Cq	Cq Mean	Std. Dev. Cq
RT PCR 13	A5	ND	33.16	0.21	ND	30.15	0.06	RT PCR 13	A7	ND	ND	ND		26.55	26.49	0.12
RT PCR 13	A5	33.02			30.11			RT PCR 13	A7	ND				26.35		
RT PCR 13	A5	33.31			30.19			RT PCR 13	A7	ND				26.56		
RT PCR 13	A6	34.23	34.30	0.12	30.12	30.10	0.02	RT PCR 13	A8	ND	ND	ND		26.87	26.77	0.21
RT PCR 13	A6	34.43			30.08			RT PCR 13	A8	ND				26.92		
RT PCR 13	A6	34.23			30.12			RT PCR 13	A8	ND				26.53		
LC 1	B5	ND	ND	ND	29.97	30.22	0.30	LC 1	B7	ND	ND	ND		26.73	26.56	0.16
LC 1	B5	ND			30.12			LC 1	B7	ND				26.42		
LC 1	B5	ND			30.56			LC 1	B7	ND				26.53		
LC 1	B6	ND	37.87	0.02	29.97	30.09	0.11	LC 1	B8	ND	ND	ND		26.44	26.55	0.16
LC 1	B6	37.89			30.19			LC 1	B8	ND				26.48		
LC 1	B6	37.86			30.12			LC 1	B8	ND				26.73		
RT PCR 4	C5	ND	ND	ND	30.33	30.27	0.13	RT PCR 4	C7	ND	ND	ND		26.69	26.75	0.17
RT PCR 4	C5	ND			30.12			RT PCR 4	C7	ND				26.94		
RT PCR 4	C5	ND			30.37			RT PCR 4	C7	ND				26.61		
RT PCR 4	C6	ND	ND	ND	30.11	30.35	0.30	RT PCR 4	C8	ND	ND	ND		26.94	26.88	0.07
RT PCR 4	C6	ND			30.26			RT PCR 4	C8	ND				26.80		
RT PCR 4	C6	ND			30.69			RT PCR 4	C8	ND				26.90		
RT PCR 19	D5	ND	ND	ND	30.22	30.26	0.11	RT PCR 19	D7	ND	ND	ND		26.75	27.02	0.36
RT PCR 19	D5	ND			30.18			RT PCR 19	D7	ND				26.88		
RT PCR 19	D5	ND			30.38			RT PCR 19	D7	ND				27.42		

RT PCR 19	D6	ND	ND	ND	30.19	30.05	0.26	RT PCR 19	D8	ND	ND	ND	27.12	26.98	0.21
RT PCR 19	D6	ND			30.19			RT PCR 19	D8	ND			26.74		
RT PCR 19	D6	ND			29.75			RT PCR 19	D8	ND			27.09		
RT PCR 3	E5	ND	ND	ND	30.09	30.14	0.26	RT PCR 3	E7	ND	ND	ND	26.87	26.77	0.13
RT PCR 3	E5	ND			29.92			RT PCR 3	E7	ND			26.82		
RT PCR 3	E5	ND			30.42			RT PCR 3	E7	ND			26.62		
RT PCR 3	E6	ND	ND	ND	30.26	30.27	0.05	RT PCR 3	E8	ND	ND	ND	26.31	26.53	0.20
RT PCR 3	E6	ND			30.23			RT PCR 3	E8	ND			26.70		
RT PCR 3	E6	ND			30.32			RT PCR 3	E8	ND			26.57		
RT PCR 11	F5	36.40	37.19	0.87	30.16	30.43	0.56	RT PCR 11	F7	34.35	34.81	0.43	26.59	26.77	0.31
RT PCR 11	F5	37.03			31.08			RT PCR 11	F7	34.86			26.58		
RT PCR 11	F5	38.13			30.06			RT PCR 11	F7	35.22			27.12		
RT PCR 11	F6	ND	ND	ND	30.75	30.93	0.27	RT PCR 11	F8	35.74	35.45	0.81	26.58	26.88	0.29
RT PCR 11	F6	ND			30.80			RT PCR 11	F8	34.52			26.93		
RT PCR 11	F6	ND			31.24			RT PCR 11	F8	36.07			27.15		
RT PCR 18	G5	ND	ND	ND	30.33	30.22	0.12	RT PCR 18	G7	ND	ND	ND	26.63	26.68	0.24
RT PCR 18	G5	ND			30.10			RT PCR 18	G7	ND			26.46		
RT PCR 18	G5	ND			30.23			RT PCR 18	G7	ND			26.94		
RT PCR 18	G6	39.53	39.15	0.54	30.19	30.25	0.37	RT PCR 18	G8	ND	ND	ND	26.55	26.62	0.07
RT PCR 18	G6	38.77	39.15	0.54	30.65			RT PCR 18	G8	ND			26.69		
RT PCR 18	G6	ND	39.15	0.54	29.92			RT PCR 18	G8	ND			26.62		
LC 6	H5	ND	ND	ND	29.32	29.16	0.14	LC 6	H7	ND	ND	ND	26.82	26.81	0.02
LC 6	H5	ND			29.07			LC 6	H7	ND			26.84		
LC 6	H5	ND			29.08			LC 6	H7	ND			26.79		
LC 6	H6	ND	ND	ND	29.22	29.31	0.13	LC 6	H8	ND	ND	ND	26.59	26.74	0.28
LC 6	H6	ND			ND			LC 6	H8	ND			26.57		
LC 6	H6	ND			29.40			LC 6	H8	ND			27.06		
RT PCR 14	I5	37.85	37.76	0.12	28.69	28.88	0.35	RT PCR 14	I7	ND	34.90	ND	26.25	26.37	0.15
RT PCR 14	I5	ND			28.68			RT PCR 14	I7	ND			26.53		

RT PCR 14	I5	37.68			29.28			RT PCR 14	I7	34.90			26.32		
RT PCR 14	I6	37.48	37.89	0.37	28.76	28.90	0.13	RT PCR 14	I8	38.46	38.46	ND	26.84	26.70	0.14
RT PCR 14	I6	38.00			29.01			RT PCR 14	I8	ND			26.69		
RT PCR 14	I6	38.20			28.92			RT PCR 14	I8	ND			26.57		
RT PCR 10	J5	31.25	31.20	0.09	28.69	29.15	0.68	RT PCR 10	J7	29.36	29.29	0.06	26.78	26.80	0.25
RT PCR 10	J5	31.25			28.83			RT PCR 10	J7	29.24			27.06		
RT PCR 10	J5	31.10			29.92			RT PCR 10	J7	29.28			26.57		
RT PCR 10	J6	31.51	31.61	0.24	28.68	29.08	0.44	RT PCR 10	J8	30.64	30.37	0.23	26.63	26.74	0.16
RT PCR 10	J6	31.88			29.00			RT PCR 10	J8	30.23			26.68		
RT PCR 10	J6	31.44			29.56			RT PCR 10	J8	30.24			26.92		
RT PCR 17	K5	31.90	32.28	0.58	29.07	28.87	0.18	RT PCR 17	K7	31.21	31.88	0.65	26.43	26.65	0.28
RT PCR 17	K5	32.01			28.73			RT PCR 17	K7	31.93			26.55		
RT PCR 17	K5	32.95			28.80			RT PCR 17	K7	32.50			26.97		
RT PCR 17	K6	31.93	32.04	0.10	28.89	28.87	0.03	RT PCR 17	K8	31.43	31.75	0.33	26.47	26.43	0.11
RT PCR 17	K6	32.06			28.88			RT PCR 17	K8	31.72			26.52		
RT PCR 17	K6	32.12			28.84			RT PCR 17	K8	32.09			26.30		
RT PCR 20	L5	37.64	37.65	0.11	28.78	28.84	0.15	RT PCR 20	L7	34.53	35.22	0.60	26.66	26.51	0.16
RT PCR 20	L5	37.56			28.73			RT PCR 20	L7	35.54			26.54		
RT PCR 20	L5	37.77			29.01			RT PCR 20	L7	35.59			26.34		
RT PCR 20	L6	36.83	36.95	0.32	28.88	29.00	0.28	RT PCR 20	L8	ND			26.75	26.94	0.39
RT PCR 20	L6	37.32			29.32			RT PCR 20	L8	37.91			26.69		
RT PCR 20	L6	36.71			28.81			RT PCR 20	L8	36.71			27.39		
LC 5	M5	ND	ND	ND	28.97	29.03	0.65	LC 5	M7	ND	ND	ND	26.54	26.44	0.12
LC 5	M5	ND			29.71			LC 5	M7	ND			26.31		
LC 5	M5	ND			28.41			LC 5	M7	ND			26.48		
LC 5	M6	ND	ND	ND	ND	29.05	0.18	LC 5	M8	ND	ND	ND	26.63	26.55	0.25
LC 5	M6	ND			28.93			LC 5	M8	ND			26.75		
LC 5	M6	ND			29.18			LC 5	M8	ND			26.26		
RT PCR 12	N5	ND	ND	ND	29.79	30.18	0.75	RT PCR 12	ND7	ND	ND	ND	26.48	26.30	0.17

RT PCR 12	N5	ND			31.04			RT PCR 12	ND7	ND			26.26		
RT PCR 12	N5	ND			29.71			RT PCR 12	ND7	ND			26.16		
RT PCR 12	N6	ND	38.91	ND	29.80	29.80	0.18	RT PCR 12	ND8	ND	ND	ND	26.22	26.33	0.09
RT PCR 12	N6	ND			29.62			RT PCR 12	ND8	ND			26.39		
RT PCR 12	N6	38.91			29.98			RT PCR 12	ND8	ND			26.37		
RT PCR 9	O5	ND	ND	ND	29.85	30.30	0.45	RT PCR 9	O7	37.16	37.16	ND	26.47	26.64	0.18
RT PCR 9	O5	ND			30.31			RT PCR 9	O7	ND			26.63		
RT PCR 9	O5	ND			30.74			RT PCR 9	O7	ND			26.82		
RT PCR 9	O6	ND	ND	ND	29.89	30.12	0.21	RT PCR 9	O8	ND	ND	ND	26.57	26.51	0.06
RT PCR 9	O6	ND			30.31			RT PCR 9	O8	ND			26.53		
RT PCR 9	O6	ND			30.15			RT PCR 9	O8	ND			26.44		
RT PCR 8	P5	ND	ND	ND	29.64	29.75	0.10	RT PCR 8	P7	ND	ND	ND	26.13	26.12	0.08
RT PCR 8	P5	ND			29.85			RT PCR 8	P7	ND			26.02		
RT PCR 8	P5	ND			29.76			RT PCR 8	P7	ND			26.19		
RT PCR 8	P6	ND	ND	ND	29.60	30.06	0.43	RT PCR 8	P8	ND	ND	ND	26.28	26.34	0.15
RT PCR 8	P6	ND			30.15			RT PCR 8	P8	ND			26.22		
RT PCR 8	P6	ND			30.44			RT PCR 8	P8	ND			26.51		
RT PCR 1	Q5	ND	ND	ND	29.68	29.67	0.12	RT PCR 1	Q7	ND	ND	ND	26.33	26.35	0.03
RT PCR 1	Q5	ND			29.79			RT PCR 1	Q7	ND			26.38		
RT PCR 1	Q5	ND			29.55			RT PCR 1	Q7	ND			26.34		
RT PCR 1	Q6	ND	ND	ND	31.47	31.14	0.35	RT PCR 1	Q8	ND	ND	ND	26.21	26.38	0.18
RT PCR 1	Q6	ND			31.19			RT PCR 1	Q8	ND			26.36		
RT PCR 1	Q6	ND			30.76			RT PCR 1	Q8	ND			26.56		
LC 4	R5	ND	ND	ND	29.65	29.76	0.21	LC 4	R7	ND	35.99	ND	26.53	26.49	0.08
LC 4	R5	ND			29.62			LC 4	R7	ND			26.54		
LC 4	R5	ND			30.00			LC 4	R7	35.99			26.40		
LC 4	R6	ND	38.52	ND	29.77	29.86	0.08	LC 4	R8	ND	ND	ND	26.53	26.49	0.22
LC 4	R6	ND			29.87			LC 4	R8	ND			26.25		
LC 4	R6	38.52			29.94			LC 4	R8	ND			26.69		

RT PCR 7	S5	ND	ND	ND	30.51	30.83	0.55	RT PCR 7	S7	ND	ND	ND	26.49	26.51	0.11
RT PCR 7	S5	ND			31.46			RT PCR 7	S7	ND			26.42		
RT PCR 7	S5	ND			30.51			RT PCR 7	S7	ND			26.63		
RT PCR 7	S6	ND	ND	ND	29.69	29.65	0.04	RT PCR 7	S8	ND	ND	ND	26.32	26.51	0.17
RT PCR 7	S6	ND			29.63			RT PCR 7	S8	ND			26.54		
RT PCR 7	S6	ND			29.62			RT PCR 7	S8	ND			26.67		
LC 2	T5	ND	ND	ND	29.66	29.88	0.19	LC 2	T7	ND	ND	ND	27.07	26.66	0.36
LC 2	T5	ND			29.97			LC 2	T7	ND			26.54		
LC 2	T5	ND			30.01			LC 2	T7	ND			26.38		
LC 2	T6	ND	ND	ND	34.61	32.59	2.87	LC 2	T8	ND	ND	ND	25.90	26.03	0.31
LC 2	T6	ND			ND			LC 2	T8	ND			26.38		
LC 2	T6	ND			30.56			LC 2	T8	ND			25.81		
RT PCR 5	U5	ND	ND	ND	30.33	30.92	1.65	RT PCR 5	U7	ND	ND	ND	26.07	26.16	0.14
RT PCR 5	U5	ND			32.78			RT PCR 5	U7	ND			26.09		
RT PCR 5	U5	ND			29.65			RT PCR 5	U7	ND			26.32		
RT PCR 5	U6	ND	ND	ND	29.73	29.78	0.07	RT PCR 5	U8	ND	ND	ND	26.02	26.11	0.11
RT PCR 5	U6	ND			29.75			RT PCR 5	U8	ND			26.08		
RT PCR 5	U6	ND			29.87			RT PCR 5	U8	ND			26.22		
RT PCR 15	V5	ND	ND	ND	29.36	29.30	0.09	RT PCR 15	V7	ND	ND	ND	26.30	26.15	0.14
RT PCR 15	V5	ND			29.19			RT PCR 15	V7	ND			26.04		
RT PCR 15	V5	ND			29.34			RT PCR 15	V7	ND			26.10		
RT PCR 15	V6	ND	ND	ND	29.36	29.60	0.29	RT PCR 15	V8	ND	ND	ND	25.99	25.96	0.03
RT PCR 15	V6	ND			29.92			RT PCR 15	V8	ND			25.93		
RT PCR 15	V6	ND			29.51			RT PCR 15	V8	ND			25.95		
LC 3	W5	ND	ND	ND	29.42	29.57	0.36	LC 3	W7	ND	ND	ND	25.92	25.90	0.03
LC 3	W5	ND			29.31			LC 3	W7	ND			25.87		
LC 3	W5	ND			29.97			LC 3	W7	ND			25.92		
LC 3	W6	ND	ND	ND	29.27	29.24	0.13	LC 3	W8	ND			26.03	26.21	0.17
LC 3	W6	ND			29.10			LC 3	W8	ND			26.37		

LC 3	W6	ND			29.35			LC 3	W8	35.31			26.23		
RT PCR 6	X5	ND	ND	ND	29.28	29.32	0.08	RT PCR 6	X7	ND	ND	ND	26.29	26.27	0.18
RT PCR 6	X5	ND			29.42			RT PCR 6	X7	ND			26.09		
RT PCR 6	X5	ND			29.28			RT PCR 6	X7	ND			26.44		
RT PCR 6	X6	ND	ND	ND	29.20	29.22	0.13	RT PCR 6	X8	ND	ND	ND	26.27	26.15	0.10
RT PCR 6	X6	ND			29.10			RT PCR 6	X8	ND			26.11		
RT PCR 6	X6	ND			29.35			RT PCR 6	X8	ND			26.09		
RT PCR 2	Y5	ND	ND	ND	29.28	29.33	0.16	RT PCR 2	Y7	ND	ND	ND	26.04	25.98	0.08
RT PCR 2	Y5	ND			29.52			RT PCR 2	Y7	ND			25.89		
RT PCR 2	Y5	ND			29.20			RT PCR 2	Y7	ND			26.01		
RT PCR 2	Y6	ND	ND	ND	29.23	29.71	0.43	RT PCR 2	Y8	ND	ND	ND	26.32	26.18	0.21
RT PCR 2	Y6	ND			30.05			RT PCR 2	Y8	ND			25.93		
RT PCR 2	Y6	ND			29.85			RT PCR 2	Y8	ND			26.29		
RT PCR 16	Z5	35.27	35.28	0.22	29.37	29.28	0.09	RT PCR 16	Z7	ND			26.01	25.97	0.18
RT PCR 16	Z5	35.06			29.28			RT PCR 16	Z7	36.32			25.77		
RT PCR 16	Z5	35.50			29.19			RT PCR 16	Z7	35.59			26.12		
RT PCR 16	Z6	35.60	35.85	0.68	29.42	29.36	0.08	RT PCR 16	Z8	ND			25.94	25.91	0.05
RT PCR 16	Z6	36.61			29.38			RT PCR 16	Z8	ND			25.85		
RT PCR 16	Z6	35.32			29.26			RT PCR 16	Z8	38.44			25.92		
PTA DNA	2ng	22.27	22.20	0.18	28.79	28.71	0.16	PTA DNDA	undiluted	14.56	14.65	0.08	26.13	26.00	0.13
PTA DNA	2ng	21.99			28.53			PTA DNDA	undiluted	14.71			25.98		
PTA DNA	2ng	22.33			28.81			PTA DNDA	undiluted	14.69			25.88		
PTA DNA	2ng	21.81	21.81	0.16	29.55	29.60	0.07	PTA DNDA	undiluted	13.92	13.88	0.18	26.03	25.99	0.13
PTA DNA	2ng	21.65			29.57			PTA DNDA	undiluted	13.68			25.85		
PTA DNA	2ng	21.97			29.68			PTA DNDA	undiluted	14.05			26.10		

* ND, not detected or amplification below limits of detection.