

Using Sigmoidal Curve-Fitting in a Real-Time PCR Detection Assay to Determine Detection Thresholds¹

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Abstract

Phytophthora ramorum, the causal agent of sudden oak death (SOD) is a quarantine pathogen that has forced the implementation of extraordinary measures to track and contain the movement of infected nursery stock both within and outside of the three western states of California, Oregon and Washington. Federal guidelines in the United States for diagnostic testing of *P. ramorum* are in place to insure the sensitivity and reliability of detection tests. PCR assays are used to determine whether the *Phytophthora* sp. detected by the initial immunoassay screening is *P. ramorum*. Most of the time definitive results can be obtained from a single PCR reaction. However, there are times when the accuracy of the results can be called into question because of low pathogen titer, a situation that can result in false negatives given the limits of detection of the marker system. In addition, experimental samples often contain significant amounts of PCR inhibitors that can also give results outside the normal detection cutoffs. Therefore the need for re-testing the samples using the same or alternative methods of pathogen detection is manifest. To understand the effect of low DNA concentration, or the role of PCR inhibitors in the extracted DNA in the sensitivity of detection of *P. ramorum*, plant and pathogen specific markers were amplified in real time PCR experiments using TaqMan® chemistry. The kinetics of amplification of the PCR reactions were modeled using a four-parametric sigmoidal curve. Standard curves of pure *P. ramorum* DNA and plant host DNA, with standard amounts of *P. ramorum* DNA added, were created and used to establish a base for data analysis. Samples having low pathogen titer were amplified and the values of the sigmoid curve parameters described. Statistical analysis of data allowed the identification of samples falling outside the proposed 95 percent confidence interval. Curve modeling also provided experimental support for determining threshold values for assessing the presence of the pathogen.

Key words: *Phytophthora ramorum*, real time PCR, PCR efficiency, 4-parametric sigmoidal regression.

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Introduction

Phytophthora ramorum, the causal agent of sudden oak death disease, is an Oomycete that can infect a large number of plant species. Given its quarantined status, special measures need to be taken to prevent the movement of the pathogen out of infested areas and in the nursery industry. Consequently, host material needs to be checked regularly for the pathogen presence, and surveys are performed at regular intervals to track the movement of *P. ramorum* pathogen in infested areas. To accurately detect the pathogen in suspected samples, a strategy based on an initial screening by immunoassay for detection of *Phytophthora* genus-specific proteins, followed, if positive, by culturing and testing by PCR is mandated. A polymerase chain reaction (PCR) assay, which specifically amplifies a portion of the ribosomal internal transcribed spacer (ITS) DNA of *P. ramorum*, is the only protocol that can be used to test samples for United States Department of Agriculture (USDA)-Animal and Plant Health Inspection Service (APHIS) regulatory purposes. Given the high importance that accurate pathogen detection has in the quarantine process, other PCR assays have been developed. Although the USDA-APHIS approved PCR tests have been optimized, the specificity and sensitivity of the assays might be variable when the quantity of the pathogen in the host sample is very low or close to detection limits. In addition, inconclusive results could be observed when mixed *Phytophthora* infections are present or when DNA contaminants that co-elute with the sample DNA during extraction affect the performance of the reactions.

In current real time PCR procedures to detect *P. ramorum* there is a cut-off value for sample amplification (Cycle threshold or Ct), above which, the sample requires a retest or is considered negative (in other words, when the signal related to specific pathogen amplification is not seen before a set number of cycles into the PCR reaction). This cut-off value is the product of the assay limits of detection and therefore a sample with very low amounts of *P. ramorum* DNA, or containing a mixture of *P. ramorum* and other *Phytophthora* species DNA or containing PCR inhibitors could be erroneously called negative. To address these possibilities in real time PCR experimentation, we used a mathematical approach based on a 4-parametric sigmoidal curve (Tichopad and others 2002) (fig. 1) to model the kinetics

$$F = Y_0 + \frac{a}{1 + e^{-\left(\frac{X - X_0}{k}\right)}}$$

Figure 1—4-parametric sigmoidal curve (Tichopad and others 2002). Absolute net fluorescence (a), background fluorescence (Y₀), first derivative of function (X₀) and slope of the sigmoid curve (k).

of amplification of *P. ramorum*-specific real time PCR reactions (fig. 2a). Experiments were designed to test the models under ideal, in other words pure pathogen DNA or real, in other words field samples (fig. 2b), with variable amounts of pathogen DNA. Statistical analysis of the sigmoidal curve parameters of the data allowed the discovery of relationships that could yield statistical support for the identification of aberrant PCR reactions, which did not follow the specific kinetics of known *P. ramorum* amplifications. A statistical approach to determine the detection limits of the assays is proposed.

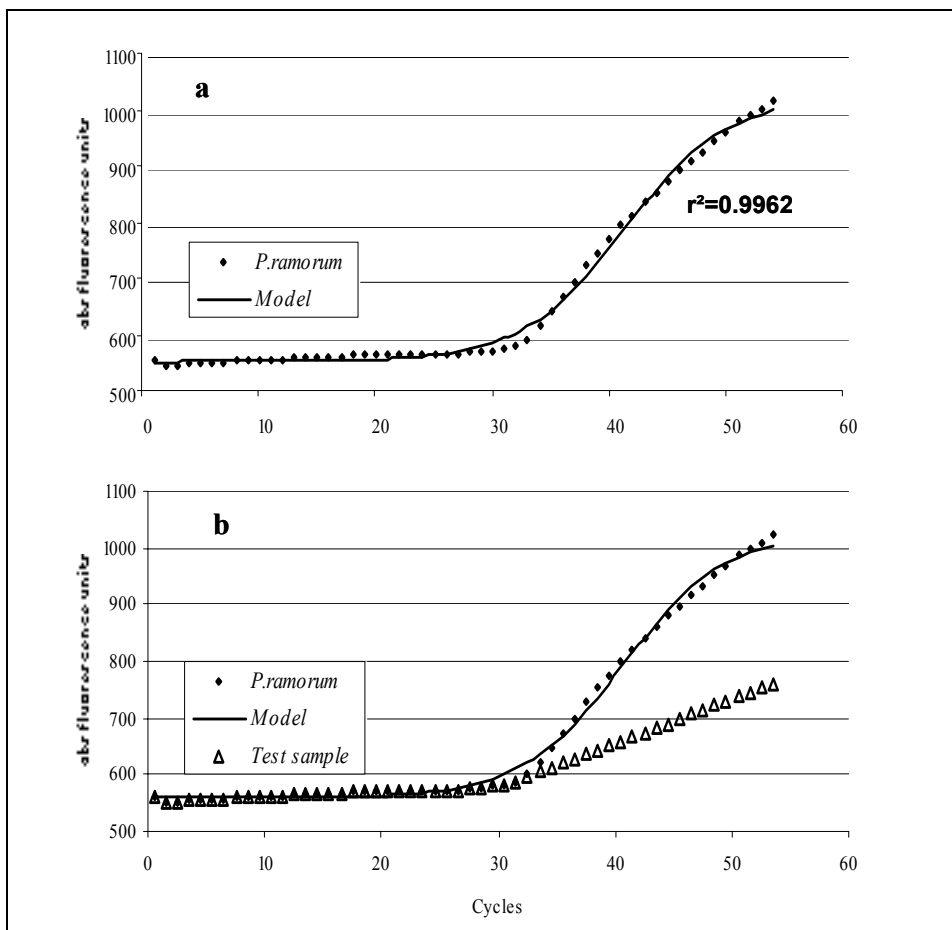


Figure 2—Standard plot of real time PCR amplifications. (a) Curve fit between sigmoidal curve model and the fluorescence output of a 20pg/μl of purified *P. ramorum* DNA. (b) *Phytophthora ramorum* suspected sample from the field (triangles) showing different PCR kinetics from the standard curve.

Materials and Methods

To extract the *P. ramorum*-infected and not infected tanoak DNA needed for the analyses, sample leaves were processed according to the official APHIS protocol for DNA extraction, based on the Dneasy DNA extraction kit from Qiagen (USDA-APHIS). Pure *P. ramorum* DNA from cultures was extracted according to Tooley and others 2004. Real time PCR amplifications were carried out in an I-cycler thermal cycler (BioRad Co., Hercules, CA) using TaqMan® master mix reagents (Applied Biosystems, Foster City, CA). *Phytophthora ramorum* specific primers

FMPPr1a and FMPPr7, amplifying a portion of the spacer region between CoxI and CoxII genes of the mitochondrial genome of this species (Tooley and others 2006), were used at a concentration of 1000 nM each. Plant specific primers FMPI2b and FMPI3b (Tooley and others 2006) were used at a concentration of 40 nM each as internal control.

To specifically detect pathogen amplification a TaqMan® probe, PrFAM (Tooley and others 2006), containing the fluorescent label FAM and the black hole quencher (BHQ1) was used at 400 nM. To detect plant DNA, the plant-specific probe, Plant Calred (Tooley and others), that carries the fluorescent reporter CalRed and the black hole quencher (BHQ2) was added at a concentration of 200 nM. Labeled probes were synthesized by Biosearch Co. (Novato CA) and primers by Invitrogen Co. (Carlsbad CA). All the PCR reactions included an additional 1 mM MgCl₂ in the standard master mix and were carried out in a final volume of 25µl. Amplification conditions used were: initial denaturation for 10 minutes at 95 °C and 55 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. A total of seven independent experiments were performed. Each experiment included an equal concentration of host DNA (tanoak) to which a dilution series of *P. ramorum* DNA had been added. Within each experiment, each sample was run in triplicate.

Pathogen concentration was initially spectrophotometrically determined using a Nanodrop (Nanodrop technologies, Wilmington DE). A total of six, 10-fold dilutions with concentrations between 200 pg/µl and 2 fg/µl were included in the experiments. To normalize PCR variability from experiment to experiment an additional standard of known concentration (50 pg/µl), called the calibrator sample, was added to the experimental set up. Threshold cycles of detection (**Ct**) were determined using the I-cycler software. For this purpose the baseline for detection was automatically set in each experiment and **Ct**s were normalized between experimental replicates by setting the software's threshold level of amplification to a value, that although different in each experiment, resulted in equal threshold cycle of detection in all the calibrator samples. Background subtracted real time PCR data was collected from the I-cycler output file and recorded in Excel (Microsoft, Redmond, WA, version 9.0 SP-3) spreadsheets and exported into Sigma Plot (Systat Software Inc, San Jose, CA, version 8.0) for modeling. Modeling process consisted in 100 iterations of the 4-parameter sigmoid curve formula (Tichopad and others 2002.). Sigmoid curve coefficients net fluorescence (**a**), slope of the sigmoid curve (**k**), first derivative of function (**Xo**), background fluorescence (**Yo**) and coefficient of determination (**r**²) were recorded and exported into Sigma Stat (Systat Software, San Jose, CA, version 3.0) and Excel software for data analysis and Sigma Plot for creating graphs. Using the values of **Xo** and **k** the initial efficiency of the reactions according to the sigmoid model (**Eo**) was calculated with the formula presented by Rutledge and others 2004 (fig. 3).

$$Eo = \frac{1 + e^{(1 + Xo / k)}}{1 + e^{(Xo / k)}} - 1$$

Figure 3—4-parametric sigmoidal curve initial efficiency formula (**Eo**) (Rutledge and others 2004).

Results and Discussion

Using background subtracted fluorescence data from replicated experiments, a database to use in the modeling of kinetics of PCR reactions was created. Each independent experiment included in the database contained the results of the real time PCR amplification of a dilution series of *P. ramorum* templates. Using Sigma Plot software, the approximation of the background subtracted fluorescence data of the reactions to the 4-parameter sigmoid curve (Tichopad and others 2002) was accomplished by repeated iterations (100 times) of the polynomial regression formula.

The values of the sigmoidal curve parameters and their corresponding coefficient of determination were recorded. The relationship between each parameter of the sigmoidal curve and the log of template concentration was compared using linear regression. Linear correlations were drawn between each of the four parameters and the log of template concentration (fig. 4). The net fluorescence values of the reaction (**a**) (data not shown) and the slope of the sigmoid curve (**k**) (fig. 4c) tended to increase with increasing template concentration, while the threshold cycle (**Ct**) (fig. 4a), and the inflection point of the sigmoid curve (first derivative of the sigmoid curve or **Xo**) (fig. 4b) decreased with increasing template concentration. In all cases the r^2 values of the linear regression analysis were above 0.96.

Equally high r^2 values of the linear regression between sigmoid curve parameters and the log of template concentration were not obtained when the relationship between the initial sigmoid curve efficiency (**Eo**) (fig. 3) and the log of template concentration of the different standards was tested (fig. 4d). Because **Eo** can be expressed solely in terms of **Xo** and **K**, it is expected that in experimental reactions involving identical

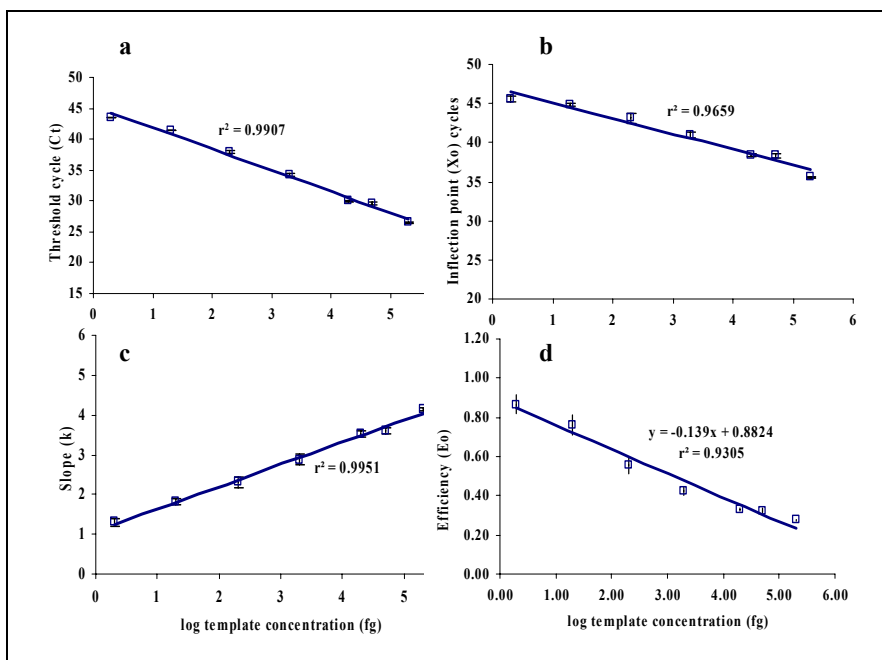


Figure 4—Linear regression analysis of four different kinetic parameters of real time PCR amplifications. (a) Threshold cycle of detection (**Ct**), (b-d) sigmoid curve parameters **k**, **Xo** and **Eo** in repeated real time PCR reactions using standard dilutions of *P. ramorum* templates.

templates X_o and K should have very similar values and hence equal or very similar E_o . (Rutledge and others 2004). Therefore by testing the behavior of E_o at different template concentrations we were able to access the effect of decreased template concentration on the value of E_o . Under our experimental conditions it was possible to detect amplification of standard templates up to the fg range (2 fg/ μ l) with an r^2 value of 0.99 (fig. 4a) and given that in the literature it is acknowledged that E_o should be identical in PCR reactions with identical templates (Rutledge and others 2004), we expected to find similar E_o values at this detection limits of the assay.

Statistical analysis of the data using repeated measures ANOVA and Tukey tests made evident that it was possible to separate the data into two different groups. The analysis showed that at high template concentration (in other words > 3 logs of pathogen concentration or > 2 pg/ μ l) there was no statistical difference between the E_o values of the tested standards (fig. 4d). On the other hand, the data also showed that at low template concentration (in other words < 2 logs of pathogen concentration or 200 fg/ μ l) the E_o values of the standards were statistically different (fig. 4d). Most of the deviation between the E_o for each of the standards and their expected value (all of them equal) appeared to occur inside a range comprising the lowest template concentrations tested (data not shown), perhaps a reflection of the inability of the assay to reliably detect the presence of *P. ramorum* templates at such low concentration (detection limits).

Removing the lowest template values that were statistically different from the other higher values and comparing the relationship between E_o values and their corresponding log of template concentration with linear correlation, resulted in higher r^2 values and regression slopes closer to the expected value of 0 (E_o of equal value, fig 5). This statistical approach allowed the placing of the detection limits of the assay at the minimum standard template concentration, which would still maintain statistically equal E_o values. Using confidence interval values established for the E_o linear regression, it is possible to predict whether a field sample, after calculating its E_o , will behave kinetically like the model curve.

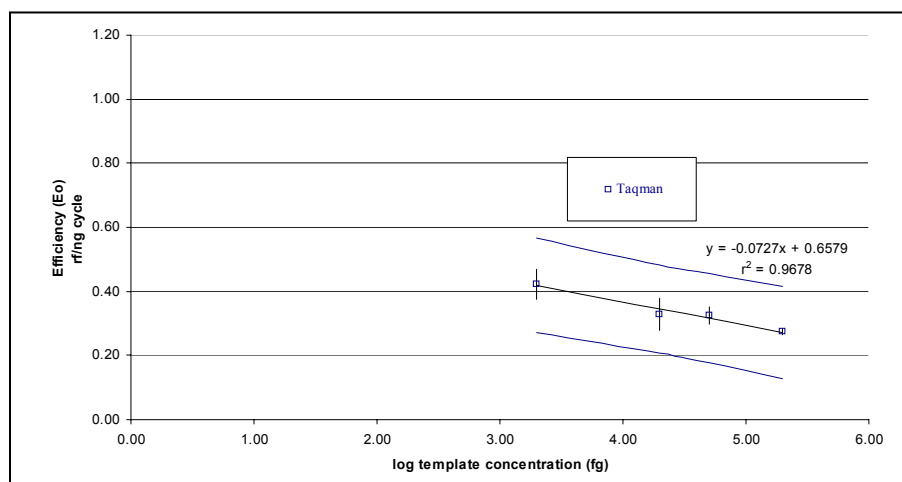


Figure 5—Amended linear regression analysis of sigmoid curve parameter E_o discarding data points shown to be statistically different in repeated real time PCR reactions from data points of greater concentrations using standard dilutions of *P. ramorum* templates. The plot also includes the 95 percent confidence intervals for analysis of suspected samples.

Using this statistically supported method it was possible to test the detection limits of the CoxI-CoxII marker system in TaqMan® real time PCR amplifications of independently determined *P. ramorum*-positive samples that were serially diluted with non-infected host tissues of equal concentration. We intentionally used this marker system instead of the USDA-APHIS approved, ITS marker system to show the usefulness and complementarity of other diagnostic systems in helping with the diagnosis of *P. ramorum*. We foresee that this methodology could be applied to any real time marker system or diagnostic technique based on real time PCR. Experiments are underway to test the system under very high template concentrations, where cross-amplification of other closely related species is observed or during mixed infections, when the interference of other DNA species can be expected. Further validation of the model is being carried out with blind samples from the field, that were independently classified as positive or negative for the presence of *P. ramorum* using the USDA-APHIS validated rDNA ITS-based PCR detection assay.

Literature Cited

- Rutledge, R.E. 2004.** Sigmoidal curve-fitting redefines quantitative real-time PCR with the prospective of developing automated high-throughput applications. *Nucleic Acids Research*. 32: e178. doi:10.1093/nar/gnh177.
- Tichopad, A.; Dzidic, A.; Pfaffl, M.W. 2002.** Improving quantitative real-time RT-PCR reproducibility by boosting primer-linked amplification efficiency. *Biotechnology Letters*. 24: 2053–2056.
- Tooley, P.W.; Martin, F.N.; Carras, M.M.; Frederick, R.D. 2006.** Real-time fluorescent PCR detection of *Phytophthora ramorum* and *Phytophthora pseudosyringae* using mitochondrial gene regions. *Phytopathology*. 96: 336–345.
- USDA Animal and Plant Health Inspection Service. 2004.** Invasive species and pest management programs (ISPM), USDA-APHIS PPQ approved laboratory diagnostic protocols, nested PCR protocol (revised August 2004). Published online. http://www.aphis.usda.gov/ppq/ispm/pramorum/pdf_files/pcrprotocol4.pdf.