

Document Control Number WI-B-T-1-7	WORK INSTRUCTION USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Quarantine Laboratory, Bldg 580, BARC-East, Beltsville, MD 20705	Revision Number Original
Effective Date: June 20, 2008	<u>Elicitin</u> Quantitative Multiplex Real-Time PCR (qPCR) for Detection of <i>Phytophthora ramorum</i> Using a TaqMan System on the Cepheid SmartCycler®.	Page 1 of 10

The purpose of this work instruction is to describe all activities concerning the detection of *Phytophthora ramorum* using the Elicitin gene, quantitative multiplex real-time TaqMan PCR assay on the Cepheid SmartCycler® II.

Please note, to make a diagnostic determination based on real-time PCR, all samples tested using this assay must also be tested using the ITS-target *P. ramorum* qPCR work instruction (WI-B-T-1-6). Elicitin qPCR test results alone are insufficient for diagnostic determinations.

I. Related Work Instructions

WI-B-T-1-6 (*ITS*) Quantitative Multiplex Real-time PCR (qPCR) for Detection of *Phytophthora ramorum* Using a TaqMan System on the Cepheid SmartCycler® and the ABI 7900/7000

WI-B-T-2-3 Extraction of DNA from Leaf Sub-samples for *Phytophthora spp.* PCR analysis

II. Introduction

This quantitative PCR (qPCR) assay provides an additional diagnostic tool for use in the APHIS-PPQ *Phytophthora ramorum* program in combination with the ITS-target, real-time PCR assay currently used (WI-B-T-1-6) for making *P. ramorum* diagnostic determinations.

This new assay targets the Elicitin genomic loci of *P. ramorum*. We multiplexed the *P. ramorum* Elicitin-specific TaqMan assay developed by Bilodeau et al. (2007) from the Canadian Forest Service (Natural Resources Canada, Forest Pest Biotechnology Laboratory), with the primers and probe used in the TaqMan assay developed by Hughes et al. (2006) from the Central Science Laboratory (Plant Health Group, UK) that targets the 5.8S region of the nuclear ribosomal (nr) RNA gene from known *Phytophthora* spp. Both assays were modified for use on a Cepheid SmartCycler® and validated for making diagnostic determinations. The *Phytophthora* spp. 5.8S internal control reaction serves to demonstrate that amplifiable DNA of *Phytophthora* spp. was extracted from the sample being tested and is different from the internal control in the ITS qPCR which is plant based.

The qPCR described in this work instruction uses DNA prepared following WI-B-T-2-3, using the Qiagen DNeasy® Plant Mini Kit. *P. ramorum* can be detected with this qPCR in a broad range of host plants over the range of detection (the interval between the upper and the lower concentration of the pathogen in environmental samples from which the agent can be reliably and reproducibly detected) greater than 50fg of genomic DNA.

Note: *This assay uses undiluted total DNA in comparison to the ITS qPCR that uses DNA at a 1:10 dilution.*

Printed copies of this document are considered for reference only. It is the responsibility of employees to ensure that they are using the correct revision of this document by checking the document revision level with that held on the I drive, folder ISO.

Document Control Number WI-B-T-1-7	WORK INSTRUCTION USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Quarantine Laboratory, Bldg 580, BARC-East, Beltsville, MD 20705	Revision Number Original
Effective Date: June 20, 2008	<u>Elicitin</u> Quantitative Multiplex Real-Time PCR (qPCR) for Detection of <i>Phytophthora ramorum</i> Using a TaqMan System on the Cepheid SmartCycler®.	Page 2 of 10

III. Equipment, Reagents and Materials

A. Equipment

1. PCR clean workstation or PCR enclosure with UV light (any vendor)
2. Cepheid SmartCycler® II (version 2)
3. Cepheid mini-centrifuge (Cepheid # 900-0020)
4. Vortex (any vendor)
5. Micro-centrifuge, bench top, capable of $\geq 10,000$ rpm (any vendor)
6. Dedicated, calibrated pipettors (P10, P50, P200)

B. Reagents and Materials

1. SmartCycler® reaction tubes, 25 μ l (Cepheid #900-0022)
2. Cepheid cooling block (Cepheid #900-0028)
3. Molecular Biology Grade (MG) Water (any vendor)
4. Platinum Taq DNA Polymerase (Invitrogen #10966-034) *Note: Platinum Taq is supplied in a set with 10X PCR Buffer and 50mM MgCl₂*
5. dNTP Mix, 10 mM solution, PCR reagent (Sigma # D7295)
6. Sterile filter (barrier) pipette tips (P10, P50, P200) (any vendor)
7. Amber-colored 1.5 ml Flex tubes (e.g.: Eppendorf # 22-36-351-4)
8. Micro-centrifuge tubes, 1.5-1.7 ml, (pre-sterilized, certified DNase & RNase free, any vendor)
9. Tube openers (any vendor,) *see note below on use*

Note: All flip-cap microcentrifuge tubes are to be opened using a decontaminated tube opener. Tube openers and reusable tube holders/racks are decontaminated by soaking 30 min to 2 hours in 10% bleach solution, followed by two rinses in diH₂O to remove bleach.

IV. Primers and Probe Preparation

Note: Primer and probe stock and working concentrations do not need to be prepared each time you conduct the PCR assay. They are generally prepared upon receipt of new reagents. Primer and probe solutions are stored in small working aliquots in a -20°C freezer until needed, and used no longer than 12 months from date of preparation. When an assay is being conducted, the analyst should use one 'working' tube of each primer mix and probe at a time to avoid contamination and the detrimental effects caused by repeated freeze/thaw of reagents.

Printed copies of this document are considered for reference only. It is the responsibility of employees to ensure that they are using the correct revision of this document by checking the document revision level with that held on the I drive, folder ISO.

Document Control Number WI-B-T-1-7	WORK INSTRUCTION USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Quarantine Laboratory, Bldg 580, BARC-East, Beltsville, MD 20705	Revision Number Original
Effective Date: June 20, 2008	<u>Elicitin</u> Quantitative Multiplex Real-Time PCR (qPCR) for Detection of <i>Phytophthora ramorum</i> Using a TaqMan System on the Cepheid SmartCycler®.	Page 3 of 10

Table 1. Primers

Primer Name	Primer Sequence (synthesized by Integrated DNA Technologies, Inc.) (Purification - Standard Desalting)
<i>P. ramorum</i> (Pram) Elicitin primers:	
Prameli-102U-F	5'-TTCAACCAGTGCGCGACC-3'
Prameli-259L-R	5'-GGCACAGTCAGCTCGCAGTC-3'
<i>Phytophthora 5.8S</i> internal control (IC) primers:	
5.8S-F	5'-TGTCTAGGCTCGCACATCGA-3'
5.8S-R	5'-GATGACTCACTGAATCCTGCAATT-3'

Table 2. TaqMan probes*

Probe name	Probe sequence (synthesized by Integrated DNA Technologies, Inc.) (HPLC purification for FAM/BHQ-1 and Dual-HPLC purification for TET/BHQ-1)
Pram Elicitin probe Taq195eli-R	5'-6-FAM-(CCGTGGACGCGCACATGAGCGAGTAC) BHQ-1-3'
<i>Phytophthora 5.8S</i> internal control (IC) probe	5'-TET-(ACGCTGCGAACTGCGATAATGC) BHQ-1-3'

***Note:** Probe may take 14-30 days from order to delivery.

- Concentrated Freezer stock solutions (100 μ M) of the probes and primers
Dissolve primers and probes in MG water (or 1x TE buffer) for a final concentration of 100 μ M and store at -20°C. Store concentrated freezer stocks of probes in amber-colored microcentrifuge tubes.
- Working solutions of primer mixes
Prepare a **4 μ M** working solution of the *Prameli* (**102U-F/259L-R**) primer pair mixture and a **1 μ M** primer pair mixture for the plant internal control 5.8S (**5.8S-F/R**) primer pair mixture. Blend each primer mixture well by vortexing and aliquot in 100 μ l volumes. Store at -20°C.
Prameli (102U-F/259L-R): to make 1 ml of a **4 μ M** mix of primers 102U-F/259L-R, add 40 μ l of the 100 μ M 102U-F primer stock and 40 μ l of the 100 μ M 259L-R primer stock to a 1.5 ml microfuge tube and bring up to 1 ml with 920 μ l of sterile molecular grade water. Mix and aliquot.
5.8S Internal Control (5.8S-F/R): To make 1 ml of a **1 μ M** mix of primers 5.8S-F/R, add 10 μ l of the 100 μ M 5.8S-F primer stock and 10 μ l of the 100 μ M 5.8S-R primer stock to a 1.5 ml microfuge tube and bring up to 1 ml with 980 μ l of sterile molecular grade water. Mix and aliquot.

Printed copies of this document are considered for reference only. It is the responsibility of employees to ensure that they are using the correct revision of this document by checking the document revision level with that held on the I drive, folder ISO.

Document Control Number WI-B-T-1-7	WORK INSTRUCTION USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Quarantine Laboratory, Bldg 580, BARC-East, Beltsville, MD 20705	Revision Number Original
Effective Date: June 20, 2008	<u>Elicitin</u> Quantitative Multiplex Real-Time PCR (qPCR) for Detection of <i>Phytophthora ramorum</i> Using a TaqMan System on the Cepheid SmartCycler®.	Page 4 of 10

- Working solutions of probes

Prepare 2 μ M solutions of each probe in molecular grade water or 1x TE buffer by diluting the 100 μ M probe concentrated freezer stock solution. Store small aliquots (i.e.: \leq 100 μ l) of probes at -20°C in 1.5 ml amber-colored microfuge tubes to protect the probe from degradation due to light exposure. (Small aliquots, suitable for single day use, of probe are desirable since probes are sensitive to freeze/thaw cycles.)

V. Preparation

1. According to WI-B-T-2-3, sample DNA extractions are either held on ice for immediate testing or stored frozen at -20°C until testing can commence. If frozen, thaw DNA samples briefly (several minutes) then vortex 5-10 seconds at a speed setting of 7-10. Briefly centrifuge the tubes 10-20 seconds at \geq 10,000 rpm to settle the liquid at the bottom of the tube. Place in ice.
2. Thaw the primers, probe, and all other reagents (*except Taq*) at room temperature.
3. When thawed, vortex for 10-15 sec at setting 7-10, then centrifuge 10-20 seconds at \geq 10,000 rpm to settle the liquid to the bottom of the tube. Place in ice.
4. *Taq* Polymerase should be kept on ice all the time. We do not recommend vortexing the enzyme but gently mixing it by flicking the tube prior to use.
5. Remove the Cepheid cooling block from the 4 °C refrigerator just prior to Master Mix preparation.

VI. Master Mix Preparation

Master Mix preparation and aliquoting must be done in a decontaminated PCR workstation /enclosure, on a new disposable paper mat.

1. Prepare the Master Mix (Table 3) for the number of the samples plus 1 positive (DNA) control and 1 non-template control (NTC) reactions. Prepare 1 extra reaction for every 10 reactions needed. For example, for 20 reactions prepare the Master Mix for 22 reactions.

Printed copies of this document are considered for reference only. It is the responsibility of employees to ensure that they are using the correct revision of this document by checking the document revision level with that held on the I drive, folder ISO.

Document Control Number WI-B-T-1-7	WORK INSTRUCTION USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Quarantine Laboratory, Bldg 580, BARC-East, Beltsville, MD 20705	Revision Number Original
Effective Date: June 20, 2008	<u>Elicitin</u> Quantitative Multiplex Real-Time PCR (qPCR) for Detection of <i>Phytophthora ramorum</i> Using a TaqMan System on the Cepheid SmartCycler®.	Page 5 of 10

Table 3. Master Mix for Elicitin-target Real-time PCR assay

Reagents	Volume (µl) for each reaction	Final concentration
10X PCR Buffer*	2.5	1x
MgCl ₂ (50 mM)*	2.0	4.0mM
dNTP mix (10 mM each)	0.5	200µM each
Primers Pram (4 µM mix)	2.5	0.4 µM each
Probe Pram (2 µM)	2.5	0.2 µM
Primers IC (1 µM mix)	2.5	0.1 µM each
Probe IC (2 µM)	2.5	0.2 µM
Platinum <i>Taq</i> (5U/µl)	0.2	1 Unit
Molecular grade water	7.8	-
Total	23.0	-

*The 10X buffer and MgCl₂ are supplied with the Platinum *Taq*.

2. Blend Master Mix well by pipetting gently up and down several times and keep on ice.
3. Pipette 23.0µl of the Master Mix into each Cepheid tube. (Label the caps either before or after pipetting the Master Mix.)
4. Place SmartCycler® tubes (25µl) into the Cepheid cooling block.

VII. Addition of Sample or Control DNA

1. Take the Cepheid rack with tubes containing Master Mix to the PCR thermocycler station and place all items on a new disposable paper mat.
2. Add **2.0µl** of each sample DNA (**undiluted**) or control to the appropriate tube and close the tubes. Total reaction volume should be 25µl.

Note 1: Each diagnostic run of this *P. ramorum* qPCR must include one NTC (containing either TE buffer or MG water) and one *P. ramorum* positive control, either a *P. ramorum*-positive environmental sample or *P. ramorum* culture, that produces a FAM Ct in the range of 23-28 and is aliquotted into small units and stored at -20°C to preserve integrity.

Note 2: Do not use 25 µl of master mix as your NTC. The NTC is used to indicate contamination of master mix reagents or contamination introduced by the analyst during

Printed copies of this document are considered for reference only. It is the responsibility of employees to ensure that they are using the correct revision of this document by checking the document revision level with that held on the I drive, folder ISO.

Document Control Number WI-B-T-1-7	WORK INSTRUCTION USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Quarantine Laboratory, Bldg 580, BARC-East, Beltsville, MD 20705	Revision Number Original
Effective Date: June 20, 2008	Elicitin Quantitative Multiplex Real-Time PCR (qPCR) for Detection of <i>Phytophthora ramorum</i> Using a TaqMan System on the Cepheid SmartCycler®.	Page 6 of 10

sample addition so it should be prepared in a similar manner including the addition of 2 µl of MG water instead of DNA.

VIII. PCR set-up

A. Thermocycler settings

Verify the following settings on the SmartCycler® II prior to use:

Step1: 95C, 120 seconds, Optics Off – One Cycle

Step2: 94C, 15 seconds, Optics Off – 45 Cycles
 68C, 60 seconds, Optics On

Use Cepheid default temperature ramping rate. (Default ramping rate, according to Cepheid, is “as fast as it can go” which is dependent on the ambient air temperature.)

B. Starting the run

1. **Turn on the SmartCycler® Block first, followed by the SmartCycler® software. If not done in this order the machine will give an error message.**
2. Centrifuge the Cepheid tubes in the Cepheid centrifuge for 10-15 seconds. Be sure no bubbles remain on the sides of the flat diamond-shaped area of the Cepheid tube. Place the tubes in the SmartCycler® in the appropriate I-core modules.
3. To begin amplification: (instructions contained in the SmartCycler® manual)
 - i. Select “Create Run”
 - ii. Give the run a name (i.e.: date and protocol)
 - iii. Select dye set (for this qPCR it is “FTTC25”)
 - iv. Select “add/Remove Sites” to select the protocol
 - v. Select the sites with the samples and click on ► symbol to add sites to the right column, and click on OKAY.
 - vi. Select “Start Run” found in the lower left-hand corner of the screen.
 (Steps i. through v. can be completed prior to Master Mix set-up)

Printed copies of this document are considered for reference only. It is the responsibility of employees to ensure that they are using the correct revision of this document by checking the document revision level with that held on the I drive, folder ISO.

Document Control Number WI-B-T-1-7	WORK INSTRUCTION USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Quarantine Laboratory, Bldg 580, BARC-East, Beltsville, MD 20705	Revision Number Original
Effective Date: June 20, 2008	<u>Elicitin</u> Quantitative Multiplex Real-Time PCR (qPCR) for Detection of <i>Phytophthora ramorum</i> Using a TaqMan System on the Cepheid SmartCycler®.	Page 7 of 10

IX. Assessment of the *P. ramorum* qPCR using the SmartCycler® software Version 2

Analyze data from the run as per the thermocycler Manufacturer's instructions.

A. Evaluate all control (positive, non-template and internal) reaction sample results and determine that they are valid before determining sample results.

B. Internal Control (TET) Cts

The entire run is invalid if the positive control DNA has an internal control $0 \leq \text{TET Ct} < 13$ or is 3 Cts greater than what you expect for a positive control. No sample results obtained in the run may be used and all samples must be retested using this work instruction.

The non-template control (NTC) has to have an internal control TET Ct = 0 at the end of 45 cycles or the entire run is invalid. No sample results obtained in the run may be used and all samples must be retested using this work instruction.

If the test sample has an internal control $0 \leq \text{TET Ct} < 13$, then that specific sample needs to be retested.

If the sample TET retest is again $0 \leq \text{TET Ct} < 13$ then the sample has failed quality control and no amplifiable *Phytophthora* DNA is present in that sample. A new DNA extraction should be prepared and tested.

C. Positive and Non-template control (FAM) Cts

If either

- the positive control DNA (environmental or pure culture DNA) has a $0 \leq \text{FAM Ct} < 13$ or is 3 Cts greater than expected for your positive control
- or
- the non-template control (MG water or TE buffer only) has a FAM Ct > 0,

then the entire run is invalid. No sample determinations can be made and all samples and controls must be retested using this work instruction.

Printed copies of this document are considered for reference only. It is the responsibility of employees to ensure that they are using the correct revision of this document by checking the document revision level with that held on the I drive, folder ISO.

Document Control Number WI-B-T-1-7	WORK INSTRUCTION USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Quarantine Laboratory, Bldg 580, BARC-East, Beltsville, MD 20705	Revision Number Original
Effective Date: June 20, 2008	<u>Elicitin</u> Quantitative Multiplex Real-Time PCR (qPCR) for Detection of <i>Phytophthora ramorum</i> Using a TaqMan System on the Cepheid SmartCycler®.	Page 8 of 10

After the retest:

- If the NTC FAM Ct is again invalid (FAM Ct >0) then prepare a new set of reagents or probes/primers and test them with a set of controls before testing the samples again.
- If the positive control FAM Ct is again invalid ($0 \leq \text{FAM Ct} < 13$) then prepare a new positive control as per Note 1 (Page 5) of this work instruction before testing the samples again.

D. Assessment of Sample (FAM) Cts

Reminder: Evaluation of sample FAM Cts cannot occur until you have first assessed the control reaction results (Sections B and C above) and determined them to be valid.

Reminder: Combined results from this work instruction and the ITS-target *P. ramorum* qPCR work instruction (WI-B-T-1-6) are required before final sample determinations can be made. Elicitin-target qPCR test results alone do not provide all the results required for final sample determinations.

1. Samples that test positive by this assay

A sample is determined to test positive for *P. ramorum* by this Elicitin qPCR assay if it produces a FAM Ct value between $13 < \text{FAM Ct} \leq 39$ **and** the positive and non-template controls give acceptable results, and the internal control for the sample is valid.

Samples which test positive for *P. ramorum* with this work instruction must also be tested with the validated ITS-target *P. ramorum* qPCR work instruction (WI-B-T-1-6) before a diagnostic determination can be made.

2. Samples that test negative by this assay

Samples that have a FAM Ct = 0 or >43 in this Elicitin qPCR assay cannot be determined to be negative until they also test negative using the more sensitive ITS-target qPCR (WI-B-T-1-6).

3. Inconclusive samples by this assay

Samples that have a FAM Ct in the range of 40 to 43 should be retested with this work instruction **and/or** tested undiluted **and** 1:10 with the ITS-target *P. ramorum* qPCR work instruction (WI-B-T-1-6).

Printed copies of this document are considered for reference only. It is the responsibility of employees to ensure that they are using the correct revision of this document by checking the document revision level with that held on the I drive, folder ISO.

Document Control Number WI-B-T-1-7	WORK INSTRUCTION USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Quarantine Laboratory, Bldg 580, BARC-East, Beltsville, MD 20705	Revision Number Original
Effective Date: June 20, 2008	<u>Elicitin</u> Quantitative Multiplex Real-Time PCR (qPCR) for Detection of <i>Phytophthora ramorum</i> Using a TaqMan System on the Cepheid SmartCycler®.	Page 9 of 10

Important Note about use of this Elicitin qPCR assay for diagnostic determinations: Under conditions tested, this assay appears to be approximately 10-20 fold less sensitive for *P. ramorum* (detection limit 50-100fg target DNA) than the ITS-target qPCR assay for *P. ramorum* (detection limit <5fg target DNA). Because of this decreased sensitivity for target DNA of *P. ramorum*, this Elicitin-targeting *P. ramorum* assay cannot be used as a stand-alone diagnostic assay. If you choose to use this assay it must be used together with the more-sensitive, ITS-target qPCR (see WI-B-T-1-6). Cycle thresholds (Cts) with this assay are approximately 6-7 cycles higher, compared to the ITS-target qPCR assay using DNA at the same concentration.

X. Samples Requiring Federal Confirmation

A **Potentially Actionable Suspect Sample (PASS)** is a sample from a potential new location, a putative new host, a previously certified nursery, a geographically distinct/unique environmental find, or other non-routine situation. For more information, read the PASS policy for SOD at http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/passpolicy4-06.pdf

A PASS sample must be forwarded to a federal lab for confirmation. Samples should be submitted by overnight delivery and must be accompanied by (1) a PPQ Form 391 (Sample Submission Form), and (2) the Ct values for each run. Prior to sending please notify Dr. Palm by e-mail (Mary.Palm@aphis.usda.gov) with the tracking number and estimated delivery date using the shipping address below:

USDA-APHIS-PPQ-PHP-MDL, BARC-East, Bldg 580, Powder Mill Rd, Beltsville, MD 20705
Phone 301-504-5700

References

- Hughes, K. J. D., Tomlinson, J. A., Griffin, R. L., Boonham, N., Inman, A. J., and Lane, C. R. 2006. Development of a one-step real-time polymerase chain reaction assay for diagnosis of *Phytophthora ramorum*. *Phytopathology* 96:975-981.
- Bilodeau, G. J., Levesque, C.A., de Cock, A.W.A.M., Duchaine, C., Briere, S., Uribe, P., Martin, F.N., and Hamelin, R. C. 2007. Molecular detection of *Phytophthora ramorum* by real-time polymerase chain reaction using TaqMan, SYBR Green, and molecular beacons. *Phytopathology* 97:632-642.

Printed copies of this document are considered for reference only. It is the responsibility of employees to ensure that they are using the correct revision of this document by checking the document revision level with that held on the I drive, folder ISO.

Document Control Number WI-B-T-1-7	WORK INSTRUCTION USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Quarantine Laboratory, Bldg 580, BARC-East, Beltsville, MD 20705	Revision Number Original
Effective Date: June 20, 2008	Elicitin Quantitative Multiplex Real-Time PCR (qPCR) for Detection of <i>Phytophthora ramorum</i> Using a TaqMan System on the Cepheid SmartCycler®.	Page 10 of 10

3. SmartCycler® II Operator Manual Copyright 1999-2002 by Cepheid.

Document Revision History

Status (Original/Revision/C ancelled)	Document Revision Number	Effective Date	Description
Original	Original	6-20-2008	Elicitin quantitative real-time PCR qPCR for <i>P. ramorum</i>

Approved: Signature on File with Original Document

Date: 6-20-2008

Approved By: Renee M. DeVries, NPGBL Quality Manager

Approved By: Laurene Levy, NPGBL Technical Manager

Printed copies of this document are considered for reference only. It is the responsibility of employees to ensure that they are using the correct revision of this document by checking the document revision level with that held on the I drive, folder ISO.