Quantitative PCR for detection of *Phytophthora* genus and species-specific: *P. sojae – P. sansomeana*

I. Reagents, primers and probes

A. Reagents

- RealMaster Mix Probe without Rox (5 Prime; VWR, Cat No. 10052-276)
  - Includes magnesium acetate 25 mM
- Molecular grade water
- Microcentrifuge amber-colored tubes
- Optical film or optical tubes for qPCR
- Plate or tube strips compatible with the qPCR platform

B. Primers

Primer	Sequence (5' - 3')	Length (bp)	Target
PhyG_ATP9_2FTail	AATAAATCATAACCTTCTTTACAACAAGAATTAATG	36	*Phytophthora*
PhyG-R6_Tail	AATAAATCATAAATACATAATTCATTTTTATA	32	*Phytophthora*
FMPI2b	GCGTGGACCTGGAATGACTA	20	Plant IC
FMPI3b	AGGTTGTATTAAAGTTTCGATCG	23	Plant IC

C. Probes

Probes	Sequence (5' - 3')	Length (bp)	Supplier
Phytophthora genus-specific TaqMan probe	[FAM] AAAGCCATC [ZEN] ATTAAACARAATAAAGC [IABkFQ]	26	IDT
P. sojae species-specific TaqMan probe	[HEX] TTGATATAT [ZEN] GAATACAAAGATAGATTTAAGTAAAT [IABkFQ]	35	IDT
P. sansomeana species-specific TaqMan probe	[Quasar670] TATTAGTACTAAYTACTAATATGCATTATTTTTAG [BHQ-2]	35	Biosearch
Plant-IC probe	[CalFluorRed610] CTTTTATTATCACTTCCGGTACTGGCAGG [BHQ-2]	29	Biosearch
Soil-IC (PPF)	[CalFluorRed610] AAAGTAAGCTTATCGATACCGTCGACCT [BHQ-2]	28	Biosearch

* IDT: Integrated DNA Technologies, Inc. (https://www.idtdna.com/)

* Biosearch: Biosearch technologies, Inc. (https://www.biosearchtech.com/)

II. Preparation of probes and primers

1. Briefly centrifuge the tubes with primers and probes for 20 secs at 10,000 rpm before opening the tubes.

2. **100 µM stock solutions:** dissolve primers and probes by adding molecular grade water for a final concentration of 100 µM. Store probes in amber-colored tubes at -20ºC or -80ºC if possible.

3. **10 µM working solutions:** Prepare working solutions by making a ten-fold dilution from the stock solutions using molecular grade water and store 100 µL aliquots at -20ºC or -80ºC if possible. Use amber-colored tubes for probes.
    a. For example, to make 500 µL of primer working stock, dilute 50 µL of the primer/probe and dissolve in 450 µL of molecular grade water.

4. **Plant Internal Control 1 µM working stocks:** the plant internal control for amplification is required to be at a lower concentration than the rest of the working stocks, hence a 100-fold dilution from the stock solution should be made using molecular grade water. Store 100 µL aliquots at -20ºC or -80ºC, using amber-colored tubes for probes.
    a. For example, to make 500 µL of plant internal control, dilute 5 µL of plant internal control primer/probe and dissolve in 495 µL of molecular grade water.

III. Master mix preparation

1. Thaw primers, probe, RealMaster mix and magnesium acetate. Vortex primers, probe and magnesium acetate (Mg++) briefly for 10 sec at setting 7, then spin for 10 sec in a microspin centrifuge. Place all the reagents on ice, do not vortex the RealMaster mix; mix it by flicking the tube.

2. Prepare the master mix in a clean area; make the mix volume using the table below, adding 5% extra reactions to account for pipetting errors. Keep master mix on ice before use.

A. Plant samples

Reagents	Initial Concentration	Plant samples - Volume per reaction (µL)		
		1X	Rxn X	Check
Primers				
PhyG_ATP9_2FTail	10 µM	1.0		
PhyG-R6_Tail	10 µM	1.0		
Probes				
Phytophthora genus-specific TaqMan probe	10 µM	0.05		
P. sojae species-specific TaqMan probe	10 µM	0.2		
P. sansomeana species-specific TaqMan probe	10 µM	0.1		
Plant Internal Control				
FMPI2b	1 µM	0.4		
FMPI3b	1 µM	0.4		
Plant-IC probe	1 µM	0.4		
Real Master Mix without Rox (5 Prime)	2.5X	8.0		
Mg^{++}	25 mM	2.0		
PCR-grade water		4.45		
Total volume		**18 µL**		

B. Soil samples

Reagents	Initial Concentration	Soil samples - volume per reaction (µL)		
		1X	Rxn X	Check
Primers				
PhyG_ATP9_2FTail	10 µM	1.0		
PhyG-R6_Tail	10 µM	1.0		
Probes				
Phytophthora genus-specific TaqMan probe	10 µM	0.05		
P. sojae species-specific TaqMan probe	10 µM	0.2		
P. sansomeana species-specific TaqMan probe	10 µM	0.1		
Internal Control (Soil)				
PPF	10 µM	0.2		
Internal Control		1.0		
Real Master Mix without Rox (5 Prime)	2.5X	8.0		
Mg^{++}	25 mM	2.0		
PCR-grade water		4.45		
Total volume		**18 µL**		

3. Mix the master mix by inverting tube up and down and spinning it down in a microspin centrifuge for 10 sec. Aliquot 18 µL into each well or tube, keep tubes or plates on ice.

4. Add 2 µL of sample DNA or 2 µL of molecular grade water for the NTC control.
    a. Include 10 pg, 1pg and 100 fg of *Phytophthora sojae* or *Phytophthora sansomeana* as positive controls in each assay.
    b. The final volume for each reaction after adding DNA should be 20 µL.

5. Seal the plates or close the tubes and spin down the reaction mix before setting the samples on the platform and keep samples on ice.

IV. qPCR cycling conditions

- Verify the following conditions on the real-time PCR platform:
    1. Stage 1: Denature at 95ºC for 2 min
    2. Stage 2: Repeat 45 cycles of:
        a. 95ºC for 15 sec
        b. 57ºC for 90 sec

- Depending on the real-time PCR platform select the appropriate channel configuration:
    - SmartCycler: dye set "FTTC25"
    - StepOne Plus: FAM, VIC, ROX
        - In this case uncheck the ROX as the background fluorescence
        - *P. sansomeana* probe will not work on this platform, so it has to be run in an independent run labeling the probe with HEX.
    - CFX96 or iQ5 systems: FAM, HEX, CalFluor610 and Quasar670

V. Result interpretation

A plant sample should have an internal control with a Ct value in the range of 13 to 30, otherwise samples should be repeated and possibly include a 1:10 diluted sample to improve amplification and help rule out PCR inhibition issues or too high of a concentration of DNA.

Target	**Label (Dye)**	**Expected range**
Phytophthora genus	FAM	Positive (Ct < 28)
Phytophthora sojae	HEX	Positive (Ct < 31)
Phytophthora sansomeana	Quasar670	Positive (Ct < 31)
Plant Internal Control	CalFluorRed 610	Ct ranging 13 - 30

VI. References

Bilodeau, G. J., Martin, F. N., Coffey, M. D. & Blomquist, C. L. Development of a multiplex assay for genus- and species-specific detection of Phytophthora based on differences in mitochondrial gene order. Phytopathology 104, 733–748 (2014).

Bilodeau, G. J., Koike, S. T., Uribe, P. & Martin, F. N. Development of an Assay for Rapid Detection and Quantification of Verticillium dahliaein Soil. Phytopathology 102, 331–343 (2012).