Real-time qPCR assay for the confirmation of *F. virguliforme*

DNA extraction from fresh soybean root samples:

1. Wash collected root samples under tap water to remove attached soil
2. Taproots and lateral roots with obvious lesions or discoloration are preferred for DNA extraction. Cut roots samples into small pieces (less than 1 cm each)
3. Grind root tissue in a purple screw-cap tube (MPBio, Cat: #5076-200, Cat: 5064-002) with one 6-mm diameter glass bead, five 2-mm diameter glass beads, and 400 µL AP1 lysis buffer (Qiagen DNeasy Plant mini Kit, cat. #69104) for 40 s at speed 6 in a FastPrep bead-beater machine.
4. Incubate the tube at 68˚C for 10 min, vortex the tube two to three times during incubation
5. Follow the manufacturer's instructions, except:
    a. Elute DNA with 100 µL TE buffer
6. The eluted DNA can be used for downstream PCR work directly or store at -20˚C.

DNA extraction from dried whole soybean root samples:

1. Dry roots at 50˚C for 48h in a convection oven
2. Grind two or more roots in a Wiley mill with a 2mm sieve
   Weigh 100 mg of root tissue for DNA extraction with FASTDNA SPIN kit (MPBIO, Catalog number: 116540600), following manufacturer's instruction, except:
    a. Lysis buffer CLS-Y is recommended for root tissue lysis
    b. Elute DNA with 100 µL DES buffer
3. Dilute DNA 10-fold with sterilize molecular grade H_2O

Important Notes:

We strongly recommend at least duplicate DNA extractions be performed for diagnoses of soybean root samples due to the spatial heterogeneity of *F. virguliforme* colonization in soybean roots.

DNA extraction from pure cultures:

1. Inoculate seven to eight plugs of mycelia or macro conidia from a *Fusarium* colony grown on potato dextrose agar (PDA) into a 150-mL Erlenmeyer flask containing 50 mL potato dextrose broth (PDB); incubate the still liquid culture at room temperature for 7-10 d.
2. Collect mycelia with a vacuum pump on a Buchner funnel covered with miracloth (Calbiochem), transfer the mycelia into an eppendorf tube, cover the opening of the tube with a small piece of miracloth and secured with a rubber band.
3. Freeze the mycelia at -20ºC overnight and lyophilize the mycelia for 24 h
4. Weigh 50 mg of lyophilized mycelia (fill close to the 100 µL line of a 1.5-mL eppendorf tube), grind lyophilized mycelia with a metal spatula. Extract DNA following the Qiagen Plant DNeasy Mini kit instructions

Real-time qPCR Protocol

1. Preparations before running qPCR assay (Wang et al., 2015)
   - Reagents and materials
     i. TaqMan Universal PCR Master Mix Cat #: 4304437 (Life Technologies, Carlsbad, CA)
     ii. All Primers (Sigma-Aldrich) (**Table 1**)
     iii. Dual labeled MGB Probe-3 (Life Technologies, Carlsbad, CA)
     iv. HHIC probe (IDT, Coralville, IA) and plasmid*
     v. Bovine serum albumin (BSA) 100X (NEB, Ipswich, MA)
     vi. Distilled water Cat #: 10977-015 (Gibco, Grand Island, NY)
   - Serially diluted *F. virguliforme* genomic DNA for standard curve
     i. Recommended 8 dilution levels: 5 ng/µL ~ 0.5 fg/µL with 1:10 dilution factor
   - Ref to Haudenshield and Hartman (2011) for internal control (IC) plasmid preparation
   - An IC included in the qPCR assay is to increase negative call veracity in routine diagnostics
   - Measuring the concentration of DNA samples
     i. either using NanoDrop spectrophotometer
     ii. or fluorophotometer (using Quant-iT dsDNA HS kit)
   - Dilute primer and probes to the stock concentration as Table 2
   - Dilute linearized HHIC DNA into stock concentration as Table 2
   - Dilute root or soil total DNA as needed, recommended dilution levels
     i. Soil total DNA: 1:10 and 1:100
     ii. Plant root total DNA: 1:1 and 1:10

Table 1 Primers and probes sequences

Names	Sequences (5'-3')	Length(nt)	Target
F6-3	GTAAGTGAGATTTAGTCTAGGGTAGGTGAC	30	
R6	GGGACCACCTACCCTACACCTACT	24	*F. virguliforme*
FvPrb-3	6FAM-TTTGGTCTAGGGTAGGCCG-MGBNFQ	19	
IC-F	CTAGGACGAGAACTCCCACAT	21	
IC-R	CAATCAGCGGGTGTTTCA	18	HHIC
IC-prb	5HEX-TCGGTGTTGATGTTTGCCATGGT-3IABkFQ	23	

2. Reagents and materials used in the following assay

   Thaw the following PCR reagents on ice:
   - Primers (F6-3, R6, IC-F, and IC-R)
   - Probes (Probe-3 and IC-Prb)
   - Linearized IC plasmid
   - Serially diluted *F. virguliforme* genomic DNA
   - BSA (100X)
   - DNA samples for testing

   Briefly vortex reagents to homogenize thoroughly

3. PCR master mix preparation

Make a PCR master mix allowing for 5% more than needed in a 2-mL centrifuge tube based on the contents composition of **Table 2** below:

Table 2. Real-time qPCR reaction recipe

Reagent	Work Conc.	Final conc.	1X (µl)	_ X (µl)
2X Buffer	2X	1X	10	
F6-3 Primer	20 µM	500nM	0.5	
R6 Primer	20 µM	500nM	0.5	
Prb3 Probe	10 µM	250nM	0.5	
IC F primer	20 µM	600nM	0.6	
IC R primer	20 µM	200nM	0.2	
IC probe	10 µM	200nM	0.4	
IC DNA	3k copies/µl	600 copies	0.2	
BSA	20 mg/µl	200ng/µl	0.2	
H_2O	N/A		4.9	
DNA	N/A		2	

Note: In the first few runs of this assay, we highly recommend you to include an eight-point standard curve of *F. virguliforme* genomic DNA with two replicates to setup a baseline for the detection sensitivity and assay efficiency for your real time PCR platform.

4. Vortex the master mix, and spin down with a mini centrifuge

StepOne Plus Real-time PCR System

5. Aliquot 18 µL of the master mix into PCR tubes / 96-well plate, and add 2 µL of each sample DNA into the corresponding tubes/wells
6. qPCR run on StepOne Plus Real-time PCR system

Table 3. Real-time PCR cycling conditions

Step	Temperature	Time	Stage
Incubation	50ºC	2 min	Holding
Denaturing	95ºC	10 min	
Denaturing	95ºC	15 s	Repeat 40 cycles
Annealing / extension	60ºC	1 min	

7. Define targets
   - *F. virguliforme* assay Reporter: FAM Quencher: NFG-MGB
   - HHIC exogenous control Reporter: JOE Quencher: None
   - **Note:** HHIC probe was labeled with HEX reporter, which shares the same excitation and emission wavelength as JOE reporter. Therefore, they are interchangeable in this case.
8. Data collection
   - Check the QC Summary
   - Check standard curve to calculate the PCR efficiency (normal range: 85 ~ 110%)

- An easy way to calculate PCR efficiency**: PCR efficiency = -3.32/slope**, where slope is the linear regression between Ct values and corresponding log10 transformed DNA quantity.
- Check exogenous control IC amplification, Ct value < 24 is expected for all samples except for the tubes/wells with the serially diluted *F. virguliforme* genomic DNA.
- Adjust the threshold line to 0.1 for *F. virguliforme* assay, reanalyze the data
- Export the results to .CSV file for further analysis
- Save the raw data file (extension: .eds) to your USB driver

9. Data analysis
   - Organize the data with Open Office
   - Statistical analysis using SAS, R or others

Cepheid Smart Cycler System

5. Aliquot 18 µL of the master mix into Cepheid PCR tubes, and add 2 µL of each sample DNA into the corresponding tubes/wells
6. Spin the tubes in the Cepheid micro centrifuge.
7. Load tubes in the Cepheid block, pushing firmly to insure they are seated in the block.
8. Set up the reaction using the following cycling conditions

Step	Temperature	Time	Stage
Incubation	50ºC	2 min	Holding
Denaturing	95ºC	10 min	
Denaturing	95ºC	15 s	Repeat 40 cycles
Annealing / extension	60ºC	1 min	

9. Select dye set FTTC25 when setting up your run.

References:

Haudenshield JS, Hartman GL (2011) Exogenous controls increase negative call veracity in multiplexed, quantitative PCR assays for *Phakopsora pachyrhizi*. Plant Disease **95:** 343-352

Wang J, Jacobs JL, Byrne JM, Chilvers MI (2015) Improved diagnoses and quantification of *Fusarium virguliforme*, causal agent of soybean sudden death syndrome. Phytopathology **105:** 378-387