Title: RPA amplification of *Fusarium virguliforme* with lateral flow detection

Purpose: Detect *Fusarium virguliforme* in diseased root tissue and/or purified DNA samples using RPA and end-point lateral flow detection chambers

Note* *If you are performing a crude DNA extract from diseased plant tissue, start at Part 1. If you are using purified DNA samples, skip ahead to Part 2.*

Part 1. Materials

1. Soybean root samples
2. Razor blade / scalpel (preferably sterile)
3. Paper towels
4. Scale
5. Mesh DNA extraction bags (Agdia, cat # ACC 00930/0100)
6. Tissue homogenizer (Agdia, cat # ACC 00900)
7. GEB2 buffer (Agdia, cat # ACC 00130)

Part 1. Crude DNA extraction

1. Prepare enough 1X GEB2 buffer from the powdered stock for your samples plus two extra to account for errors. Each sample requires 0.279g of GEB2 powder mixed in 5 mL of sterile distilled water.
2. Gently rinse any excess soil off of infected soybean roots. Set the rinsed roots onto a layer of paper towels, and pat them dry with additional paper towels.
3. Examine the roots for any obvious lesions or discolorations, and use the razor blade to collect 500mg of tissue. If no obvious lesions or discolorations are present, select a random 500mg of root tissue.
4. Place the 500mg of root tissue into a mesh DNA extraction bag. Add 5mL of fresh GEB2 buffer. Fold and seal the mesh bag.
5. Carefully use the homogenizer to grind the root sample into small bits in the GEB2 extraction buffer, making sure not to break through the mesh bag.
6. Crude extract of DNA can be used directly, or an aliquot (~1mL) can be stored in a 1.5mL microcentrifuge tube at -20°C for later use.

Stop Point – *Crude extraction of DNA can be stored at -20°C until used in RPA*

Part 2. Materials

1. RPA reaction kits
   AmplifyRP Acceler8 – Agdia cat # ACS 98800/0048
   or TwistAmp Nfo – TwistDx cat # TANFO01KIT

Each kit contains reaction strips, rehydration buffer, and magnesium acetate (MgOAc).

2. Heat block
3. Bench top centrifuge
4. 8-well PCR tube strip, or x number of PCR tubes for your purposes
5. 1.5mL microcentrifuge tubes, clear and amber colored
6. Primers and probe

Primer/Probe	Sequence (5' - 3')	Length (bp)
SDS – F. virguliforme multiplexed assay		
*FvF30	Not available at this time	n/a
*FvR30	Not available at this time	n/a
*FvNfo	Not available at this time	n/a

*These primers and probes are ordered through Sigma

Part 2. RPA and lateral flow detection

1. Obtain RPA reagents (molecular grade water, FvF30, FvR30, FvNfo, target DNA) and thaw on ice. Rehydration buffer and MgOAc may be stored at room temperature.
2. Calculate the volume of reagents you need for all of your reactions. Add extra reagents for an additional ½ reaction to account for pipetting errors. Create a master mix by adding the following reagents together, in order, in a 1.5 mL microcentrifuge tube and mix by briefly vortexing.

Note: *rehydration buffer is viscous, pipette slowly to ensure you obtain the full volume desired*

Reagents	Initial concentration	1X	8.5 X	___.5 X	Check
Rehydration buffer	-	14.75µL	125.375 µL		
Water	-	5.6µL	47.6 µL		
FvF30	10 µM	1.05 µL	8.925 µL		
FvR30	10 µM	1.05 µL	8.925 µL		
FvNfo	10 µM	0.3 µL	2.55 µL		
Crude Plant Extract/DNA		1.00 µL	-		
Total Volume*			**25 µL**		

** Total volume is 25 µL after adding the Magnesium acetate, but this is added just before the start of the reaction.*

3. Obtain an 8 well PCR tube strip (or x number of PCR tubes for your number of reactions) and place in an ice block. Pipette 22.75µL of master mix into each tube.
4. Pipette 1µL of template DNA (from crude extract or purified DNA) into appropriate tube. Place caps on tubes and mix by inversion. Use a bench top centrifuge to spin down. Place back on ice.
5. Obtain an 8 well Acceler8 tube strip and place on ice. Carefully open all caps, making sure not to disrupt the lyophilized pellet. Mix the MgOAc by vortexing and pipette 1.25µL MgOAc into top of each open cap.

6. Use a multichannel pipette (if available) to transfer 23.75µL from all 8 tubes of the PCR strip to the pellets in the TwistAmp strip simultaneously. Pipette up and down vigorously to resuspend all pellets.

Note* *when resuspending the pellet, it will become bubbly or foamy. This is normal.*

7. Carefully close caps containing MgOAc onto the Acceler8 tubes so that the MgOAc does not fall into the reaction. Make sure one end cap is labeled.

Note* Addition of the MgOAc to the reaction mixture initiates the reaction, even at room temperature. Make sure MgOAc does not fall into a reaction mixture before this point.

8. Spin the Acceler8 reactions in a bench top centrifuge so that the MgOAc in the caps falls into the reaction simultaneously. Mix the reactions very well by inverting 10 times. Spin briefly again in the bench top centrifuge and immediately place in a 39°C heat block for 4 minutes.
9. After the 4 minutes, remove from the heat block and mix by vortexing or inverting 10 times. Spin in a bench top centrifuge and immediately place samples back in heat block for 16 minutes.
10. After the 16 minutes, the reactions have used up most or all of the reagents. Remove the samples from the heat block and proceed to lateral flow detection.

Part 3. Materials

1. Detection chamber (Agdia – cat # ADC 98800/0001)
2. Milenia HybriDetect 2T (Milenia Biotec – cat # MGHD2 1)

Part 3. Lateral flow detection

1. Carefully detach the reaction tube of interest from the rest of the strip using a sharp razor blade or scissors.
2. Open the grey apparatus from the detection chamber. Place the reaction tube into the slot next to the plastic round bottom tube containing running buffer. Close the grey apparatus.
3. Place the grey apparatus back into the detection chamber, making sure that the reaction tube is facing the lateral flow strip.
4. Push down on the white handle with a firm grip until both tubes are crushed, and make sure the white handle is pressed all the way down and snaps shut.
5. Wait for 10 minutes, and interpret the results. The image shows a positive reaction (left) and a negative reaction (right). Repeat this process for all samples.