Interpretation of results

Well	Signal Strength (Cq)	Interpretation	Troubleshooting
Foc TR4 or Banana COX PAC	25 or less	Pass	
	Above 25 (or no Cq)	Fail	Repeat Real-Time PCR assay
Foc TR4 or Banana COX NAC	No Cq	Pass	
	less than 40	Fail	NAC well is contaminated. Possible sources of contamination should be identified and removed. Also, repeat the Real-Time PCR assay.
Banana COX sample	35 or less	Pass	
	Above 35 (or no CQ)	Fail	Assuming plant tissue was present in the sample, failure to detect a Banana COX gene signal might be due to inhibitors or by failed DNA extraction. To remove inhibitors a 20-fold dilution is recommended. If a problem has occurred during the extraction or purification of plant DNA, please repeat the DNA extraction
Foc TR4 sample	35 or less	Pass	
	Cq between 35 and 40	Inconclusive	Compare the melting curve with the PAC/NAC samples If the melting curve has a similar shape as the PAC, but below the threshold, Foc TR4 might be present. Test a lower dilution of the sample If the melting curve shows background comparable to NAC, there is no detectable Foc TR4 If the melting curve is not similar to PAC or NAC, results are inconclusive. Repeat Real-Time PCR assay with increased DNA concentration
	Above 40 (or no CQ)	Foc TR4 not detected	

Notices and disclaimers

Despite the utmost care in the development and preparation of this protocol, ClearDetections cannot take any responsibility for errors, omissions and/or future changes herein.

This kit is designed for general laboratory and research use only. For the legal notices & disclaimer see website, www.cleardetections.com, or contact ClearDetections at info@cleardetections.com.

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ClearDetections
revolutionary diagnostics

For use of the Real-Time PCR diagnostic kit

Fusarium oxysporum f. sp. cubense (Foc) Tropical Race 4 (TR4) (Panama disease)

RT-F-D-0901/0902

For general laboratory and research use only

Introduction

The ClearDetections' all-inclusive Real-Time PCR identification kit contains a Real-Time PCR assay for simple, sensitive, and rapid detection *of Fusarium oxysporum f. sp. cubense* tropical race 4 (Foc TR4) in purified DNA samples obtained from various sources. For DNA extraction from banana plant tissue, please consider the ClearDetections kit EX-P-T/P-PDEP. The specificity of the ClearDetections Foc TR4 primers is based on a unique mutation in the Intergenic Spacer (IGS) region. The use of a control to verify successful DNA extraction and purification is highly recommended. For banana plant tissue it is recommended to include a Real-Time PCR assay designed for the detection of Cytochrome C Oxidase (COX) gene, present in banana plant tissue, to be used as an extraction control. This option is included in kit RT-F-D-0902. For further information or questions please contact our technical experts via info@cleardetections.com.

We recommend that you read the entire manual before starting the procedure.
Feel free to contact us at info@cleardetections.com for questions regarding this protocol, laboratory set-up or equipment specifications.

Kit components, storage conditions, and shelf life

Components	Code	Storage conditions
All-inclusive ClearDetections Foc TR4 PCR mix †	PMD_0901_50	Room temp*
Foc TR4 Positive Amplification Control (Foc TR4 PAC)	PAC_0901_02	Room temp*
Resuspension buffer	RSB_2	Room temp*
All-inclusive ClearDetections Banana COX PCR mix** †	PMD_2601_50	Room temp*
Banana Cox gene Positive Amplification Control (COX PAC)**	PAC_2601_02	Room temp*

* Supplied as lyophilized reagent. It should be stored in their original packaging at room temperature in a dry environment. After resuspension, store at -20˚C until their next use. If properly stored, the kit components can be used until the expiry date printed on the box.

** Included in the RT-F-D-0902-050 Real-Time PCR Diagnostic Kit for Panama Disease (Foc TR4) for banana plant tissue.

Reagents and equipment to be supplied by the user

- Centrifuge for microcentrifuge tubes and/or for 96-well plates
- Pipettes and corresponding filter-tips for volumes of 5-1000 µl
- Microcentrifuge tubes or 96-wells plates for DNA dilution
- PCR plates & seals or PCR tubes appropriate for Real-Time PCR
- Real-Time PCR machine with FAM or SYBR/FAM channel †
- Nuclease free water
- Vortex

† The ClearDetections PCR mixes do not contain a passive reference dye. If applicable, please deactivate any passive reference dye option in your Real-Time PCR software. Feel free to contact us for advice and support if this situation applies to you.

Sample preparation

Diluting DNA extracts is an essential step to further reduce the presence of compounds that inhibit Real-Time PCR. Traces of inhibitors might be encountered in DNA samples obtained from for instance (infected or not) banana plant material. Diluting each DNA extract 10 - 20 fold (in nuclease free water) will normally ensure proper PCR performance.

Resuspension of the Real-Time PCR components:

1. Gently tap the vials containing the Foc TR4 (and Banana COX) PCR mix, to settle any contents that may have moved during shipping.
2. Unscrew the cap of the mix vial(s) and discard their rubber stoppers.
3. Add 790 µL of resuspension buffer to each vial.
4. Close the vial(s) and incubate 5 minutes at room temperature.
5. Briefly spin down the PAC tube(s) before opening.
6. Unscrew the caps of the Foc TR4 (and Banana COX) PAC tube(s), and add 300 µL of resuspension buffer to each tube.
7. Close the tubes and incubate 5 minutes at room temperature.
8. Mix by vortexing and spin down the content of the tubes.

Note: Failing to properly mix the components will result in poor assay performance.

9. Proceed with the Real-Time PCR assay.

Tip: It is possible to make aliquots of all resuspended reagents in order to prevent accidental contamination of master stocks.

Foc TR4 and Banana COX PCR

1. Design the layout of your PCR plate. We recommend using two wells for each sample to be screened for Foc TR4 and Banana COX DNA, plus four wells for the following controls:
    - Foc TR4 Negative amplification control (Foc TR4 NAC)
    - Foc TR4 Positive amplification control (Foc TR4 PAC)
    - Banana COX Negative amplification control (Banana COX NAC)
    - Banana COX Positive amplification control (Banana COX PAC)
2. Mix the PCR mix in the vial by pipetting up and down.
3. Pipette 15 µL of each mix into their designated wells.
4. Vortex the Foc TR4 and Banana COX PAC tubes for 1 – 2 sec, and briefly spin down the content of the PAC tubes.
5. Pipette 5 µL of each PAC into each PAC well.

Note: Take great care to avoid contaminating neighbouring wells.

6. Pipette 5 µL nuclease free water into each NAC well.
7. Pipette 5 µL of each diluted DNA sample into its designated well.

Note: The final volume in each well should be 20 µL.

8. Seal the plate and centrifuge for 1 min at maximum speed.

Note: No liquid should remain on the seal or sides of each well, and avoid the presence of air bubbles.

9. Transfer the plate to the Real-Time PCR machine and start the run using the following settings :

	Step	Time	Temperature
Enzyme activation		3 minutes	95°C
Amplification (40 cycles)	DNA denaturation	10 seconds	95°C
	Primer annealing	60 seconds	63°C
	Primer extension*	30 seconds	72°C
Melt curve*		0.2 - 0.5°C steps	72°C → 95°C

* Measure the fluorescent signal, using the FAM or SYBR/FAM channel, after every cycle and after every temperature increment of the PCR melt curve.

Note: Run times, temperatures, and volumes have been strictly optimized and must not be altered.