Diagnostic of Foc TR4 with Real-Time PCR, a complete overview from sample collection to results interpretation

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This document presents an overview of the diagnostic of *Fusarium oxysporum f. sp. cubense* tropical race 4 (Foc TR4) pathogenic agents responsible for Panama Disease via Real-Time PCR using ClearDetections diagnostic products for Panama Disease. If you want to know more about Panama Disease check our white paper here[1].

The present document aims to be a guide for a successful diagnostic with Real-Time PCR while also covering the pre-diagnostic step of sample collection and preparation. In addition, this document includes an extensive result interpretation and troubleshooting section, as an aid for result interpretation for accurate diagnostics. Diagnosis with Real-Time PCR assay will allow for an accurate and sensitive detection of Foc TR4. Our kit is based on the primers reported by Dita et al 2010, and are able to detect 0,2 pg of Foc TR4 DNA in clean samples.

[1] https://www.cleardetections.com/white-paper-panama-disease-tr4-an-unstoppable-threat/

Sample collection

Sampling the banana tissue is a crucial step that greatly influences the success of downstream processes like Foc TR4 detection by Real-Time PCR (Video of Foc TR4 infected tissue[2]) (Figure 1). On an infected plant, the fungi is mostly present in the roots and in the necrotic meristem and pseudomeristem. Leaf tissue can also be used for Foc TR4 detection during late infection. For Foc TR4 diagnostic purposes, use 250 mg of vein tissue isolated manually from the inner leaf of the pseudostem or 250 mg of corm tissue, where the infection is most pronounced. Samples can be used fresh after recollection for best results, but can also be stored at -20 ºC or in liquid nitrogen. To store fresh samples, we recommend to place the samples between 2 sheets of clean fibre paper and on a clean plastic bag. Is important to remove moisture to prevent degradation of sample. This step is not needed if you store your samples in liquid nitrogen.

Figure 1. Example of a transversal cut of infected banana stem (left) and healthy (right above) and Foc TR4 infected (right below) banana inner leaf.

[2]https://bit.ly/3t2cSZF

DNA extraction

For the DNA extraction and purification of banana plant tissue we recommend the use of ClearDetections Banana Plant Tissue DNA Extraction & Purification Kit (EX-P-T-PDEP). The advantage of the use of this kit is that it is a complete, simple, and ready-to-go solution that is tested for this application by ClearDetections. Nevertheless, other DNA extraction methods are possible, in this case we recommend the execution of a verification step with reference material prior to use.

Kit contents

Table 1. Components for the DNA extraction and purification kit from banana plant tissue

Components	Storage
Extraction buffer	4 °C
Protease	4 °C
Homogenization solution	4 °C
Equilibration solution	4 °C
RNase A	4 °C
DTT	4 °C (-20 °C after first use)
SDS	15-30°C
DNA purification columns	15-30°C
DNA collection tubes	15-30°C

Equipment required

- Vortex
- Centrifuge
- Temperature controlled incubator/heat block or water bath
- Bead beater and stainless-steel beads (2 to 3 mm), or pestle and mortal.
- Pipettes and corresponding filter-tips for volumes of 5-1000 μL
- Optional: liquid nitrogen.

Sample preparation

For the DNA extraction using the ClearDetections Banana Plant Tissue DNA Extraction & Purification Kit (EX-P-T-PDEP) follow the next steps:

1. Pre-heat the incubator or water bath to 60 °C.
2. Prepare homogenization mix sufficient for all samples:

Table 2. Reagents to prepare the homogenization mix for one and ten samples

The homogenization mix:	per sample (μL)	x 10 samples*
Homogenization solution	185	2035
DTT	3	33
RNase	12	132

* When calculating the volume of the homogenization mix, we advise increasing the number of samples by approximately 10% to account for pipette error, or 1 extra sample

The sample can be grinded using a mortar, pestle, and liquid nitrogen or via bead-beating. Bead-beating is a faster and tidy process, but it requires a bead-beating machine. On the other hand, grinding with liquid nitrogen and mortar is a widely used procedure, but the chances of contamination between samples are higher and it is highly time consuming. To proceed with DNA extraction using a bead beater go to section A. To proceed with DNA extraction using pestle & mortar go to section B. A scheme of both methods is represented in the figure 2.

A. Grinding with bead beater & stainless-steel beads:

a. Transfer up to 250 mg banana plant tissue into tube and add 1 or 2 beads.

Note: to increase DNA extraction efficiency, the grinding of the plant tissue can be done in liquid nitrogen. For this, chill the tubes containing plant material in liquid nitrogen before bead beating. Be sure the lid is closed properly, to not let liquid nitrogen enter in the tube.

b. Grind 1 minute at 30Hz in the bead beater. Afterwards, spin down briefly (5 seconds) to make sure all material is at the bottom of the tube, so the lid can be opened without losing the sample.

c. Add 200 µL of freshly prepared **homogenization mix** and grind again 1 minute at 30Hz in the bead beater. Afterwards, spin down briefly (5 seconds).

Note: Do not freeze samples again in liquid nitrogen at this step. The sample including buffer should not be frozen, as this would block efficient bead beating.

d. Proceed to point 4 of DNA extraction procedure.

B. Grinding using a mortar & pestle:

a. Transfer up to 250 mg banana plant tissue into a mortar pre-chilled with liquid nitrogen.

Note: to increase DNA extraction efficiency, the grinding of the plant tissue can be done in liquid nitrogen. For this, chill the mortar with liquid nitrogen before grinding. After adding the banana plant tissue, add enough liquid nitrogen to freeze your sample, and grind plant tissue vigorously using a pestle.

b. Add 200 µL of freshly prepared **homogenization mix** and grind plant tissue vigorously using a pestle.

Note: Do not add liquid nitrogen after adding the buffer.

c. Transfer the content of the mortar to a 1.5 ml Eppendorf tube, compatible with the heat block.

Note: Be careful not to transfer any liquid nitrogen to the 1.5 ml Eppendorf tube when transferring the sample. If this is the case, let the liquid nitrogen evaporate before closing the tube.

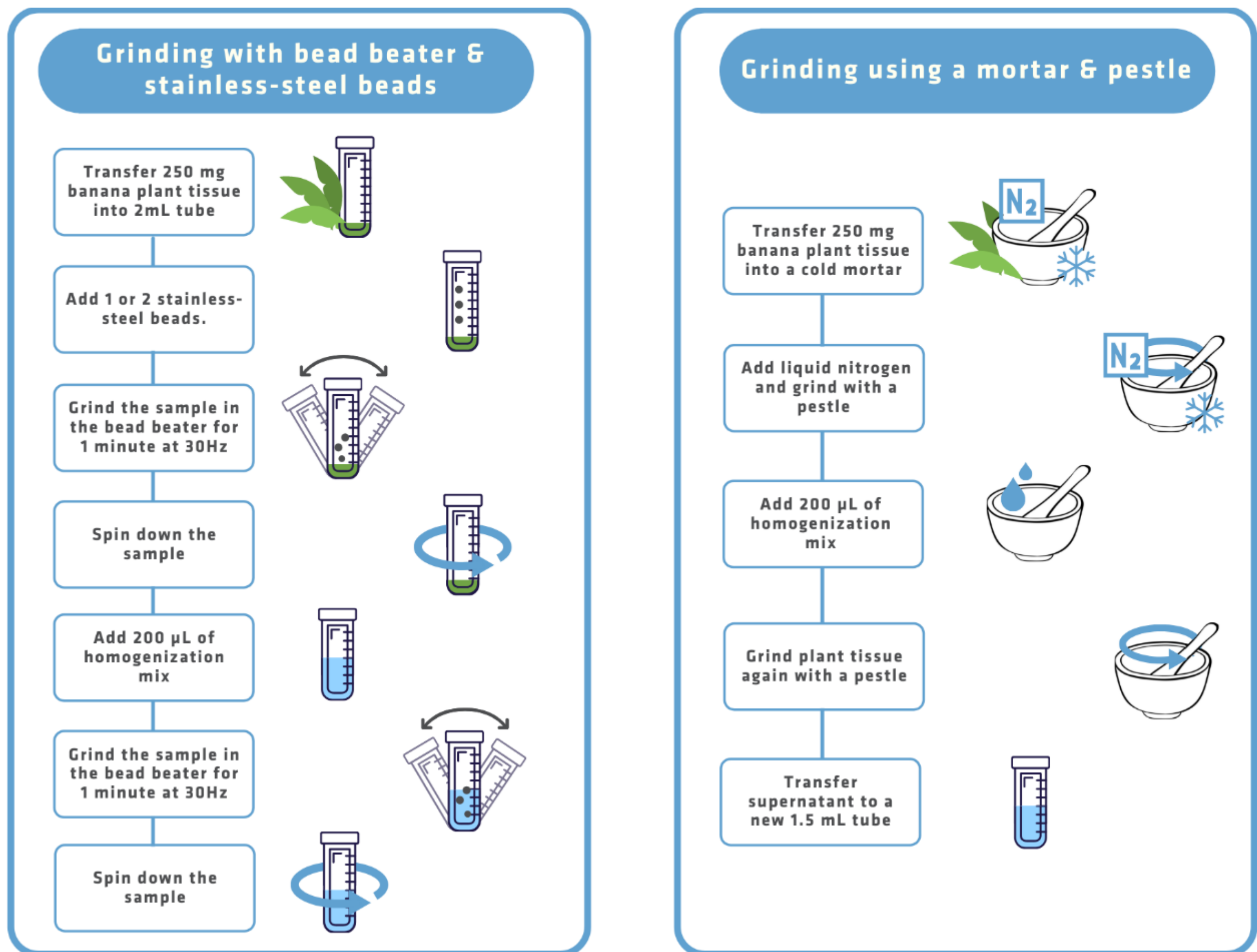


Figure 2. Scheme of sample preparation using the bead beater method or the liquid nitrogen method.

DNA extraction

3. Prepare extraction mix sufficient for all samples:

Table 3. Reagents to prepare the extraction mix for one and ten samples

The extraction mix:	per sample (µL)	x 10 samples*
DNA extraction buffer	70	770
Protease	20	220
SDS	10	110

* When calculating the volume of the extraction mix, we advise increasing the number of samples by approximately 10% to account for pipette error.

4. Add 100 µL of freshly prepared extraction mix to each sample and vortex samples thoroughly.
5. Incubate the tubes for 1 hour at 60 °C and briefly vortex tubes every 10-15 minutes.

Note: Continue with equilibration of the DNA purification columns during this 1 hour incubation step.

6. Vortex tubes thoroughly and centrifuge for 1 min at 10.000 x *g*. After this step, samples are ready to be loaded on the equilibrated purification column

Note: To facilitate easy pipetting of the supernatant, samples should not stand long after centrifugation. It is advisable to work with batches of 8 samples, so immediately after spinning down the supernatant can be loaded onto the equilibrated column.

1. Take the DNA purification columns with attached waste collection tubes.
2. Add 350 µL equilibration solution to each column for every crude DNA extract to be purified.
3. Stand at room temperature for 5 min and then centrifuge for 1 min at 350 x *g*.
4. Discard the waste collection tubes and place the DNA purification column(s) to the DNA collection column(s).
5. Transfer 100 µL of each DNA extract to the equilibrated DNA purification column(s).

Note: Be careful, try not to disturb the pellet.

6. Stand the DNA purification column for 3 min at room temperature.
7. Centrifuge 1 min at 700 x *g*.
8. The flow-through captured by the DNA collection tube contains purified DNA ready for Real-Time PCR or storage.
9. Store the DNA between 4-8 °C for immediate use. For long-term storage (>48h) keep at -20 °C.

For a visual information of this protocol please watch our step by step instruction video.

Real-Time PCR Diagnostics

Preparation of Real-Time PCR

For the detection of Foc TR4 in banana samples, we recommend the use of Real-Time PCR diagnostic kit for *Fusarium oxysporum f.sp. cubense* (Foc) Tropical Race 4 (TR4) (Panama disease) (RT-F-D-0902). The first diagnostic kit for Panama Disease with a trust network of users, recommended by different PPO organisations as a reference kit for TR4 diagnostics.

This kit is validated against reference material, includes all reagents and controls to perform diagnostic of Foc TR4 from banana plant tissue and it is a ready to use solution with easy implementation.

Kit contents

Table 4. Components of the Real-Time PCR diagnostic kit for banana tissue (RT-F-D-0902).

RT-F-D-0902 Real-Time PCR Diagnostic Kit for Foc TR4 with Banana Cox gene extraction control		
Components	Code	Storage
All-inclusive ClearDetections Foc TR4 PCR mix †	PMD_0901_050	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_050	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.

†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

Before starting the Real-Time PCR assay, it is essential to dilute your DNA extract samples from banana plant 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. We recommend diluting each DNA extract 10 - 20 fold in nuclease free water will normally ensure proper Real-Time PCR performance. Further dilutions are rarely necessary. See troubleshooting if the DNA extract contains a high concentration of inhibitors.

Use instructions

After the dilution of your samples, you can continue with the 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 mins 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.

Now you can proceed with the Real-Time PCR:

1. Design the layout of your Real-Time PCR plate. An example layout is shown in Figure 3.

		Foc TR4			COX Banana gene		
		1	2	3	4	5	6
Healthy	A	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
	B	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
	C	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
	D	PAC Foc TR4	PAC Foc TR4		PAC COX	PAC COX	
Infected	E	Sample 4	Sample 5	Sample 6	Sample 4	Sample 5	Sample 6
	F	Sample 4	Sample 5	Sample 6	Sample 4	Sample 5	Sample 6
	G	Sample 4	Sample 5	Sample 6	Sample 4	Sample 5	Sample 6
	H	NAC	NAC		NAC	NAC	

Figure 3. Example of Real-Time PCR plate layout for Foc TR4.

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 PCR PAC tubes for 1 – 2 sec, and briefly spin down the content of 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:

Table 5. Real-Time PCR program for the diagnosis of Foc TR4 and COX banana gene

	Step	Time	Temperature
Enzyme activation		3 min	95°C
Amplification (40 cycles)	DNA denaturation	10 sec	95°C
	Primer annealing	60 sec	63°C
	Primer extension*	30 sec	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, please verify your settings before starting and save the program for posterior use.

Results interpretation

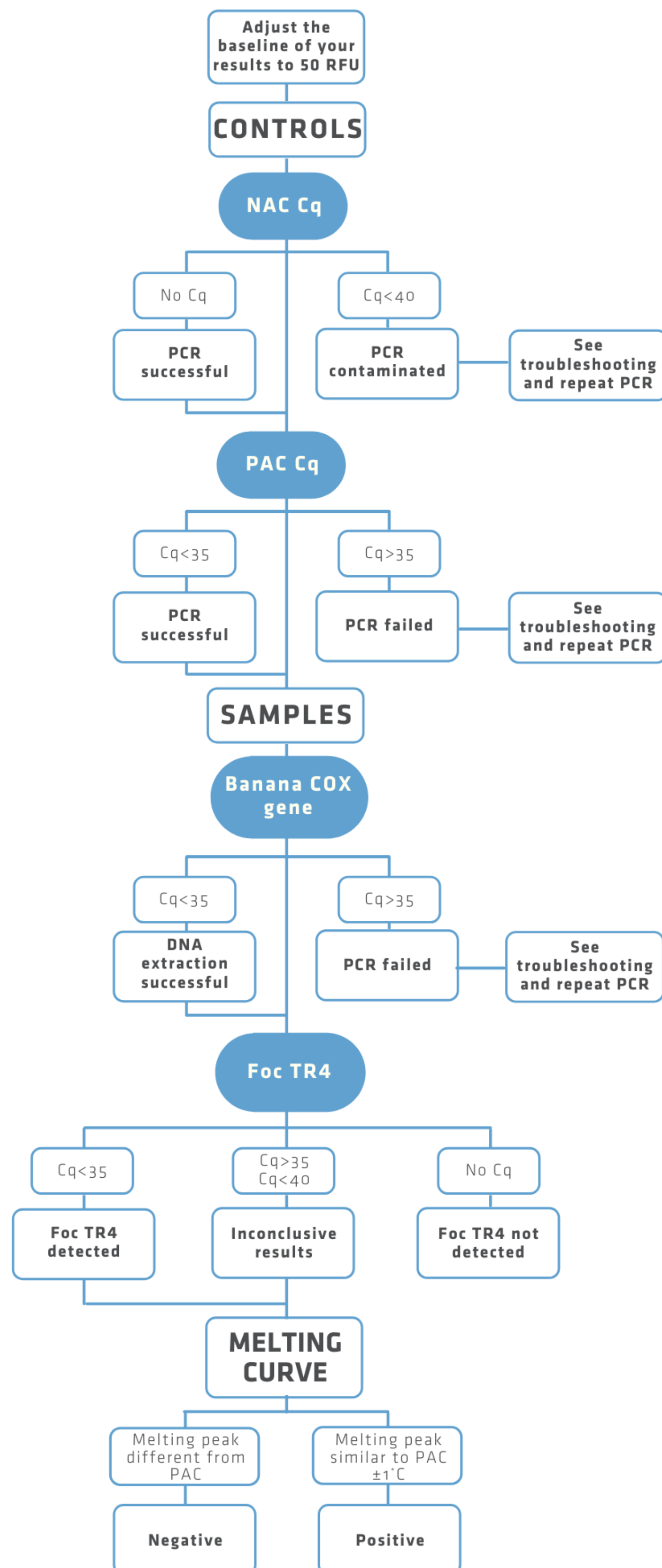


Figure 4. Scheme of results interpretation

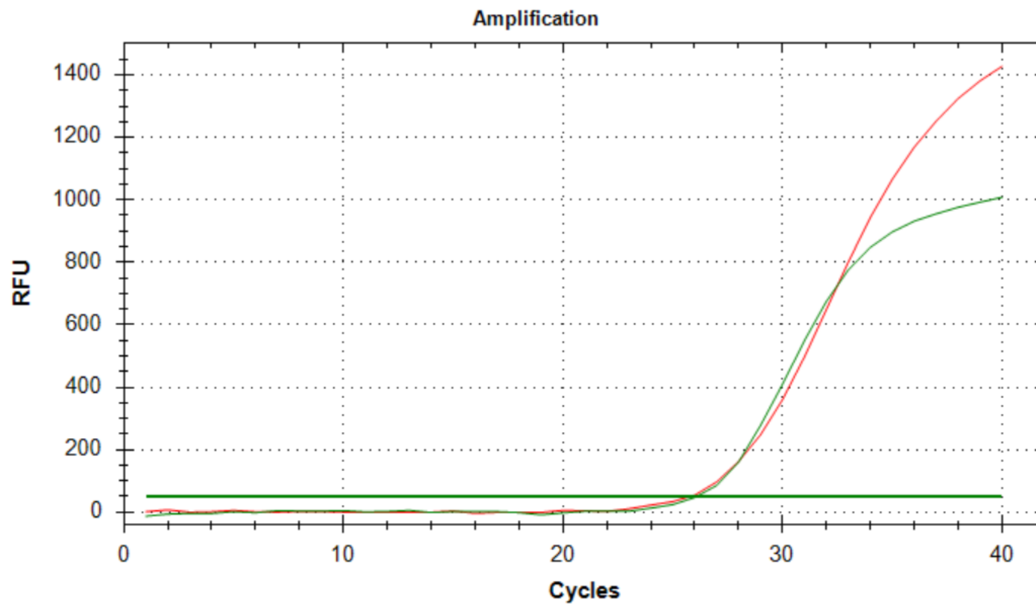
Troubleshooting

Failed positive amplification control (PAC) well

A successful Foc PAC and banana COX gene PAC has a signal strength (Cq) of 25 or lower (Figure 5A). The banana COX gene has a melting peak at 83 °C and Foc TR4 has a characteristic double melting peak at 83 °C and 89 °C (Figure 5B).

Note: The expected melting curve pattern could change depending on the machine used for the PCR.

A



B

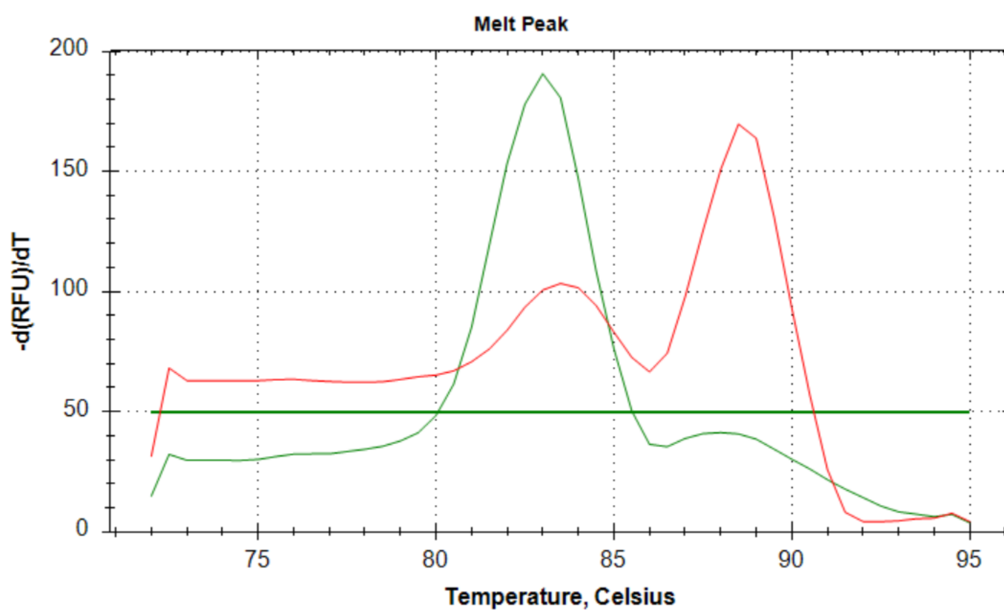


Figure 5. Amplification (a) and melting curve (b) of banana COX gene PAC (green) and Foc TR4 PAC (red)

If any of the positive controls has a Cq above 25 it means that the PCR has failed. Consider the following points and then repeat the PCR.

- Ensure that the open PCR mix vial is stored at -20 ºC.
- De-frost completely the PCR mix vial prior to the PCR reaction.
- Vortex the PCR mix vial for 5 seconds prior to the PCR reaction.
- Vortex the PAC vials for 5 seconds prior to the PCR reaction.
- Ensure that the selected program corresponds to the one recommended in the protocol.
- Ensure that there are no bubbles in the well or the seal prior to the PCR reaction.

Failed negative amplification control (NAC) well

A successful NAC has no signal strength (Cq) or a signal strength above 40. In addition, the NAC should not present a melting curve similar to the PAC. If there is any amplification in the NAC, it means that there is contamination in your PCR. Consider the following points and then repeat the PCR.

- Clean your working space and pipettes with 15% bleach solution and then with 70% alcohol solution.
- Prepare a new nuclease free water stock to use in the experiment.
- Pipette the NAC well after the PAC well and before pipetting your samples.
- Ensure that the NAC well is not contaminated by surrounding wells due to micro-spraying of the samples.

Banana COX result above 35

A successful detection of the Banana COX gene with a signal strength below 35 indicates that the DNA extraction from plant tissue is successful. To confirm the results, the melting curve of each sample must have the same melting peak ± 1 °C than the corresponding PAC.

If the signal strength for Banana COX gene in your samples is between 35 and 40 it may indicate inhibition in your DNA sample or failed DNA extraction. Consider the following points and then repeat the PCR.

- If the sample is contaminated, dilute your samples to 1:20 to reduce the inhibitor concentration.
- If the DNA extraction failed, try a lower dilution of your samples.
- If a lower dilution does not give signal, repeat the DNA extraction and purification.

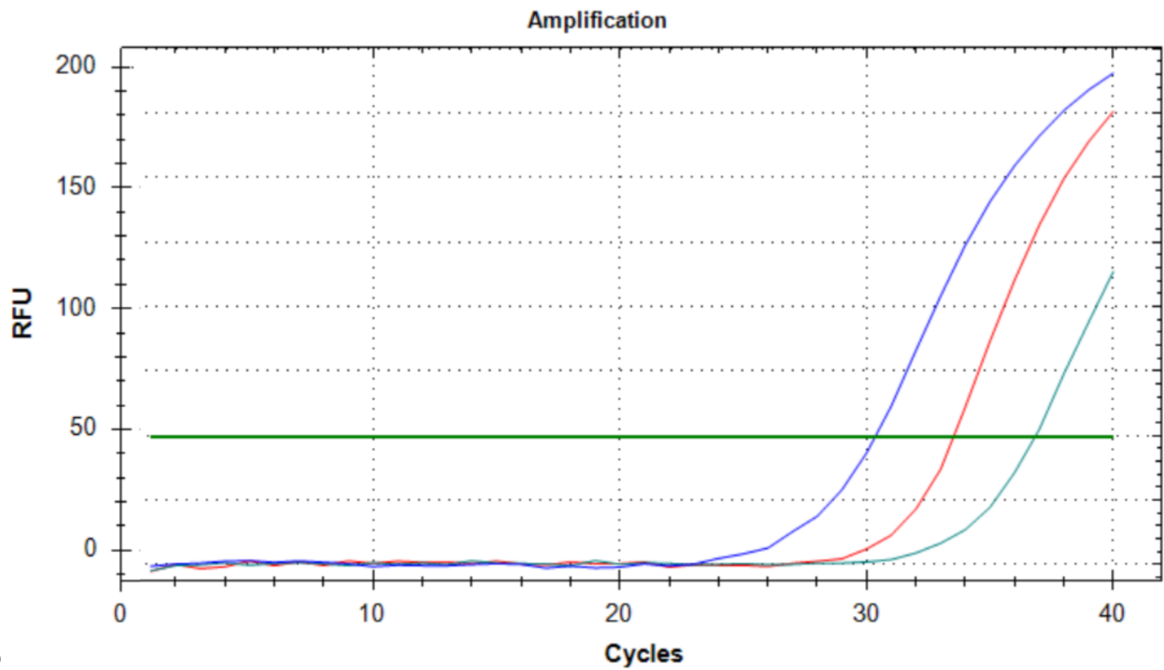
Foc TR4 result between 35 and 40

A successful detection of Foc TR4 with a signal strength below 35 indicates that DNA from the fungus is present in the sample. To confirm the results, the melting curve of each sample must have the same melting peak ± 1 °C than the corresponding PAC.

The absence of a signal indicates there was no DNA from the Foc TR4 in the sample.

If the signal strength for Foc TR4 in your samples is between 35 and 40 it may indicate an inconclusive result (Figure 6).

A



B

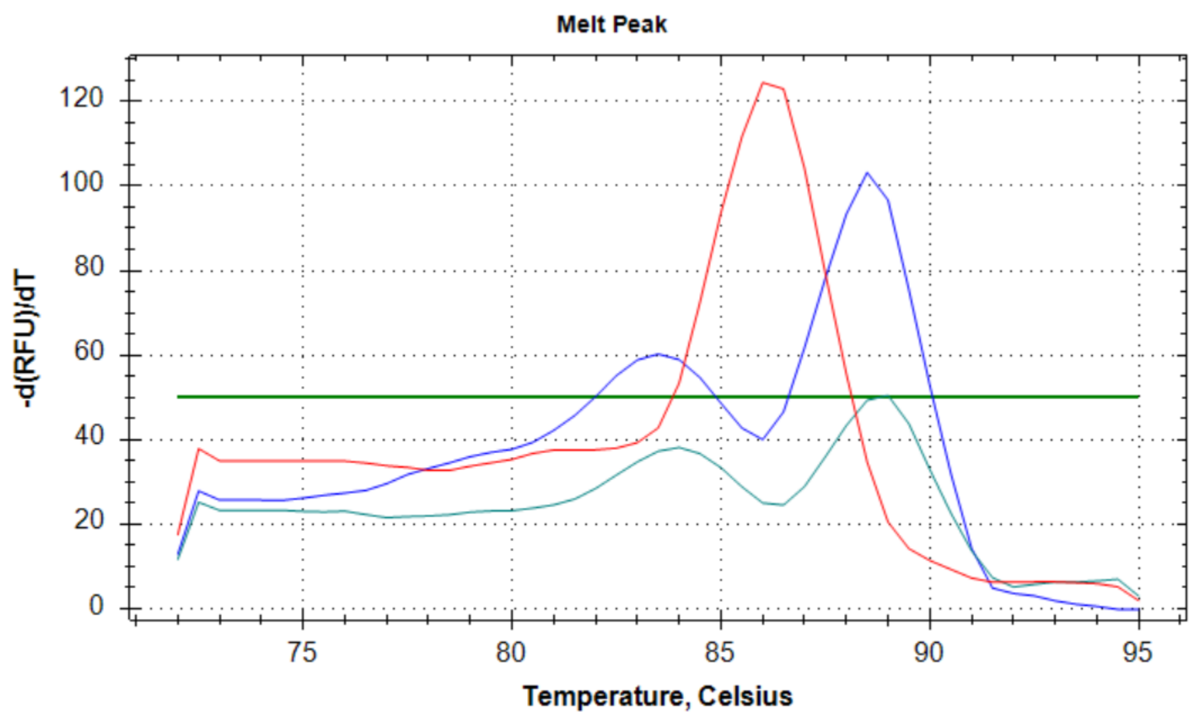


Figure 6. Amplification (A) and melting curve (B) of Foc TR4 PAC (blue), a positive sample with a Cq above 35 (green) and a negative sample with a Cq below 35 (red).

Consider the following points:

- If the melting curve is the same as the PAC, including the double peak, the DNA may be degraded, consider repeating the PCR with a lower dilution
- If the melting curve is different from the PAC, it may be a false positive amplification due to contamination of the sample.
- Identify false positives and false negatives by comparing the results with other diagnostic methods such like plating.

For more information please watch the instruction video.

Disclaimer

The information presented in this document reflects the accumulated knowledge from ClearDetections with the use of ClearDetections Diagnostic products for Panama Disease and tries to enlighten answers for frequently asked questions from users of our products. All information presented here is based on internal documents produced by ClearDetections and is available upon request. With sharing this information the authors hope to contribute to an accurate use of molecular tools for Foc TR4 diagnostics to contribute for the containment and early detection of the disease. For more information about the document or the products listed on it please contact the authors directly: Irene Cabal Blanco (in Spanish or English) and Marta Santos Paiva (in Portuguese or English) or consult www.cleardetections.com.