Interpretation of results

Well	Signal Strength (Cq)	Interpretation	Troubleshooting
PAC sample	25 or less	Pass *	
	Above 25 (or no Cq)	Fail	Repeat Real-Time PCR assay.
NAC sample	No Cq	Pass *	
	Less than 35	Fail	NAC well is contaminated. Possible sources of contamination should be identified and removed and the Real-Time PCR assay should be repeated.
DNA sample	35 or less	Pass *	
	Above 35 (or no Cq)	Fail	The target nematode is not present. Assuming nematode DNA was present in the sample, failure to detect the target nematode might be due to inhibitors or by a failed DNA extraction. To remove inhibitors a 20-fold dilution is recommended. If detection is unsuccessful, additional dilution might be necessary. If a problem has occurred during the extraction or purification of nematode DNA, it is recommended to repeat the DNA extraction using the ClearDetections Plant DNA extraction & purification kit. Furthermore, one may confirm the presence of Nematode DNA in a sample, regardless of the species, by using the ClearDetections' General Nematode Real-Time PCR diagnostic kit.

* The detection of nematodes should be confirmed by analyzing the melting curve of the PAC sample. A melting temperature that differs from the melting temperature of the PAC by more than 1 °C indicates that the sample does not contain DNA of the nematode of interest.

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

Real-Time PCR diagnostic kit for specific plant pathogenic nematode species

RT-N-D-XXXX

For general laboratory and research use only

Introduction

This ClearDetections Nematode DNA identification and detection kit offers a simple, rapid, and reliable Real-Time PCR assay for detecting DNA of a specific plant pathogenic nematode species. This assay will accurately diagnose the presence of specific nematode species using DNA prepared from nematode suspensions. Note that poor quality DNA will affect the results of this assay.

The performance of each ClearDetections Real-Time PCR nematode identification and detection assay has been optimized for use with the ClearDetections 'Nematode DNA extraction & purification kit' (EX-N-B-SNDE and EX-N-T/P-NDEP) and 'Nematode DNA identification and detection kit for general nematode DNA' (RT-N-D-GENS). We strongly recommend the use of both kits and all available controls. For further information please email 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
ClearDetections PCR mix †	PMD_NOP	Room temp.*
Nematode specific DNA primer set(s)	PS_XXXX_XX	-20˚C**
Positive amplification control (PAC)	PAC_XXXX_XX	Room temp.*
Resuspension buffer	RSB_2	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.

** During the shipment, Nematode species specific DNA primer set(s) are stable at room temperature. After arrival store at -20 ˚C. If properly stored, the kit components can be used until the expiry date printed on the box.

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 from which nematodes are isolated, including diseased plant tissues and peaty soils. 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 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 680 µL of resuspension buffer to each vial.
4. Close the vial(s) and incubate 5 minutes at room temperature.
5. Allow the primer set tube to thaw and briefly spin down the primer set tube.
6. Add 105 µL of Nematode specific primer set into the ClearDetections PCR mix and mix gently by pipetting the content up and down. Mark the vial once the Nematode specific primer set is added.
7. Briefly spin down the PAC tube containing dried PAC DNA before opening.
8. Unscrew the cap of the PAC tube, and add 300 µL of resuspension buffer to dissolve the PAC DNA.
9. Incubate 5 minutes at room temperature.
10. Mix by vortexing and spin down the content of the PAC tube.

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

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

Nematode species specific Real-Time PCR

1. Design the layout of your PCR plate. We recommend using two wells for each sample, plus two wells for the following controls:
   - Negative amplification control (NAC)
   - Positive amplification control (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 PAC tubes for 1 – 2 sec, and briefly spin down the content of PAC tubes.
5. Pipette 5 µL nuclease free water into each NAC well and then pipette 5 µL of each PAC into each PAC well. Load your samples by pipetting 5 µL of each diluted DNA sample into its designated well.

Note: Take great care to avoid contaminating neighbouring wells.

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

6. 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.

7. 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 (35 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.