User Manual

Real-Time PCR identification kit

Scope: External Control DNA

For general laboratory and research use only

We recommended 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.

1. Introduction

The extraction buffer included in our Nematode DNA extraction & purification kit contains a known amount of external control DNA (EC DNA), which consists of non-nematode DNA. The EC DNA Real-Time PCR assay can be used to assess the efficiency of the purification procedures.

Failure to detect the EC DNA using the Real-Time PCR assay indicates that there is either no DNA present in the sample, or that the sample contains compounds that inhibit Real-Time PCR. A weak EC DNA signal similarly indicates a problem and the sample can be flagged for attention.

2. Kit components, storage conditions and shelf life

Components	Storage
ClearDetections'PCR mix	Room temp*
EC specific DNA primer set	-20˚C**
Positive amplification control (PAC)	-20˚C**
Resuspension buffer	Room temp

* Supplied as lyophilized reagent. 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 DNA primer set(s) and PAC(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.

3. Additional reagents and equipment needed

- Centrifuge for either microcentrifuge tubes 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
- Real-Time PCR machine*
- Nuclease free water
- Vortex

*Passive reference dye:
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.

4. Detecting EC DNA in samples by Real-Time PCR

4.1 Preparation of DNA samples and reagents for Real-Time PCR

Diluting DNA extracts is an **essential step** to further reduce the presence of compounds that inhibit Real-Time PCR. Traces of inhibitors are frequently encountered in materials from which nematodes are isolated, including diseased plant tissues and peaty soils.

Diluting each DNA extract **20 fold** (*e.g.*, 1 µL DNA in 19 µL nuclease free water) will normally ensure proper Real-Time PCR performance. Rarely, further dilutions are necessary if the DNA extract contains a high concentration of inhibitors.

Resuspension of the Real-Time PCR Components

1. Gently tap the vial containing the ClearDetections PCR Mix, to settle any contents that may have moved during shipping.
2. Unscrew the cap of ClearDetections PCR mix vial and discard its rubber stopper.
3. Add 680 µL of resuspension buffer.
4. Incubate 5 mins at room temperature.
5. Add 105 µL of EC primer set into vial containing the ClearDetections PCR mix and mix gently by pipetting the content of vial up and down 5 times.

   Note: Mark the vial once the EC specific primer set is added.

Tip: We recommend making aliquots of all re-suspended reagents in order to prevent accidental contamination of master stocks.

4.2 External control DNA Real-Time PCR

1. Design the layout of you Real-Time PCR plate: one well for each sample to be screened for EC plus two wells for the following controls:

   - Negative amplification control (NAC)
   - Positive amplification control (PAC)

2. Vortex the ClearDetections' PCR mix containing the EC specific primer set for 1 – 2 seconds.

3. Pipette 15 µL into each well.

4. Dilute the EC PAC to match the dilution factor of the samples (See 4.1, Sample preparation).

5. Pipette 5 µL diluted EC PAC into the EC PAC well.
   Take care to avoid contaminating neighbouring wells/tubes.

6. Pipette 5 µL of nuclease free water into the NAC well.

7. Pipette 5 µL of each diluted sample into its designated well.

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

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

   No liquids should remain in the lids or sides of each well.

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

	Step	Time	Temperature
Enzyme activation		3 min	95°C
Amplification (35 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.

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

5. Interpretation of results

Important note: for a correct interpretation of results, always:
1) check if results of controls pass;
2) combine amplification curves analysis with melting curves analysis;
3) confirm that the samples melting temperatures match the PACs melting temperatures.

Quick interpretation scheme

1) Check EC PAC and NAC controls

Control	Signal strength (Cq)	Interpretation
(diluted) EC PAC	27 or less	Pass
	above 27 (or no Cq)	Fail*
NAC	No Cq	Pass
	35 or less	Fail*

*See Troubleshooting

If both NAC and EC PAC results pass

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2) Check Cq values in the sample wells

⇒ Analyse the Cq values obtained for each sample and compare them with the Cq value obtained for the PAC diluted to the same dilution factor.

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3) Compare the melting temperature of sample(s) and EC PAC signals.

Explanation

Positive amplification control (EC PAC)
The EC PAC control will give a Cq value of 27 or less if the assay was performed correctly.

Negative amplification control (NAC)
The presence of an EC signal in the NAC indicates contamination of the Real-Time mix or NAC well by EC DNA.

Sample EC
The strength of each EC signal is a measurement of sample quality. Strong Cq values indicate good DNA recovery and high sample purity. Weak Cq values (or no signal) indicate poor DNA recovery and/or poor DNA purity (the presence of inhibitors).

It is essential to check the melting temperature of each EC signal and compare it to the melting temperature of the EC PAC signal (below).

Melting temperature
Signals from the sample and the EC PAC must have the same melting temperature. This useful feature of Real-Time PCR technology is used to confirm that an EC signal is produced only by EC DNA. If the melting temperature of the sample and the EC PAC differ by more than 1 °C, the EC signal is false.

Remember: The lower the Cq value, the stronger the signal!

6. Troubleshooting

- Failed EC PAC (weak or no signal)

  Solution:
  > The assay should be repeated, ensuring that all protocol steps are carefully performed.

- Failed NAC (signal with a Cq value of 35 or less)

  - Cq value of 35 or less indicates contamination of the Real-Time PCR mix or NAC well with EC DNA (from a sample or the EC PAC).

  Signals with a Cq value of 35 or less can sometimes occur. These are easily identified as false by comparing their melting temperature to that of the EC PAC control.

  If the melting temperature of the sample and EC PAC signals match (+/- 1°C) then DNA contamination has occurred and the NAC has truly failed.

  The NAC passes if the melting temperature of the sample and EC PAC signals differ by **more** than +/- 1°C, or if the melting temperature cannot be determined.

  Solution:
  > All sample data must be ignored and the assay must be repeated.
  > Possible source(s) of contamination should be identified and removed.

For further questions or assistance, please do not hesitate to contact us at info@cleardetections.com.

7. 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 for general laboratory and research use only.

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