Alternaria cucumerina - Real Time DNA

BioinGentech Biotechnology

50 /100 /150 tests (Ready to use kit)

A. cucumerina DNA Real Time PCR Kit is a screening assay for a rapid and accurate detection of Alternaria cucumerina.

Principles of the test:

One Step Bioingentech® PCR Kits provide components for “onestep” real time PCR detection in a convenient format that is compatible with both rapid and standard qPCR cycling conditions.

The One Universal qPCR DNA Master Mix include Bioingentech® all reagents for an optimized qPCR. Specific primers and probe for Alternaria cucumerina are provided in the kit and can be detected through your Real Time thermal cycler by the 5’ nuclease PCR detection method. During PCR amplification, forward and reverse primers hybridize to the Alternaria cucumerina target genomic DNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled with a 5-reporter FAM and 3-quencher which can be detected through green channel.

To confirm extraction of a valid biological template, internal control primers and probe mix are included, which consist of a DNA probe labeled with a 5-reporter HEX and a 3-quencher which hybridize inside a specific housekeeping endogenous target gene. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. As a result, a fluorescence increase can be detected on a range of Real Time thermal cyclers through yellow channel.

Our kits also include Positive and Negative Controls which are detailed in the FAQ section.

Principle and use:

This amplification kit has been manufactured by Bioingentech Ltd. to detect Alternaria cucumerina via Real Time PCR. This allows absolute quantification or a qualitative assay.

Real Time PCR is based on fluorogenic dyes. Ct values between 0 - 36 should be taken as positive. Values between 36 - 40 Ct should be taken as marginal positive. Ct values above 40 must be considered as negative **(for more details see Table 5).** This kit needs DNA as a which can be isolated from blood, serum, faeces, respiratory fluid, cerebrospinal fluid, digestive system, tissues, egg yolk, milk, swabs, bacterial cultures, cell lines, among others.

Table 1. Kit Components:

Reactions Tubes	50 test	100 test	150 test
Universal qPCR Master Mix	(1 vial)	(1 vial)	(1 vial)
Primer, Probes and Internal Control Universal Mix	(1 vial)	(1 vial)	(1 vial)
A. cucumerina Positive Control	(1 vial)	(1 vial)	(1 vial)
A. cucumerina Negative Control	(1 vial)	(1 vial)	(1 vial)
PCR grade Water	(1 vial)	(1 vial)	(1 vial)

Table 2. Instrument Compatibility:

- ABI 7300
- ABI 7500FAST
- ABI 7900
- AB Step One
- AB Step One Plus
- Agilent Mx3005P
- CFX96 & CFX384
- ExiCyclerTM 96
- iQ5 & MyiQ Cycler
- Illumina Eco
- LightCycler Nano
- CFX Opus
- Bioer Quant gene 9600
- Mastercycler® ep realplex
- LightCycler 2.0 and 480
- Mx3000P QPCR System
- Mx3005P QPCR System
- RotorGene 3000
- RotorGene 6000
- RorotGeneQ
- SLAN® Real-Time PCR
- Smartcycles II
- CFX Opus 96
- CFX Opus 384
- Quantstudio 3
- Quantstudio 5

Procedure:

Please read through the entire procedure before starting.

Before Starting

- Pulse-spin each tube in a centrifuge before opening.
- Homogenize the solutions for 5 seconds prior to pipetting
- Use different tips in order to avoid cross contamination.
- Use only sterile, RNAse, DNAase, and pyrogen-free tips.

Step 1

Prepare a Master mix according to the following reaction table 3.

Table 3. Reaction components for PCR

Reaction Tubes	Sample and Internal Control	Positive Control	Negative Control
Universal qPCR Master Mix	7,5 µL	7,5 µL	7,5 µL
Primer, Probes and Internal Control Universal Mix	0,6 µL	0,6 µL	0,6 µL
PCR grade Water	3,4 µL	3,4 µL	3,4 µL
DNA Sample	3,5 µL		
A. cucumerina Positive Control		3,5 µL	
A. cucumerina Negative Control			3,5 µL
Total Volume	15 µL	15 µL	15 µL

Step 2

Place the tubes in a thermal cycler and perform One Step qPCR according to the program outlined in **Table 4.**

Table 4. Recommended PCR Cycling table

Cycle	Steps	Time	Temp.(°C)
1 Cycle	Denaturation	3 min	95°C
40 Cycles	Denaturation	10 seg	95°C
	Annealing	1 min	60°C
	Hold	∞	4°C

Note1: Colect data in 60 °C
Note 2: Hold Step is optional.

Interpretation of the test

1) Qualitative analysis:

Ct (Threshold cycle) value of each sample can be interpreted as follows.

Table 5. Ct value result

Ct value	Result
0 - 36	Positive
36 - 40	Marginal Positive
> 40	Negative

1.1.-Ct values over 40 are considered Negative. If the Ct value is in the 0 - 36 range, it must be considered as Positive. This is depending of the sample initial concentration used for each reaction. Note that the sample real concentration could be altered by the sample purity when it is quantified.

1.2.- For more technical information you must request the **QUALITY CONTROL** for each kits. Also you can request more information writing to our email **info@kitpcr.com**

2) Quantitative analysis:

Table 6. Preparation of standard curve dilution series. A. cucumerina positive control:

Average Positive Control Concentration	
A. cucumerina	See Quality Control

Standar curve	Preparation series a fresh dilution	Concentration	Copy Number
Tube N°1:	2uL A. cucumerina Positive Control (2 ng/µL) + 18 µL de PCR grade Water	See quality control	See quality control
Tube N°2:	2uL Tube N°1 + 18 µL de PCR grade Water	See quality control	See quality control
Tube N°3:	2uL Tube N°2 + 18 µL de PCR grade Water	See quality control	See quality control
Tube N°4:	2uL Tube N°3 + 18 µL de PCR grade Water	See quality control	See quality control
Tube N°5:	2uL Tube N°4 + 18 µL de PCR grade Water	See quality control	See quality control
Tube N°6:	2uL Tube N°5 + 18 µL de PCR grade Water	See quality control	See quality control
Tube N°7:	2uL Tube N°6 + 18 µL de PCR grade Water	See quality control	See quality control

2.1. Important Note: Don't forget Homogenize the tubes.
2.2. We will send a Quality Control report for each purchase.
2.3. If you want to obtain less DNA copies you must include a new dilution tube **(Tube N°8).**
2.4. Final DNA copy number will depend of the DNA concentration **(you can see it in Quality Control Report).**

Table 7. Standard curve set up

For each of the tubes (tube 1 to tube 7), take 3,5 µl of the final 15 µl mixture.

	Tube A	Tube B	Tube C	Tube D	Tube E	Tube F	Tube G
Universal qPCR Master Mix	7,5 µL	7,5 µL	7,5 µL	7,5 µL	7,5 µL	7,5 µL	7,5 µL
Primer, Probes and Internal Control Mix	0,6 µL	0,6 µL	0,6 µL	0,6 µL	0,6 µL	0,6 µL	0,6 µL
PCR grade Water	3,4 µL	3,4 µL	3,4 µL	3,4 µL	3,4 µL	3,4 µL	3,4 µL
Tube N° 1 (Positive Control)	3,5 µL						
Tube N° 2		3,5 µL					
Tube N° 3			3,5 µL				
Tube N° 4				3,5 µL			
Tube N° 5					3,5 µL		
Tube N° 6						3,5 µL	
Tube N° 7							3,5 µL
Total Volume	15 µL	15 µL	15 µL	15 µL	15 µL	15 µL	15 µL

Take table N°6

2.5.- Assess the Ct value when the amplification curve of Standard tubes 1, 2, 3, 4, 5, 6, 7 passes the threshold line. Four tubes are sufficient for a standard curve (tubes 1 to 4).
2.6.- Calculate the quantitative value in order to compare the Ct value of the unknown samples with the standard curve.
2.7.- When visualizing the result for the Positive Control in the Real Time thermal cycler, there will be only one amplification curve for the target gene. It will not have an amplification curve for the Internal Control.

3) Test validation:

3.1.- Each Ct value standard should be as follows. Standard 1 < Standard 2 < Standard 3 < Standard 4 < Standard 5 < Standard 6 < Standard 7.
3.2.- R-value of standard curve should be 0.900 - 0.999. R-value represent how well the experimental data fit the regression line. A significant difference in observed Ct values between replicates will lower the R-value.
3.3.- The standard curve slope result should be all negative.
3.4.- The desired amplification efficiencies vary from 90% to 110%. The the oretical maximum of 100% indicates that the polymerase enzyme is functioning at its maximum capacity. Low reaction efficiencies may be caused by poor primer design or by suboptimal reaction conditions. Reaction efficiencies >110 may indicate pipetting error in your serial dilutions or coamplification of non specific products, such as primer-dimers.

Visual explanation FAQ:

1.-Positive control:

The Positive control assay uses a FAM dye and should be detected through the green channel of your Real Time thermal cycler **(see Table 8).**

For copy number determination and as a positive control for the PCR setup, the kit contains a plasmidial positive control template. This can be used to generate a standard curve of Alternaria cucumerina copy number / Ct value. Alternatively, the positive control can be used at a single dilution if full quantitative analysis of the sample is not required. Each time the kit is used, at least one positive control reaction must be included in the run. We recommend running a positive control for each 12 samples.

A positive result indicates that the primer and probes for the target gene worked properly in that particular experimental scenario. If a negative result is obtained the test results should be invalid and must be repeated **(see Table 11).** Seal all other samples and negative controls before pipetting the positive control into its well.

2.-Internal Control:

The internal control is included in the Primer, Probes and Internal Control Mix alongside the target pathogen detection. The internal control assay uses a HEX dye and should be detected through the Yellow channel of your Real Time PCR instrument and should result in a Ct value of 28 (+/-5), depending on the level of sample dilution and concentration. The refore, a positive result through the yellow channel indicates that PCR conditions are suitable for detection of the target pathogen gene.

If a negative result is obtained through the green channel, the results should be analyzed following the **Table 11**

3.-Negative control:

To confirm absence of contamination a negative control reaction should be included every time the kit is used. **Particularly, due to amount of this reagent, you should run a negative control for each 12 samples.** In this instance the Alternaria cucumerina Negative Control should be used in place of template. A negative result indicates that the reagents have not become contaminated. If a positive result and Ct value less than 36 is obtained, the results should be analyzed and check if a correct amplification curve was obtained. When you obtain a clear amplification curve you should consider repeat your assay due to probably the sample was contaminated **(see Table 11).**

* Remember: We recomment running a Positive control and Negative control for each 12 samples. For reaction mix you must use Universal qPCR Master Mix.

Table 8. Fluorogenic probes, Channels and Dyes

* In order to setup your software to read the probes, **(see Table 8 and 9)** and run the corresponding channel:

Channel	Source	Detector	Dyes
Green	470(Nm)	520(Nm)	**FAM**, Sybr green1, Fluorescein, Eva green, Alexa fluor 488
Yellow	530(Nm)	550(Nm)	Joe, Vic,**HEX**, Tet, Cal Fluorgold 540, YaKima Yellow
Orange	585(Nm)	610(Nm)	Rox, Cal Fluor Red 610, Cy3.5, Texas Red, Alexa Fluor 568
Red	625(Nm)	660(Nm)	Cy5, Quasar 670, Lightcycler, Red 640, Alexa Fluor 633, Aeon Zw™.
Crimson	680(Nm)	710(Nm)	Quasar 705, Lightcycler Red 705, Alexa Fluor 680

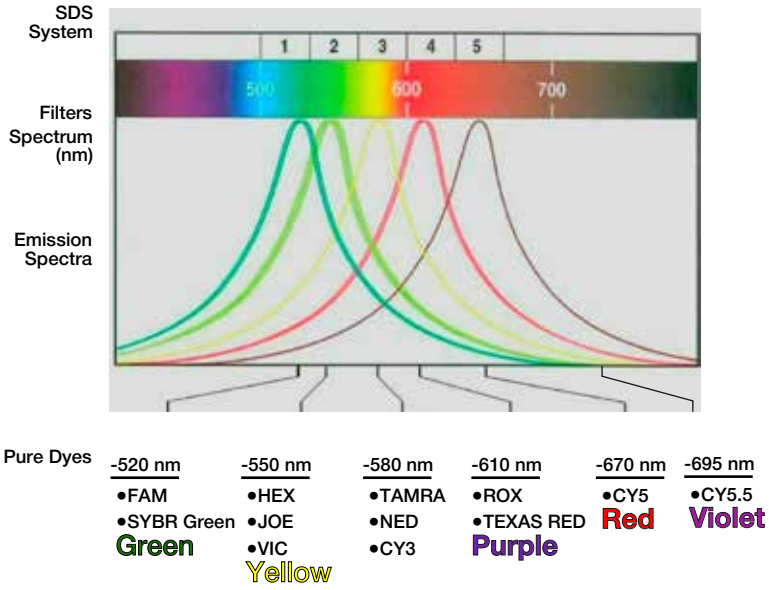

Table 9. Similitary of our fluorophores with HEX and FAM dyes.

	Channel	Dyes
1.- Positive Control	Green	FAM
2.- Internal Control	Yellow	HEX
3.- Sample Target	Green	FAM

Important Note:

Request a Certificate of Analysis when you purchase one of our PCR Kits.

Is important mentioned that we can develop special request for other pathogens or multiplex detection according client's specifications.

We strongly recommend don't use or combine our products with reagents from another kits or unknown provenance. We can't assure good result if incompatibility problems occur.

Temperature

All our reagents are made through protein engineering and are stable at room temperature, the label temperature is just a recommendation after the product is open.

Table 10. Store Temperature Kits

	Store temperature	The label temperature
	Shipping temperature	At room temperature

Table 11. Interpretation of Results

Sample	I.C	N.C	P.C	Result
+	+	-	+	POSITIVE
+	-	-	+	POSITIVE
+	+	+	+	Check Ct and Consider repeat assay*
+	-	+	+	Check Ct and Consider repeat assay*
+	-	-	-	NEGATIVE
+	+	-	-	NEGATIVE
-	+ / -	+ / -	+ / -	NEGATIVE

* Sometimes amplification curves for Negative or Internal control with Ct < 30 can be seen, however it might not be a Positive result. If the amplification curve is not sigmoid, you should consider it as a Negative result.

Table 12. Other Products

Products
OneAgroqPCR-realtime™ A. cucumerina Alternaria cucumerina Real Time 50 tests (Ready to use kit) / Cat. No: Oneq-A628-50D
OneAgroqPCR-realtime™ A. cucumerina Alternaria cucumerina Real Time 100 tests (Ready to use kit) / Cat. No: Oneq-A628-100D
OneAgroqPCR-realtime™ A. cucumerina Alternaria cucumerina Real Time 150 tests (Ready to use kit) / Cat. No: Oneq-A628-150D

Table 13. Products Specifications

Technology	5' nuclease probe based real time PCR assay
Type of nucleic acid Kit	DNA
Kit storage	Shipped at room temperature, the label temperature is just a recommendation after the product is open.
Detection Limit	See Quality Control file. Request it!
Sensitivity & Specify	Ct value between 0 – 36 should be taken positive. Value between 36-40 Ct should be taken as marginal positive. Ct values above 40 must be considered as negative.
Controls included	Internal control, Positive control and Negative control included.
Channels	**FAM** Green channel detect pathogen aplicons
	HEX Yellow channel detect internal control amplicons.
	FAM Green channel detect Positive Control.

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Please visit our web site for more information: www.kitpcr.com