PCRmax Ltd ™ qPCR test

Botrytis cinerea

Species-specific fragment

150 tests

For general laboratory and research use only

Introduction to Botrytis cinerea

Botryotinia cinerea is a spore forming fungus of the Botryotinia genus with a haploid DNA genome of approximately 38 Mb. The asexual form is called Botrytis cinerea, while the sexual form is Botryotinia cinerea (also known as Botryotinia fuckeliana), although they are the same organism. The anamorphic form blights many fruit crops causing rot.

The organism over-winters in one of two ways depending on the conditions. The first is as mycelia – the mass of branching hyphae living on decaying plant matter; while the second is as sclerotia – which is where the mycelia harden and compact, then detaches from the fungi. When conditions improve in the spring, new growth begins with the production of condiophores bearing conidia. These conidia detach and are dispersed by wind to attack new hosts. Initial infection usually occurs in a previously damaged tissue that has increased susceptibility although seedlings are also vulnerable. New mycelia then invade the tissue causing cells death which results in tissue softening and eventually decay. These mycelia ultimately produce new condiophores and the cycle continues. Fungicide can often be used to prevent B. cinerea infection. Preventing the spread of this disease usually involves removing infected plants or parts of the plant during dry conditions.

In grapes – the most commercially affected plant – the fungus causes two conditions: grey rot, in wet or humid conditions which results in the loss of infected bunches; and noble rot, in dry conditions which can result in sweetening the grapes. The fungus also infects many other plants including vegetables and fruit. In these plants it can cause leaf spots, shoot or bud blights, bud blats or cankers depending on the specific part of the plant that is infected.

Specificity

The PCR Max qPCR Kit for Botrytis cinerea (B.cinerea) genomes is designed for the in vitro quantification of B.cinerea genomes. The kit is designed to have the broadest detection profile possible whilst remaining specific to the B.cinerea genome.

The primers and probe sequences in this kit have 100% homology with a broad range of B.cinerea sequences based on a comprehensive bioinformatics analysis.

The intergenic spacer region has previously been identified as a highly specific market for Botryotinia cinerea (Plant Physiol Biochem. 2005 Sep;43(9):890-9) and the primers have 100% homology with all reference sequences for Botryotinia cinerea in the NCBI database (DQ000001.1, AY674786.1, AY694147.1, AY694146.1, AJ422103.1, AJ539088.1).

If you require further information, or have a specific question about the detection profile of this kit then please send an e.mail to help@pcrmax.com and our bioinformatics team will answer your question.

Kit Contents

- **B.cinerea specific primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **B.cinerea positive control template (for Standard curve RED)**
- **Internal extraction control primer/probe mix (150 reactions BROWN)**
  VIC labelled as standard
- **Internal extraction control DNA (150 reactions BLUE)**
- **Endogenous control primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **RNAse/DNAse free water (WHITE)**
  for resuspension of primer/probe mixes and internal extraction control DNA
- **Template preparation buffer (YELLOW)**
  for resuspension of positive control template and standard curve preparation

Reagents and equipment to be supplied by the user

Real-Time PCR Instrument

DNA extraction kit
This kit designed to work well with all processes that yield high quality DNA with minimal PCR inhibitors.

Lyophilised 2x qPCR Mastermix
This kit is designed to be compatible with all commercially available Mastermixes that run with standard cycling conditions.

Pipettors and Tips

Vortex and centrifuge

Thin walled 1.5 ml PCR reaction tubes

Kit storage and stability

This kit is stable at room temperature but should be stored at -20ºC on arrival. Once the lyophilised components have been resuspended they should not be exposed to temperatures above -20ºC for longer than 30 minutes and unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from the date of resuspension under these circumstances.

If a standard curve dilution series is prepared this can be stored frozen for an extended period. If you see any degradation in this serial dilution a fresh standard curve can be prepared from the positive control.

PCRmax does not recommend using the kit after the expiry date stated on the pack.

Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and DNA integrity (An internal PCR control is supplied to test for non specific PCR inhibitors). Always run at least one negative control with the samples. To prepare a negative-control, replace the template DNA sample with RNAse/DNAse free water.

Dynamic range of test

Under optimal PCR conditions PCRmax B.cinerea detection kits have very high priming efficiencies of >95% and can detect less than 100 copies of target template.

Notices and disclaimers

Trademarks

Principles of the test

Real-time PCR

A B.cinerea specific primer and probe mix is provided and this can be detected through the FAM channel.

The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the B.cinerea DNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of real-time PCR platforms.

Positive control

For copy number determination and as a positive control for the PCR set up, the kit contains a positive control template. This can be used to generate a standard curve of B. cinerea copy number / Cq value. Alternatively the positive control can be used at a single dilution where full quantitative analysis of the samples is not required. Each time the kit is used, at least one positive control reaction must be included in the run. A positive result indicates that the primers and probes for detecting the target B.cinerea gene worked properly in that particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false-positive results. This can be achieved by handling this component in a Post PCR environment. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

Negative control

To validate any positive findings a negative control reaction should be included every time the kit is used. For this reaction the RNAse/DNAse free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run.

Internal DNA extraction control

When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis buffer. This control DNA is then co-purified with the sample DNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the control DNA also indicates that PCR inhibitors are not present at a high concentration.

A separate primer and probe mix are supplied with this kit to detect the exogenous DNA using real-time PCR. The primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control DNA does not interfere with detection of the B.cinerea target DNA even when present at low copy number. The Internal control is detected through the VIC channel and gives a Cq value of 28+/-3.

Endogenous control

To confirm extraction of a valid biological template, a primer and probe mix is included to detect an endogenous gene. Detection of the endogenous control is through the FAM channel and it is NOT therefore possible to perform a multiplex with the B.cinerea primers. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

Carry-over prevention using UNG (optional)

Carry over contamination between PCR reactions can be prevented by including uracil-N-glycosylase (UNG) in the reaction mix. Some commercial Mastermix preparations contain UNG or alternatively it can be added as a separate component. UNG can only prevent carry over from PCR reactions that include deoxyuridine triphosphate (dUTP) in the original PCR reaction. PCRmax recommend the application of 0.2U UNG per assay with a 15 minute incubation step at 37°C prior to amplification. The heat-labile UNG is then inactivated during the Taq polymerase activation step.

Reconstitution Protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Filter tips are recommended for all pipetting steps.

1. **Pulse-spin each tube in a centrifuge before opening.**
   This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

2. **Reconstitute the kit components in the RNAse/DNAse free water supplied, according to the table below:**
   To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in water	Volume
Pre-PCR pack	
B.cinerea primer/probe mix (BROWN)	165 µl
Internal extraction control primer/probe mix (BROWN)	165 µl
Endogenous control primer/probe mix (BROWN)	165 µl
Pre-PCR heat-sealed foil	
Internal extraction control DNA (BLUE	600 µl

3. **Reconstitute the positive control template in the template preparation buffer supplied, according to the table below:**
   To ensure complete resuspension, vortex the tube thoroughly.

Component - resuspend in template preparation buffer	Volume
Post-PCR heat-sealed foil	
B.cinerea Positive Control Template (RED) *	500 µl

* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

DNA extraction

The internal extraction control DNA can be added either to the DNA lysis/extraction buffer or to the DNA sample once it has been resuspended in lysis buffer.

DO NOT add the internal extraction control DNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal.

1. **Add 4µl of the Internal extraction control DNA (BLUE) to each sample in DNA lysis/extraction buffer per sample.**

2. **Complete DNA extraction according to the manufacturers protocols.**

Real-time PCR detection protocol

1. **For each DNA sample prepare a reaction mix according to the table below:**
   Include sufficient reactions for positive and negative controls.

Component	Volume
Lyophilised 2x qPCR Mastermix	10 µl
B.cinerea primer/probe mix (BROWN)	1 µl
Internal extraction control primer/probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	3 µl
Final Volume	**15 µl**

2. **For each DNA sample prepare an endogenous control reaction according to the table below (Optional):**
   This control reaction will provide crucial information regarding the quality of the biological sample.

Component	Volume
Lyophilised 2x qPCR Mastermix	10 µl
Endogenous control primer/probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	4 µl
Final Volume	**15 µl**

3. **Pipette 15µl of each mix into individual wells according to your real-time PCR experimental plate set up.**

4. **Prepare sample DNA templates for each of your samples.**

5. **Pipette 5µl of DNA template into each well, according to your experimental plate set up.**
   For negative control wells use 5µl of RNAse/DNAse free water. The final volume in each well is 20µl.

6. **If a standard curve is included for quantitative analysis prepare a reaction mix according to the table below:**

Component	Volume
Lyophilised 2x qPCR Mastermix	10 µl
B.cinerea primer/probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	4 µl
Final Volume	**15 µl**

7. Preparation of standard curve dilution series.

1) Pipette 90µl of template preparation buffer into 5 tubes and label 2-6
2) Pipette 10µl of Positive Control Template (RED) into tube 2
3) Vortex thoroughly
4) Change pipette tip and pipette 10µl from tube 2 into tube 3
5) Vortex thoroughly

Repeat steps 4 and 5 to complete the dilution series

Standard Curve	Copy Number
Tube 1 Positive control (RED)	2×10^5 per µl
Tube 2	2×10^4 per µl
Tube 3	2×10^3 per µl
Tube 4	2×10^2 per µl
Tube 5	20 per µl
Tube 6	2 per µl

8. Pipette 5µl of standard template into each well for the standard curve according to your experimental plate set up.
The final volume in each well is 20µl.

Amplification Protocol

Amplification conditions using Lyophilsed 2x qPCR Mastermix.

	Step	Time	Temp
	UNG treatment (if required) **	15 mins	37 °C
	Enzyme activation	2 mins	95 °C
Cycling x50	Denaturation	10 secs	95 °C
	DATA COLLECTION *	60 secs	60 °C

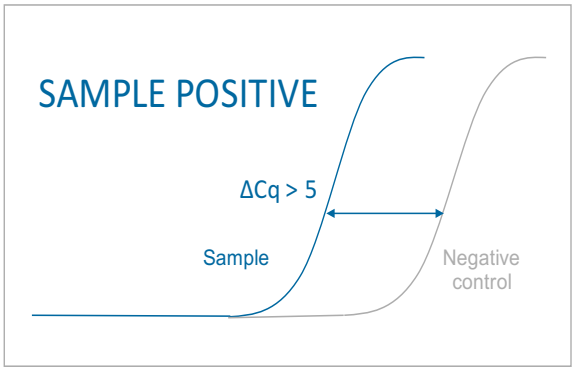
* Fluorogenic data should be collected during this step through the FAM and VIC channels
** Required if your Mastermix includes UNG to prevent PCR carryover contamination

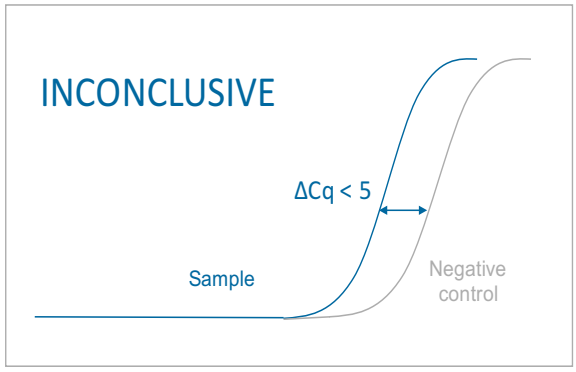
Interpretation of Results

Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
≤ 30	**+ / -**	**+**	**-**	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	**+**	**+**	**-**	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	**-**	**+**	**-**	**POSITIVE QUALITATIVE RESULT** do not report copy number as this may be due to poor sample extraction
-	**+**	**+**	**-**	**NEGATIVE RESULT**
+ / -	**+ / -**	**+**	**≤ 35**	**EXPERIMENT FAILED** due to test contamination
+ / -	**+ / -**	**+**	**> 35**	*
-	**-**	**+**	**-**	**SAMPLE PREPARATION FAILED**
+ / -	**+ / -**	**-**	**+ / -**	**EXPERIMENT FAILED**

Positive control template (RED) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

*Where the test sample is positive and the negative control is positive with a Cq > 35, the sample must be reinterpreted based on the relative signal strength of the two results:





If the sample amplifies > 5 Cq earlier than the negative control then the sample should be reinterpreted (via the table above) with the negative control verified as negative.

If the sample amplifies < 5 Cq earlier than the negative control then the positive sample result is invalidated and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.

Internal PCR control

The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of DNA added to the PCR reaction and the individual machine settings. Cq values of 28±3 are within the normal range. When amplifying a B. cinerea sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Endogenous control

The signal obtained from the endogenous control primer and probe set will vary according to the amount of biological material present in a given sample. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample.