User Guide
PCR *Agrobacterium vitis* kit

Version 04 - 01/11/2020

Information pest: *Agrobacterium vitis*

Agrobacterium vitis is the primary causal agent of grapevine crown gall worldwide. Symptoms of grapevine crown gall disease include tumor formation on the aerial plant parts, whereas both tumorigenic and nontumorigenic strains of *A. vitis* cause root necrosis.
Agrobacterium vitis pathogenicity is mainly determined by a large conjugal tumor-inducing plasmid (pTi) characterized by a mosaic structure with conserved and variable regions. During infection, part of this pTi, the transferred DNA (T-DNA), is transferred to the host plant cell, where it becomes stably integrated in the nuclear genome. The T-DNA contains genes necessary for expression of the tumorous phenotype and genes encoding opines, serving as specific nutrients for the bacteria.
Besides *Agrobacterium vitis*, *Agrobacterium tumefaciens* may also occur on grapevines as causative agent of crown gall disease. Infected vines can harbor both pathogenic and nonpathogenic strains and remain symptomless until the vines are injured. Injury can cause galls which interfere with the function of the vascular system of the plant and reduce its vigor and productivity. To limit the spread of crown gall in vineyards, it is important to test propagation materials and ensure they are free from pathogenic *Agrobacterium* prior to planting.

Introduction

The PCR *Agrobacterium vitis* kit has been developed by Qualiplante and permits to discriminate *Agrobacterium vitis* from *Agrobacterium tumefaciens*. This Triplex End-Point method uses a combination of 3 specific primers sets that permit to amplify:

- the chromosomal polygalacturonase gene *pehA* used to identify *A.vitis* strains and to distinguish them from *A. tumefaciens* (466 bp DNA fragment),
- a region of *virF* gene coding for *A. vitis* nopaline and octopine pTi (382 bp),
- a region of *virD* gene coding for *A. vitis* vitopine.

This product should be used only for research purposes.

Intended use

The PCR kit offers a sensitive diagnostic method to detect and identify agrobacteria that affect grapevine and permit to discriminate *A. vitis* from *A. tumefaciens*. Suitable samples are grapevine tumors tissues, roots or wood canes.
The performance of this kit has been demonstrated using different bacteria strains: *A. tumefaciens* A348, C58 and A281, *A. vitis* from Hungary, from Tunisia, AT6, Tm4, AB3, Zw2, B10/7, AT1, AT66, AB4, Rr4, Ni1, CG49, S4, Sz1, NW221, SF93 and F2/5. This Triplex End-Point PCR kit was also successfully used for the detection of *A. vitis* from symptomless cuttings.

Kit format and content

Article N°	Product name
PCR AgVit 96	PCR *Agrobacterium vitis* 96 tests

Content	96 tests
Direct Master Mix	2x48 tests
Positive Control	8 tests
Negative Control	8 tests

Storage conditions

This kit can be shipped at room temperature but upon receipt it should be stored immediately at the recommended storage temperature **from -30 ° C to -10 ° C**.
Avoid prolonged exposure to light and repeated freeze and thaw cycles.

Shelf life

If the kit is correctly stored, at constant-temperature freezer, its performance is guaranteed until the expiration date indicated on the tubes label.

Materials and equipment (not provided)

- DNA extraction tools and reagents
- Nuclease-free filter tips and micropipettes
- Optical grade nuclease-free tubes/plate
- Disposable latex or vinyl gloves
- DNA ladder and loading-dye buffer
- PCR thermal cycler
- Agarose gel reagents and apparatus

Nucleic acids extraction

Extract DNA from samples according to your usual protocol. Upon request, Qualiplante can recommend you an extraction method.

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Reaction set-up

a) Slowly thaw Direct Master Mix by placing it on ice or at 4°C.
b) Shake briefly Direct Master Mix and spin down the liquid.
c) Add 17 µl of Direct Master Mix (without DNA template) to each PCR tubes or wells of an optical-grade PCR plate.
d) Add 3 µl of DNA template to the Direct Master Mix. Do not forget to prepare a PCR tube or well of a PCR plate for the Positive Control and the Negative Control.

Components	Volume/PCR tube or well
DNA template or Positive control or Negative control	3 µl
Direct Master Mix	17 µl
Total Volume / PCR tube or well	20 µl

In order to confirm the absence of any reagent's contamination, we strongly recommend including a no-template control (e.g. DEPC water) in the assay.

Run and thermal cycling

a) Seal carefully the PCR tubes or PCR plate. Centrifuge briefly to collect components at the bottom of the PCR tubes or wells of the plate. Protect from light before thermocycling.
b) Load the PCR tubes or plate into the PCR thermal cycler and follow the thermal cycling below:

Steps	Temp (°C)	Time	Cycle(s)
Enzyme activation	95°C	12 min	1
Denaturation	95°C	30 sec	30
Annealing	60°C	1 min	
Elongation	72°C	1 min	
Final elongation	72°C	10 min	1
Storage	4°C	∞	-

Agarose gel electrophoresis

Prepare an agarose gel **at 2-2,5% w/v in 1X-TBE buffer.**
Gel loading:
- load the DNA ladder (for example 500 bp DNA step ladder).
- load 10 µl of PCR products from the previous step adding the loading dye buffer (*not provided in the kit)*.

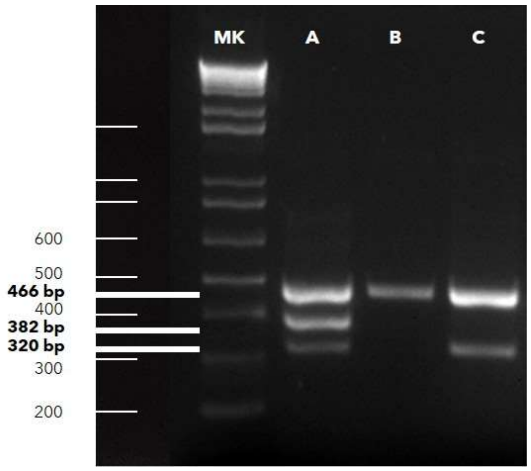
Run: run the gel electrophoresis for 40-45 minutes at 80V.

Results analysis

ANALYSIS VALIDATION

The gene that permit to detect *A. vitis* and to discriminate *A. tumefaciens* is reflected by one DNA fragment of 466 bp.
The genes expressing the virulence of *A.vitis* are reflected by two DNA fragments of 382 bp and 320 bp.
The analysis is validated when:
- ✓ 2 DNA fragments of 466 bp and 382 bp are visible in the positive control lane.
- ✓ No DNA fragment are visible in the negative control lane.

The picture below represents a 1X-TBE 2,5% agarose gel showing the DNA amplification of *A. vitis:*



MK: DNA ladder - **A**: *A.vitis* virulent strain - **B**: *A.vitis* non virulent strain - **C**: *A.vitis* virulent strain

RESULTS INTERPRETATION

The table below permits to determinate the infection status of a sample and the virulence of the bacteria:

Fragment size			Interpretation	Virulence	
466 bp	382 bp	320 bp		Yes	No
			NEGATIVE	-	-
✓	✓	✓	**POS** *A.vitis*	✓	
✓	✓		**POS** *A.vitis*	✓	
✓		✓	**POS** *A.vitis*	✓	
✓			**POS** *A.vitis*		✓
	✓	✓	**POS** *A. tumefaciens*	✓	
	✓		**POS** *A. tumefaciens*	✓	
		✓	**POS** *A. tumefaciens*	✓	

POS: infected sample – NEGATIVE: healthy sample

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Special handling instructions

This kit was designed to be used by laboratory staff trained to follow the usual molecular biology precautions. Always perform the tests in a nuclease-free work environment. Always wear gloves when handling samples containing DNA/RNA and the components of the kit. Do not touch any kit components with an ungloved hand. Use appropriate laboratory disposable parts. Use nuclease-free tubes and filter tips to avoid degradation and cross-contamination. Do not use components from kits with different batch numbers in the same test procedure. Do not interchange reagents with other kits. To avoid cross-contamination, use separate rooms for (a) nucleic acids extraction, (b) preparation of the Master Mix and (c) amplification. To avoid cross-contamination and obtain reliable results, it is essential to strictly follow the protocol in this manual. Avoid unnecessary freeze-thaw cycles of the kit components. Do not use reagents after their expiration date.

Troubleshooting

Post-PCR data analysis shows no amplification, or amplification plots look grossly abnormal:

Possible causes	Corrective actions
Evaporation of the sample due to inadequate sealing of the plate	Repeat the test using the appropriate tools to seal correctly the plate
Consumables are not appropriate for the method	Repeat the test using consumables recommended by the thermal cycler supplier
The quality of nucleic acid extracted is low	Repeat the extraction step. Ensure that the method of extraction has been performed correctly. In any doubt, please, contact us
Abnormal amplification	Centrifuge the plate briefly to spin down the contents and eliminate any air bubbles

No amplification reaction is observed in the positive control well, while other samples are positive:

Possible causes	Corrective actions
The positive control provided with the kit was not added into the reaction well	Repeat the test. If the problem persists, please, contact us

An amplification plot is observed in the negative control well:

Possible causes	Corrective actions
Contamination of the negative control or the Master Mix with target-positive nucleic acid	Repeat the test by applying appropriate quality procedures to prevent contamination. Seal the plate correctly

Warranty and Responsibilities

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Kits components are intended, developed, designed, and sold for Research Purpose Only. Product claims are subject to change.

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