User Guide

PCR ArMV, GFLV, GFkV, GLRaV-1, -2, -3, GVA woRT kit

Version 03 – 30/08/2021

Information pest: Grapevine viruses

Grapevines are affected by many viral diseases; the most harmful and widespread ones are fanleaf degeneration, leafroll, rugose wood, and fleck. The most important strategy to control viral diseases in grapes is preventive and consists of planting virus-free vines during vineyard establishment. The European regulation for grapevine plant material propagation (Directive 2005/43/CE 23/06/2005), included the certification as plant viruses-free for: Grapevine fanleaf virus and Arabis mosaic virus (infectious degeneration), Grapevine leaf-roll associated virus-1 and -3, Grapevine fleck virus (for rootstock only). In Italy, Grapevine virus A has been added for the grapevine certification.

Introduction

The Multiplex End-Point PCR 7 grapevine viruses kit has been developed by Qualiplante based on Gambino and Gribaudo, 2006. A verification was performed by Qualiplante (data not published) and the performance characteristics of the kit are the same as the original publication. This PCR kit allows to detect and to distinguish Arabis mosaic virus (ArMV), Grapevine fanleaf virus (GFLV), Grapevine leafroll-associated virus 1, -2 and -3 (GLRaV-1, -2 and -3), Grapevine fleck virus (GFkV) and Grapevine virus A (GVA).

This method was compared to ELISA method (3 different reagents) during Arnadia Italian project on 122 infected grapevine samples tested by 13 laboratories. The results of the ring-test were presented during the 17th Congress of ICVG, Davis, California, in 2012.

This product should be used only for research purposes.

Intended use

The PCR kit is validated for the detection of ArMV, GFLV, GLRaV-1, GLRaV-2, GLRaV-3, GFkV and GVA in Two-Step Multiplex RT-PCR. Suitable tissues are grapevine leaves and bark scrapings from dormant canes.

Kit format and content

Article N°	Product name
PCR 7GV wo RT 96	*PCR ArMV, GFLV, GFkV, GLRaV-1, -2, -3, GVA woRT 96 tests*

Content	96 tests
Direct Master Mix	2x48 tests
Positive Control A	8 tests
Positive Control B	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)

- RNA 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 RNA from samples according to your usual protocol. Qualiplante recommend you to use RNeasy Plant Mini kit from Qiagen (Ref. 74904) according to MacKenzie protocol (MacKenzie D.J., McLean M.A., Mukerji S., Green M., 1997. Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. Plant disease 81:222-226).

Reverse transcribe the RNA extracted from your samples into complementary DNA (cDNA) according to your usual protocol.

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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 23 µl of Direct Master Mix (without cDNA template) to each PCR tubes or wells of an optical-grade PCR plate.
d) Add 2 µl of cDNA template to the Direct Master Mix. Do not forget to prepare a PCR tube or well of an optical-grade PCR plate for the Positive Controls A and B and the Negative Control.

Components	Volume/PCR tube or well
cDNA template or Positive control A or Positive control B or Negative control	2 µl
Direct Master Mix	23 µl
Total Volume / PCR tube or well	25 µl

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)
Initial denaturation	95°C	15 min	1
Denturation	95°C	30 sec	35
Annealing	55°C	1 min 30 sec	
Elongation	72°C	1 min 30 sec	
Final elongation	72°C	10 min	1
Storage	4°C	∞	-

Agarose gel electrophoresis

Prepare an agarose gel **at 2-2,8% w/v in 0,5X-TAE buffer.**

Gel loading:
- load the DNA ladder (for example 1'000 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 minutes to 1 hour at 80V.

Results analysis

ANALYSIS VALIDATION

The different DNA fragment size obtained are specific for each virus and are indicated in the table 1.

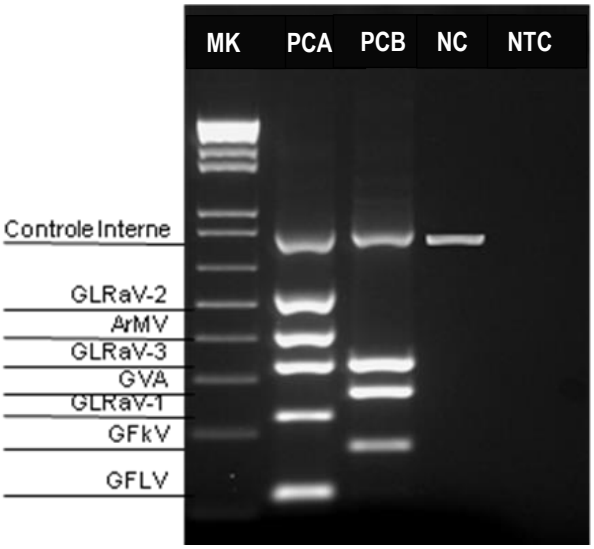
Target	Fragment size (bp)	PC A	PC B	NC
IC	844	X	X	X
GLRaV-2	543	X	-	-
ArMV	416	X	-	-
GLRaV-3	336	X	X	-
GVA	272	-	X	-
GLRaV-1	232	X	-	-
GFkV	179	-	X	-
GFLV	118	X	-	-

IC: Internal Control / PC: positive control / NC: negative control

A DNA fragment of 350 bp can appear in the Positive Control A but is not applicable with this PCR kit.

The analysis is validated when:
- ✓ 7 DNA fragments of 844 bp (Internal Control), 543 bp (GLRaV-2), 416 bp (ArMV), 336 bp (GLRaV-3), 232 bp (GLRaV-1) and 118 bp (GFLV) are visible in the Positive Control A lane.
- ✓ 4 DNA fragments of 844 bp (Internal Control), 336 bp (GLRaV-3), 272 bp (GVA) and 179 bp (GFkV) are visible in the Positive Control B lane.
- ✓ Only one DNA fragment of 844 bp is visible in the Negative Control lane corresponding to the Internal Control.
- ✓ All lanes must contain a DNA fragment of 844 bp in order to validate the nucleic acids extraction and the amplification.

The picture below represents a 0,5X-TAE 2,5% agarose gel showing the cDNA grapevine viruses*:*



MK: DNA MW ladder **- PC1:** Positive control A including amplicons relative to the Internal Control, GLRaV-2, ArMV, GLRaV-3, GLRaV-1 and GFLV - **PC2:** Positive control B including amplicons relative to the Internal Control, GLRaV-3, GVA and GFkV - **NC:** Negative control including the Internal Control - **NTC:** no template control (nuclease free water)

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RESULTS INTERPRETATION

In order to determinate the infection status of a sample, compare the size of DNA fragment(s) obtained in the sample lane with those corresponding to the Positive Controls and Negative Control.

A sample is declared as infected when the DNA fragment corresponds to the specific amplicon for one (or more) virus(es).

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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