User Guide

nPCR Universal Phytoplasma kit

Version 03 – 22/07/2022

Information pest: Universal phytoplasma

The phytoplasma are obligate plant prokaryotic plant pathogens that do not possess cell walls. On the basis of the conserved 16S rDNA gene sequence similarity, the currently known phytoplasmas are classified into a number of different 16S ribosomal (16Sr) groups and subgroups (Duduk & Bertaccini, 2011; Dickinson et al., 2013). The phytoplasmas are found in the phloem cells of host plants and occur worldwide. Symptoms that are characteristic of diseases caused by phytoplasmas include yellowing of leaves, reduction of the leaf size, stunting of the plant and proliferation of axillaries buds. Phytoplasmas are transmitted by insect vectors and by vegetatively propagated plant material, causing economical losses especially on fruit tree production.

Introduction

A number of universal PCR primers have been designed that allow amplification of the 16S rRNA gene of any known phytoplasma. The most commonly used primers are the P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) and R16F2n/R16R2 (Lee et al., 1993; Gundersen and Lee, 1996) primer pairs, which are used in this PCR Uniphy kit. The primer pair used for the 1st amplification amplifies a PCR product that contains the entire 16S rRNA gene as well as the 16S/23S rRNA spacer region. This primer pair was used by Smart et al. (1996) to amplify the 16S/23S rRNA spacer region of 10 different plytoplasma groups. Gundersen and Lee (1996) evaluated the R16F2n/R16R2 primers with representative phytoplasmas for 10 distinct 16 rRNA groups. Since then, both primer pairs have been used to characterize numerous new phytoplasma groups.

This product should be used only for research purposes.

Intended use

The PCR kit is validated for the detection of a large number of phytoplasmas in Nested End-Point PCR. Best suitable tissues are leaves with symptoms knowing that phytoplasmas reside almost exclusively in the phloem sieve elements (cambium, petioles, midveins). Although phytoplasmas can be detected in roots and bark scrapings of dormant trees, generally it is best to test for phytoplasmas at the end of summer.

Kit format and content

Article N°	Product name
nPCR Uniphy 96	Nested End-Point PCR Universal phytoplasma 96 tests

Content	96 tests
Direct Master Mix	2x48 tests
Nested 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.

Qualiplante SAS
4 rue du Mas de Lépôt
FR-34880 Lavérune

www.qualiplante.eu
info@qualiplante.eu

FIRST PCR

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 DNA template) to each PCR tubes or wells of an optical-grade PCR plate.
d) Add 2 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	2 µl
Direct Master Mix	23 µl
Total Volume / PCR tube or well	25 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)
Initial denaturation	94°C	2 min	1
Denaturation	94°C	30 sec	40
Annealing	53°C	30 sec	
Elongation	72°C	2 min	
Final elongation	72°C	10 min	1
Storage	4°C	∞	-

Dilution of 1st PCR products

Using DNase and RNase free PCR tubes, **dilute to 1:30** in sterile water each PCR product for samples, Positive Control and Negative Control obtained during the previous step.

Shake and spin down each tube.

SECOND PCR

Reaction set-up

a) Slowly thaw Nested Master Mix by placing it on ice or at 4°C.
b) Shake briefly Nested Master Mix and spin down the liquid.
c) Add 23 µl of Nested Master Mix (without DNA template) to each PCR tubes or wells of an optical-grade PCR plate.
d) Add 2 µl of **diluted 1st PCR products** to the Nested Master Mix. Do not forget to prepare a PCR tube or well of a PCR plate for the 1st PCR products obtained with Positive Control and Negative Control.

Components	Volume/PCR tube or well
PCR products from the 1st PCR diluted to 1:30 (*)	2 µl
Nested Master Mix	23 µl
Total Volume / PCR tube or well	25 µl

(*) See the section "Dilution of 1st PCR products"

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	94°C	2 min	1
Denaturation	94°C	30 sec	40
Annealing	50°C	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 1% w/v in 1X-TAE buffer.**

Gel loading:
- load the DNA ladder (for example 1'500 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.

Qualiplante SAS
4 rue du Mas de Lépôt
FR-34880 Lavérune

www.qualiplante.eu
info@qualiplante.eu

Results analysis

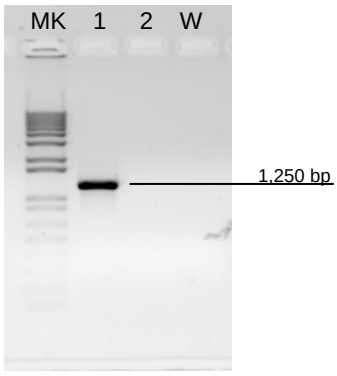
ANALYSIS VALIDATION

The specific product of Universal phytoplasma is a **1'250 bp fragment**.

The analysis is validated when:

- ✓ 1 DNA fragment of 1'250 bp is visible in the positive control lane.
- ✓ none DNA fragment are visible in the negative control lane.

The picture below represents a 1X-TAE 1% agarose gel showing the DNA amplification of Flavescence dorée phytoplasma extracted from symptomatic grapevine samples.



MK: DNA ladder - **1:** Positive Control of the kit or sample infected by Flavescence dorée - **2:** Healthy sample or Negative Control of the kit - **W**: No template control.

RESULTS INTERPRETATION

- ✓ A sample is **positive** when a 1'250 bp DNA specific fragment is present in the PCR reaction; in this case, the sample is infected by a phytoplasma.
- ✓ A sample is negative when no fragment is present in the PCR reaction.

The table below summarizes the results interpretation:

Fragment size **1'250 bp**	Interpretation
-	NEGATIVE
✓	**POSITIVE** Phytoplasma infection

POSITIVE: infected sample – NEGATIVE: healthy sample

To identify the phytoplasma present in positive samples, the amplicon will need to be sequenced.

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 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.

Qualiplante SAS
4 rue du Mas de Lépôt
FR-34880 Lavérune

www.qualiplante.eu
info@qualiplante.eu

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

Qualiplante SAS guarantees the buyer exclusively concerning the quality of reagents and of the components used to produce the Kits. Any product not fulfilling the specifications included in the product sheet will be replaced. This warranty limits Qualiplante SAS responsibility to the replacement of the product. No other warranties, of any kind, express or implied-are provided by Qualiplante SAS.
Qualiplante SAS is not responsible and cannot anyway be considered responsible or jointly responsible for possible direct and indirect damages resulting of the use and/or the misuses of the Kits. The user consciously and under her/his own responsibilities decides for the utilization purposes of the Kits and uses it the way she/he considers most suitable in order to reach her/his goals and/or objectives. Qualiplante SAS is not responsible for the data resulting from the use of the Kits, for the utilization that the user independently decides to make of them or for the direct or indirect damages possibly resulting from the disclosure or transmission of the data themselves to third parties under any form or circumstance. This clause is automatically accepted by the user when purchasing the Kits.
Some of the applications which may be performed with this product may be covered by applicable patents in certain countries. The purchase of this product does not include or provide a license to perform patented applications. Users may be required to obtain a license depending on the country and/or application. Qualiplante SAS does not encourage the unlicensed use of patented applications. The Kits may require the use of Taq Polymerase enzyme, DNA binding components and fluorochromes/quencher, often registered as trademark by companies. The product, equipment and information included in the Kits consist of assembled reagents. The Kits are designed for the services supply, quality control or any other application that is not exclusively an internal company's research and requires a specific license for PCR and Real-Time PCR use. The license and authorization for PCR and Real-Time PCR use are not included in the Kits. The user is responsible for setting prefixed goals, choosing whether or not to perform the PCR or Real-Time PCR reaction and to apply for register her/his own license.
The Kits have been internally tested by our quality control. Any responsibility is waived if the warranty of quality control does not refer to the specific Kits. The user is personally responsible for data that she/he will obtain and/or she/he will supply to third parties using these Kits. Once the sealed package is opened the user accepts all the conditions without fail; if the package is still sealed the kit can be returned and the user can be refunded.
Kits components are intended, developed, designed, and sold for Research Purpose Only. Product claims are subject to change.

Qualiplante SAS
4 rue du Mas de Lépôt
FR-34880 Lavérune

www.qualiplante.eu
info@qualiplante.eu