User Guide

qPCR Universal phytoplasma kit

Version 07 – 17/01/2024

Information pest:

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; Disckinson 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

The qPCR Uniphy kit has been developed by Qualiplante based on Christensen et al., 2004. A verification was performed by Qualiplante (data not published) and the performance characteristics of the kit are the same as the original publication.

Probes and primers for phytoplasma detection were based on alignments of 16S rDNA from a range of phytoplasma strains (one of each phytoplasma 16Sr group), bacteria, and mycoplasmas. Primers were designed to amplify DNA from a broad range of phytoplasma strains, excluding amplification of bacterial DNA.

A broad range of phytoplasma strains belonging to different subgroups of the phytoplasma 16S rDNA *gene* were detected using the assay. In details, the assay is specific for 16SrI-B (American aster yellows), 16SrI-C (Clover phyllody), 16SrII-A, (Sesame phyllody), 16SrIII-A (Green valley X), 16SrIII-B (*Crepis biennis* yellows), 16SrIII-H (Poinsettia branch-inducing), 16SrV-A (Elm yellows), 16SrV-B (Jujube witches' broom), 16SrV-C (Alder Yellows, Grapevine yellows), 16SrV-D (Grapevine yellows), 16SrV-E (Rubus stunt), 16SrVI (Lucerne virescence), 16SrVII (Ash yellows), 16SrIX (*Pichris echioides* yellows), 16SrX-A (Apple proliferation), 16SrX-B (German stone fruit yellows), 16SrXI (Flower stunting), 16SrXII-A (Bois noir, Sour cherry).

No cross reactions were observed for the following bacteria: *Agrobacterium radiobacter, Arthrobacter globiformis, A. oxydans, Bacillus gibsonii, B. megaterium, Clavibacter michiganense, Paenibacillus macerans, Pseudomonas putida, Ralstonia pickettii* and *Rhodococcus equi.*

The Universal phytoplasma specific probe is labelled with FAM® fluorophore.

This method was evaluated by testing phytoplasmas from 18 subgroups and was found to have an analytical sensitivity equal to or up to ten times higher than conventional nested PCR, depending on the host–phytoplasma combination. A test performance study was realized during the EUPHRESCO project FruitPhytoInterlab (2011).
The real-time PCR test from Christensen et al., 2004 is recommended by the European and Mediterranean Plant Protection Organization (www.eppo.int) - PM7/133, Bulletin (2018) 48 (3), 414–424.
This product should be used only for research purposes.

Intended use

The qPCR kit is validated for the detection of a large number of phytoplasmas in Real-Time PCR. Suitable tissues are leaves with symptoms (leaf petioles and midveins, stems or inner bark). 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
qPCR UNIPhy 96	qPCR Universal phytoplasma 96 tests

Content	96 tests
Nuclease free water	2 x 1,25 ml
Primers/probe mix	96 tests
Taq polymerase	2 x 48 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
- Thermal-cycler for Real-Time PCR with filters calibrated for FAM®

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Nucleic acids extraction

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

Reaction set-up

a) Do a short spin in a centrifuge to collect the pellet at the bottom of the **Primers/probe mix** tube.
b) Add 1.300,00 µl (one thousand three hundred) of **nuclease free water** provided with the kit in the **Primers/probe mix** tube. Vortex vigorously up and down the **Primers/probe mix** tube. Spin down the liquid.
c) Slowly thaw **Taq polymerase** by placing it on ice or at 4°C.
d) Shake briefly **Taq polymerase** and spin down the liquid.
e) For each reaction, in a new tube (not provided), prepare the **Master mix** by mixing 4 µL of **Taq polymerase** in 13 µl of **Primers/probe mix**. Do not forget the Positive Control and Negative Control of the kit which must be added to check that the reaction is working correctly.

   Example: if you have 8 samples to test, you will need 10 reactions (8 samples + 1 Positive Control + 1 Negative Control) of 17 µl each. In a tube, please, mix 40 µL of **Taq polymerase** in 130 µl of **Primers/probe mix**.

f) Add 17 µl of the freshly prepared **Master mix** (13 µl of **Primers/probe mix** + 4 µl of **Taq polymerase**) to each PCR tubes or wells of an optical-grade PCR plate.
g) Add 3 µl of DNA template. Do not forget to prepare a PCR tube or well of an optical-grade 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
Master mix (13 µl of Primers/probe mix + 4 µl of Taq polymerase)	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.
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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 thermal-cycler and follow the thermal cycling below:

Steps	Temp (°C)	Time	Cycle(s)
Enzyme activation	95°C	12 min	1
Denaturation	95°C	15 sec	40
Annealing and elongation	60°C	60 sec	

Results analysis

The reaction for Universal phytoplasma will generate a specific FAM®-labeled amplification curve.

Fig.1: Example of amplifications curves relative to a sample infected by phytoplasma and a healthy sample

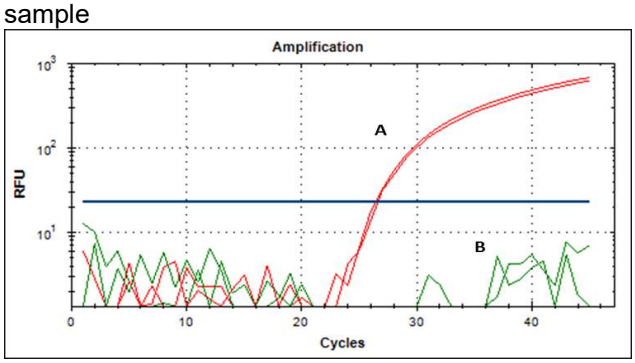

fig.1 shows the amplification curves associated to a sample infected by *Candidatus Phytoplasma ulmi* or Positive Control (red curve) and to a healthy sample or Negative Control (green curve).

ANALYSIS VALIDATION

The PCR plate is validated only when:
- ✓ the Positive Control generates an amplification curve for the FAM® fluorophore. The Cycle threshold (Ct) value of the FAM®-labeled amplification curve should be below to 35 (***fig.1***).
- ✓ the Negative Control does not generate any curve associated to the fluorophore FAM® or a Ct value higher or equal to 35.

RESULTS INTERPRETATION

When all the previous conditions are performed, the amplification results are interpreted as indicated in the **tab. 1**.

Ct < 35	Positive
Ct ≥ 35	Negative

tab.1 shows the results interpretation

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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 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, 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, 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.
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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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