User Guide
qPCR *Xylella fastidiosa* kit
Version 02 – 10/06/2022

qualiplante

Information pest: *Xylella fastidiosa*

Xylella fastidiosa is a regulated plant pathogen in many parts of the world and was included in European legislation as a quarantine pest.
Xylella fastidiosa is a Gram-negative bacterium which causes many important plant diseases such as Pierce's disease in grapevine, citrus veinal chlorosis, almond leaf scorch, phony peach and leaf scorch on a large range of ornamental plants and shade trees. Leafhoppers of the subfamily *Cicadellinae* and family *Cercopidae* (*Hemiptera*) are the most common known vectors.
Xylella fastidiosa was first described by Newton Pierce in 1892 on grapevine in USA. Until recently, it was mainly distributed throughout the Americas but there have now been reports of outbreaks in Asia and Europe.

Introduction

The qPCR *Xylella fastidiosa* kit has been developed by Qualiplante based on Harper and al., 2010, Erratum 2013. A verification was performed by Qualiplante (data not published) and the performance characteristics of the kit are the same as the original publication.
The *X. fastidiosa* target sequence is located in the gene coding for the 16S rRNA-processing *RimM* protein. The Internal Control (IC) has been designed on the COX gene.
The qPCR *Xylella fastidiosa* kit enables the detection of all the 4 main subspecies of *Xylella fastidiosa*:

- *X. fastidiosa* subsp. *fastidiosa,*
- *X. fastidiosa* subsp. *multiplex,*
- *X. fastidiosa* subsp. *sandyi,*
- and *X. fastidiosa* subsp. *pauca.*

without any distinction, by Real-Time PCR.
No cross reactions were observed from other bacterial species as *Xanthomonas axonopodis* pv. *aurantifolii, X. campestris* pv. *citri, X. arboricola* pv. *fragariae, Pseudomonas syringae* pv. *persicae, Pantoea agglomerans, Agrobacterium tumefaciens* and *Spiroplasma citri.*
The *X. fastidiosa* specific probe is labelled with FAM® fluorophore. An internal control (IC) specific probe, labelled with Cy5® fluorophore, permits user to validate the DNA extraction step from the plant.
The real-time test of Harper et al., 2010, Erratum 2013 is recommended by the European and Mediterranean Plant Protection Organization (www.eppo.int) - PM7/24, Bulletin (2019) 49 (2), 175–227.
This product should be used only for research purposes.

Intended use

The qPCR kit is validated for the simultaneous detection of *Xylella fastidiosa* (Xfast) and internal control (IC) in Real-Time PCR. Suitable tissues are plant petioles and central veins, non-woody branches or stems, vascular tissues of woody-branches (xylem).
If the sample is symptomatic, sample organs with leaf burns and/or chlorosis and/or desiccants.

- for plant species with large petioles (e.g., coffee trees, mulberry trees, grapevines...), sampling is carried out on different leaves, branches or stems (minimum 5 leaves).
- for plant species with small petioles (ex: polygales, olive trees…) or without petiole and with small central vein (ex: rosemary...), sampling is carried out on different leaves, branches or stems (minimum 25 leaves).

During the period from March to July (in the European area), **in the absence of symptoms**, for *Prunus spp*. with deciduous leaves, it is recommended to collect the vascular tissues of woody branches (xylem), by removing the bark with a scalpel.

Kit format and content

Two kits are available for 24 and 96 tests.

Article N°	Product name
qPCR Xfast 24	qPCR *Xylella fastidiosa* 24 tests
qPCR Xfast 96	qPCR *Xylella fastidiosa* 96 tests

Content	24 tests	96 tests
Direct Master Mix	24 tests	2x48 tests
Positive Control	3 tests	8 tests
Negative Control	3 tests	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® and Cy5®

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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) 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 18 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 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	2 µl
Direct Master Mix	18 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 thermal-cycler and follow the thermal cycling below:

Steps	Temp (°C)	Time	Cycle(s)
Enzyme activation	95°C	12 min	1
Denaturation	94°C	10 sec	40
Annealing and elongation	62°C	40 sec	

Results analysis

The reaction for *Xylella fastidiosa* will generate a specific FAM®-labeled amplification curve.
The reaction for Internal Control will generate a specific Cy5®-labeled amplification curve in all the wells except those that contains the no-template control.

Fig.1: Example of an amplification curve relative to a *Xylella fastidiosa* positive sample.

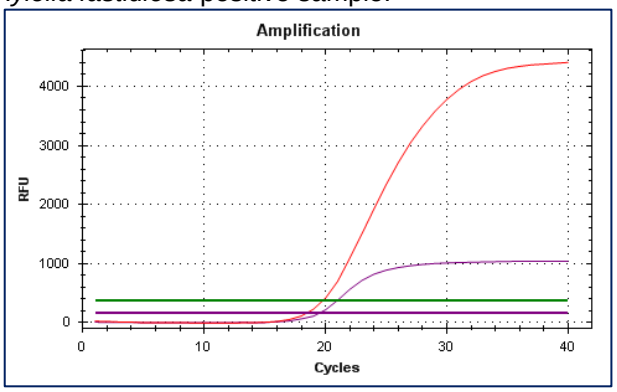

fig.1 shows the amplification curves associated to a *Xylella fastidiosa*-infected sample or Positive Control (red curve) and the relative Internal Control (violet curve).

Fig.2: Example of an amplification curve relative to a healthy olive tree sample.

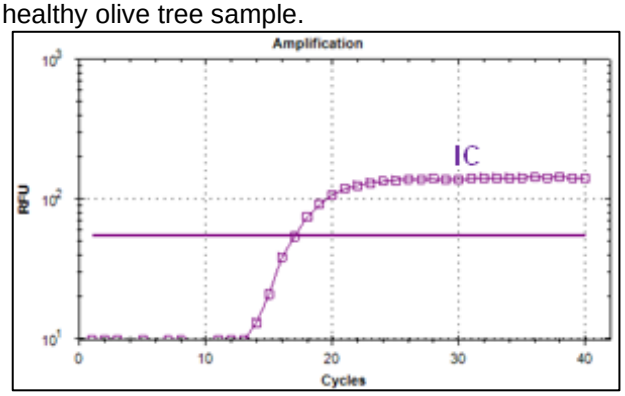

fig.2 shows the amplification curve associated to a healthy sample or the Negative Control (violet curve). Only the Internal Control curve must appear (violet curve).

ANALYSIS VALIDATION

The PCR plate is validated only when:

- ✓ the Positive Control generates an amplification curve for the two fluorophores FAM® and Cy5®. The Cycle threshold (Ct) value of the FAM®-labeled amplification curve should be below or equal to 35 (***fig.1***).
- ✓ the Negative Control does not generate any curve associated to the fluorophore FAM® but it generates a curve associated to the fluorophore Cy5® (***fig. 2***).
- ✓ the amplification plot associated to the IC is present in all the wells except in the no-template control(*).
- ✓ the no-template control does not generate any curve for the two fluorophores FAM® and Cy5® (*).

(*) An amplification curve may sometimes appear for the fluorophore Cy5® in the no-template control. In this case, the experiment is validated only if the Ct value is higher than 28.

RESULTS INTERPRETATION

When all the previous conditions are performed, the amplification results are interpreted as indicated in the **tab. 1**, by considering for each sample the Ct of the

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curve generated by the FAM® fluorophore specific to *Xylella fastidiosa* and the Cy5® fluorophore specific to the IC.
The columns of the **tab. 1** refer to the Ct of the IC amplification and the lines refer to the Ct of *Xylella fastidiosa* amplification.

	Cy5 fluorophore	
FAM fluorophore	**Ct IC < 25**	**Ct IC ≥ 25**
Ct ≤ 35	**Positive**	**Positive**
35 < Ct ≤ 38	**Indeterminate** (*)	*Unreliable* (**)
Ct > 38	**Negative**	*Unreliable* (**)
No Ct	**Negative**	*Unreliable* (**)

tab.1 shows the results interpretation
(*) The concentration of bacteria could be very low in this sample, approaching the limit of detection. The results may not be reproducible.
(**) Some inhibitors of the *Taq* polymerase might be present in this sample. Repeat the analysis by diluting the DNA of this sample to 1:5 or to 1:10. If the Ct value of IC is still **≥ 25** after repeating the analysis on diluted sample, the DNA extraction must be repeated.

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

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