User Guide
qPCR Flavescence dorée/Bois noir kit

Version 06 - 01/11/2020

Information pest: *Candidatus phytoplasma vitis* and *Candidatus phytoplasma solani*

Flavescence dorée (FD) is one of the greatest threats for grapevine in Europe. It is caused by the phytoplasma *Candidatus phytoplasma vitis*, belonging to the elm yellows group (16SrV group), efficiently transmitted by the vector *Scaphoidus titanus*. This phytoplasma is a regulated plant pathogen in Europe and is included in European legislation as a quarantine pest.
Bois noir (BN) is caused by the phytoplasma *Candidatus phytoplasma solani,* belonging to the 16SrXII-A group, transmitted principally by the vector *Hyalesthes obsoletus.*
Disease symptoms develop mainly in summer. Leaves turn yellow or red depending on the cultivar. They roll down-ward and become brittle; the interveinal area of leaves may become necrotic. Shoots show incomplete lignification.
Flavescence dorée's symptoms are similar to those caused by other yellows diseases of grapevine, in particular Bois noir; molecular diagnostic is the only way to differentiate these two phytoplasmas.

Introduction

The qPCR FD BN kit is based on an analysis method developed by the company International Plant Analysis and Diagnostics (www.ipadlab.eu). The primers and probes were designed on the *rpl14* FD gene and the *rpl22-rps3* BN gene. The primers and probes sequences and their use in diagnostic tests are the subject of a PCT patent application by IpadLab (PCT/IB2010/053563). This Triplex Real-Time PCR kit offers a specific and sensitive method to detect simultaneously 16SrV phytoplasmas including FD and 16SrXII phytoplasmas including BN, as well as an endogenous control designed on the *COX* gene.

Validation data of the method are available from a test performance study realized in 2013 (Euphresco Grafdepi project - report available from this link: http://www.euphresco.net/media/project_reports/grafdepi_final_report.pdf). This method is also referred in the Appendix 6 of the PM7/079 (2) Grapevine Flavescence dorée phytoplasma, European and Mediterranean Plant Protection Organization Bulletin (2016) 46 (1), 78-93 available from this link: https://onlinelibrary.wiley.com/doi/epdf/10.1111/epp.12280.
This product should be used only for research purposes.

Intended use

The qPCR kit is validated for the simultaneous detection of Flavescence dorée, Bois noir and Internal Control (IC) in Triplex Real-Time PCR. Suitable tissues are grapevine leaves, preferably primary veins and petioles. Other parts of grapevine tissues can be analyzed, for example vine stocks, or insects' vectors, using specific nucleic acids extraction's methods.

It is preferable to test plant tissues with vine yellows symptoms, sampled from an early stage of the veraison until the beginning of the senescence (from end of August to end of October in the Europe zone).

Kit format and content

Two kits are available for 24 and 96 tests.

Article N°	Product name
qPCR FDBN 24	qPCR FD BN 24 tests
qPCR FDBN 96	qPCR FD BN 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®, VIC® and Cy5®

Nucleic acids extraction

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

Qualiplante SAS
Cap Alpha – Avenue de l'Europe
FR-34830 Clapiers

www.qualiplante.eu
info@qualiplante.eu

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	10 min	1
Denaturation	95°C	15 sec	45
Annealing and elongation	60°C	60 sec	

Results analysis

The reaction for Flavescence dorée will generate a specific FAM®-labeled amplification curve.
The reaction for Bois noir will generate a specific VIC®-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 Flavescence dorée positive sample.

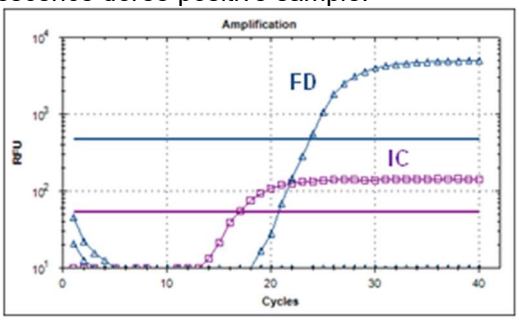

fig.1 shows the amplification curves associated to a FD-infected sample (blue curve) and the relative Internal Control (violet curve).

Fig.2: Example of an amplification curve relative to a Bois noir positive sample.

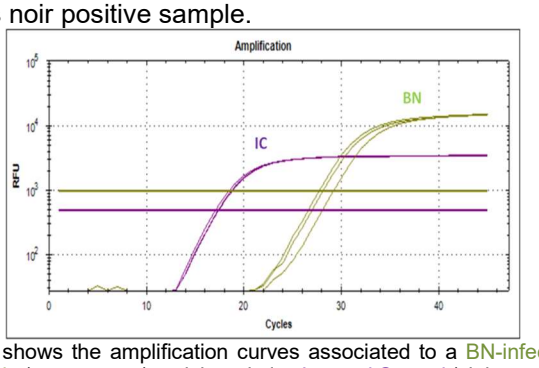

fig.2 shows the amplification curves associated to a BN-infected sample (green curve) and the relative Internal Control (violet curve).

Fig 3: Example of an amplification curve relative to a Flavescence dorée / Bois noir positive sample.

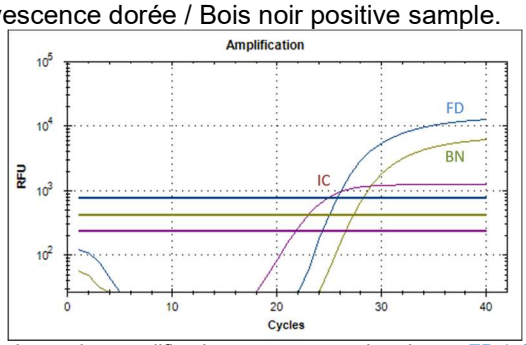

fig.3 shows the amplification curves associated to a FD infected sample (blue curve), a BN-infected sample (green curves) and the relative Internal Control (violet curve). This figure shows the Positive Control amplification curves of the kit.

Fig 4: Example of amplification curve relative to a healthy FD and BN sample

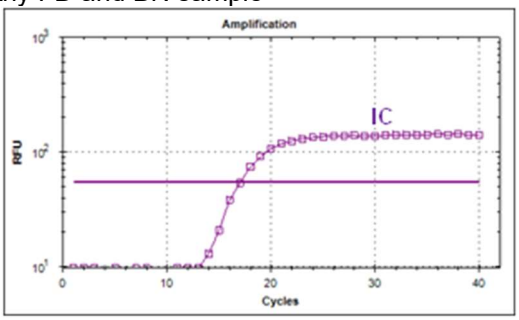

fig. 4 shows the amplification curve associated to a healthy sample and to the Negative Control of the kit. Only the Internal Control curve must appear (violet curve).

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ANALYSIS VALIDATION

The PCR plate is validated only when:

- ✓ the Positive Control generates an amplification curve for the three fluorophores FAM®, VIC® and Cy5® higher than the respective threshold lines ***(fig. 3)***.
- ✓ the Negative Control does not generate any curve associated to the fluorophores FAM® and VIC®, but it generates an amplification curve associated to the fluorophore Cy5® for the Internal Control ***(fig. 4)***.
- ✓ the amplification plot associated to the Internal Control is present in all the wells, except in the no-template control(*).
- ✓ the no-template control does not generate any curve for the three fluorophores FAM®, VIC® 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 curve generated by the FAM® fluorophore specific to FD, the VIC® fluorophore specific to BN 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 FD and BN-specific amplifications.

	Cy5 fluorophore	
Fluorophore	**Ct IC < 22**	**Ct IC ≥ 22**
Ct FD ≤ 40	Positive	Positive
Ct BN ≤ 40	Positive	Positive
40 < Ct FD < 45	Uninterpretable(*)	*Unreliable* (**)
40 < Ct BN < 45	Uninterpretable(*)	*Unreliable* (**)
No Ct	Negative for FD and BN	*Unreliable* (**)

tab.1 shows the results interpretation
(*) The concentration of phytoplasma could be very low in this sample, approaching the limit of detection. The results may not be reproducible. We recommend you to use another method to analyze this sample (PCR.FD BN kit for example).
(**) 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 **≥ 22** 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.

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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.
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.
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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.

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