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 PCR FD BN set has been developed by Qualiplante according to the previous French official method MOA006A-1b: "*détection des phytoplasmes de la vigne du groupe 16SrV (flavescence dorée) et du groupe 16SrXII (bois noir) – PCR multiplex gigogne*", currently replaced by a Real-Time PCR method. This method was evaluated during the EUPHRESCO Grafdepi I project (results available from www.euphresco.net/media/project_reports/grafdepi_final_report.pdf).

This Duplex Nested End-Point PCR method includes for the 1st amplification specific primer sets for the detection of each phytoplasma groups (FD9f/FD9r and STOL11f2/STOL11r1) and for the 2nd amplification specific primer sets for each of the phytoplasma groups searched with specific sequences for the 1st PCR product (FD9r2/FD9f3b and STOL11f3/STOL11r2).

This product should be used only for research purposes.

Intended use

The PCR set is validated for the simultaneous detection of Flavescence dorée and Bois noir in Duplex Nested End-Point PCR. Suitable tissues are grapevine leaves, preferably primary veins and petioles and bark scrapings from dormant canes.

It is preferable to test plant tissues with vine yellows symptoms.

Set format and content

Two sets are available for 24 and 96 tests.

Article N°	Product name
7FDBN-P2	PCR Flavescence dorée/Bois noir set 24
7FDBN-P9	PCR Flavescence dorée/Bois noir set 96

Content	set 24	set 96
Direct Master Mix	24 tests 7FDBN-P2-DM-	2x48 tests 7FDBN-P9-DM-
Nested Master Mix	24 tests 7FDBN-P2-NM-	2x48 tests 7FDBN-P9-NM-
Positive Control	3 tests 7FDBN-P2-PC-	8 tests 7FDBN-P9-PC-
Negative Control	3 tests 7FDBN-P2-NC-	8 tests 7FDBN-P9-NC-

Storage conditions

This set 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 set 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.

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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 20 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	20 µl
Total Volume / PCR tube or well	22 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	3 min	1
Denaturation	94°C	1 min	35
Annealing	55°C	1 min	
Elongation	72°C	1 min 30 sec	
Final elongation	72°C	10 min	1
Storage	4°C	∞	-

Dilution of 1st PCR products

Using DNase and RNase free PCR tubes, **dilute to 1:1.000** 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 20 µl of Nested Master Mix (without DNA template) to each PCR tubes or wells of an optical-grade PCR plate.
d) Add 5 µl of **diluted PCR products** to the Nested Master Mix. Do not forget to prepare a PCR tube or well of a PCR plate for the PCR products obtained with Positive Control and Negative Control.

Components	Volume/PCR tube or well
PCR products from the 1st PCR diluted to 1:1.000 (*)	5 µl
Nested Master Mix	20 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	3 min	1
Denaturation	94°C	1 min	35
Annealing	56°C	1 min	
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 100-2'000 bp DNA step ladder).
- load 10 µl of the PCR products from the previous step adding the loading dye buffer (not provided in the set).

Run: run the gel electrophoresis for 40-45 minutes at 80V.

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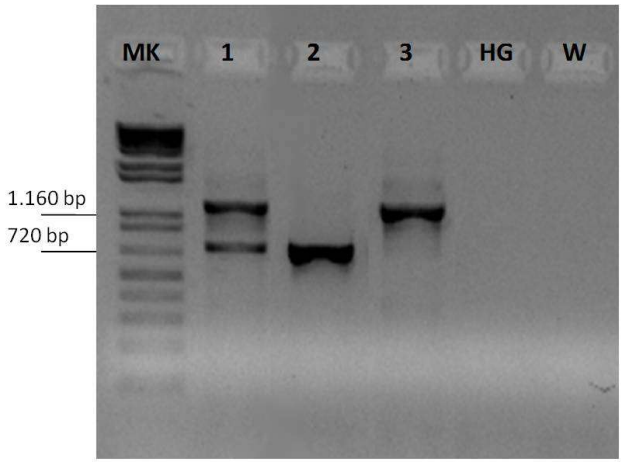
Results analysis

ANALYSIS VALIDATION

Flavescence dorée is detected when a **1.160 bp** DNA fragment is observed. Bois noir is detected when a **720 bp** fragment is observed.

The analysis is validated when:
- ✓ 2 DNA fragments, one of 1.160 bp and one of 720 bp, are visible in the positive control lane.
- ✓ no DNA fragment is visible in the negative control lane.

The picture below represents a 1X-TAE 1% agarose gel showing the DNA amplification of Flavescence dorée and Bois noir in grapevine:



MK: DNA ladder - **1:** Positive Control or sample infected by FD and BN - **2:** Sample infected by BN - **3:** Sample infected by FD - **HG:** Negative Control or healthy sample - **W:** No template control

RESULTS INTERPRETATION

The specific product of Flavescence dorée is a **1.160 bp** DNA fragment. The specific product of Bois noir is a **720 bp** fragment.

- ✓ A sample is infected *by* Flavescence dorée if a 1.160 bp specific fragment is present in the PCR reaction.
- ✓ A sample is infected by Bois noir if a 720 bp specific fragment is present in the PCR reaction.
- ✓ A sample is infected by Flavescence dorée and Bois noir if 2 DNA fragments, one of 1.160 bp and one of 720 bp, are present in the PCR reaction.
- ✓ A sample is not infected by Flavescence dorée and Bois noir if none DNA fragment is present in the PCR reaction.

The table below summarizes the results interpretation:

Fragment size		Interpretation	
1.160 bp	**720 bp**	FD	BN
-	-	Negative	Negative
✓		**POSITIVE**	Negative
✓	✓	**POSITIVE**	**POSITIVE**
	✓	Negative	**POSITIVE**

Special handling instructions

This set 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 set. Do not touch any set 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 sets with different batch numbers in the same test procedure. Do not interchange reagents with other sets. 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 set 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

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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 set 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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