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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/079 (2)

Diagnostics
Diagnostic

PM 7/079 (2) Grapevine flavescence doree phytoplasma

Specific scope

This Standard describes a diagnostic protocol for Grapevine flavescence doree phytoplasma.

Specific approval and amendment

Approved in 2005-09. Revised in 2015-10.

1. Introduction

Grapevine flavescence doree phytoplasma (FD) belongs to the elm yellows group (16SrV). It is one of a complex of diseases affecting *Vitis vinifera*, known as grapevine yellows, associated with the presence of phytoplasmas (Caudwell *et al.*, 1971). Though characterized by similar symptomatology, these diseases are caused by different phytoplasmas belonging to five out of the thirty groups so far distinguished on the basis of molecular evidence (Table 1) (Zhao *et al.*, 2009; USDA data base consulted 2nd December 2014). These pathogens have irregular distribution in the plant and are usually present at very low concentration, which makes diagnosis more difficult. The other yellows diseases of grapevine occurring in Europe (Table 1) are widespread in other hosts, and are not regulated as quarantine pests. Spread of flavescence doree occurs through infected grapevine planting material and through its main vector, the cicadellid *Scaphoideus titanus* Ball.

A flow diagram describing the procedures for detection and identification is presented in Fig. 1.

2. Identity

Name: Grapevine Flavescence doree phytoplasma.

Taxonomic position: *Bacteria, Firmicutes, Mollicutes, Acholeplasmatales, Acholeplasmataceae.*

Provisional taxon: Phytoplasma Elm Yellows (EY) Group or 16SrV.

EPPO code: PHYP64.

Phytosanitary categorization: EPPO A2 list no. 94; EU Annex designation II/A2.

3. Detection

3.1. Detection in symptomatic plants

3.1.1. Disease symptoms

Most grapevine cultivars are affected by flavescence doree. Disease symptoms develop mainly in summer (July onwards). They are similar to those caused by other yellows diseases of grapevine in particular bois noir. Leaves turn yellow or red depending on the cultivar. They roll downward and become brittle (Figs 2–4). The interveinal areas of leaves may become necrotic. Shoots show incomplete lignification and rows of black pustules develop on the green bark along the diseased branches; they are thin, rubbery and hang pendulously. During winter they blacken and die. The inflorescences dry out and fall off. Fruit setting is reduced. In later infections, bunches are irregular and berries become shrivelled. They have a significantly lower sugar content and higher acidity compared to healthy grapes. Flavescence doree spreads randomly in vineyards (this is perhaps associated with vine-to-vine transmission by the vector). It may occur in rootstocks, but without conspicuous symptoms.

3.1.2. Sampling

The titer of phytoplasmas is usually low in woody hosts (Berges *et al.*, 2000) and it varies in grapevine according to organ and season (Constable *et al.*, 2003). Samples should be collected in July–October, selecting leaves showing symptoms but in good condition (no necrotic areas) and not affected by other pests. Approximately 20 leaves per plant should be randomly collected, and midribs and veins (to a total of about 1–1.5 g) separated. Material for testing should be used fresh, or stored at 20°C (or lower depend-

Table 1. Current status of molecular characterization, biology and vectors of phytoplasmas causing grapevine yellows diseases. Based on Boudon-Padieu (2003, 2005), Reisenzein & Steffek (2011), Sousa *et al.* (2011), Seruga Music *et al.* (2011), EPPO (2013)

Grapevine yellows disease	Phytoplasma name	Ribosomal group (subgroup)	Known insect vector to grapevine	Preferred host plants of vector	Occurrence
Flavescence doree	FD	16SrV (-C, -D) or EY	*Scaphoideus titanus* Ball	*Vitis* sp.	Austria, Croatia, France, Italy, Hungary, Portugal, Spain, Serbia, Slovenia, Switzerland
Palatinate grapevine yellows	PGY	16SrV or EY	*Oncopsis alni* Schrank	*Alnus glutinosa* L.	Germany
Bois noir, Legno nero, Vergilbungskrankheit Schwarzholzkrankheit	'*Candidatus* Phytoplasma solani'	16SrXII-A or stolbur	*Hyalesthes obsoletus* Sign	*Convolvulus arvensis* L. *Urtica dioica* L. *Ranunculus*, *Solanum, Lavandula*	Europe, Israel, Lebanon
Australian grapevine yellows	'*Candidatus* Phytoplasma australiense'	16SrXII-B	Not defined	Not defined	Australia
Australian grapevine yellows	'*Candidatus* Phytoplasma australasia'	16SrII FBP	Not defined	Not defined	Australia
Buckland valley grapevine yellows (Aus)	BVGY	16SrI-related or AY-	Not defined	Not defined	Australia
Grapevine yellows	Aster yellows	16SrI (-B, -C) or AY	Not defined	Not defined	Italy, Chile
North American grapevine yellows (NAGY)	Virginia grapevine yellows I (VGY I)	16SrI-A or AY	Not defined	Not defined	Virginia (UA)
	Western X Virginia grapevine yellows III (VGYII)I	16SrIII-I or WX	Not defined	Not defined	New York (US) Virginia (US)

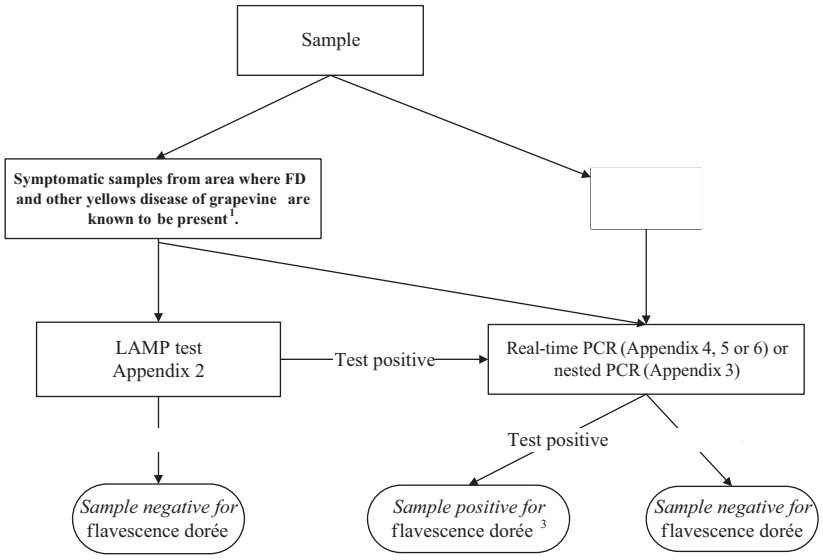

Fig. 1 Flow diagram for the detection and identification of Elm yellows group (16SrV) phytoplasmas (including FD phytoplasma).
[1]Testing of asymptomatic samples and vectors is possible but no validation data is available.
[2]For asymptomatic samples or samples from areas where FD is not known to occur performing one of the real-time PCR or nested PCR tests instead of the LAMP test is recommended.
[3]For confirmation/specific identification of FD phytoplasma (in particular for a first detection in an area and in non-grapevine samples) sequencing of PCR products is recommended, since the specificity of all methods is to the Elm yellows group (16SrV).

Fig. 2 Leaves of grapevine cv. Chardonnay affected by flavescence doree showing typical yellowing of the laminar blade and rolling of margins. Leaves present a triangular shape [photograph courtesy of Dr. Federico Bondaz, Plant Protection Unit of Val d'Aosta region (IT)].

Fig. 3 Leaves of grapevine cv Bellone affected by Stolbur phytoplasmas. Symptoms are undistinguishable from the ones observed in grapevine affected by FD. To perform molecular diagnosis is the only way to discriminate phytoplasmas belonging to different groups.

Fig 4 Leaves of red grapevine cultivars affected by FD showing rolling and reddening of the blade. Often symptoms are present on a single shoot of the plant.

ing on the storage time e.g. 80°C for more than 2 years). Pooling leaves from up to five plants is possible.

3.2. Detection in asymptomatic plants

It is possible to test asymptomatic plants (rootstocks, canes, asymptomatic leaves) but there is no recommended sampling regime available so far, nor validation data.

3.3. Detection in vectors

It is possible to test vectors but there is no validation data available. Vectors should be collected and immediately transferred into ethanol 70% or stored at 20°C before testing. Ethanol should be washed off before DNA extraction.

3.4. Molecular methods

Since confusion between symptoms of flavescence doree and other yellows diseases of grapevine is possible, loop-mediated isothermal amplification (LAMP) can be used for on-site detection as well as for screening in laboratories for symptomatic samples collected from areas where flavescence doree and other yellows diseases of grapevine are known to be present. This test is described in Appendix 2. Positive samples should be confirmed with the other included molecular methods (Fig. 1).

For asymptomatic samples or samples originating from areas where FD is not known to occur performing one of the real-time PCR or nested PCR tests instead of the LAMP test is recommended.

4. Identification of Elm yellows group (16SrV) phytoplasmas (including FD phytoplasma)

4.1. Molecular methods

Seven molecular tests have been included in the test performance study in 2013–2014 (Pasquini *et al.*, 2014): (1) a universal nested-PCR test followed by RFLP analysis with *Taq*I (Martini *et al.*, 1999); (2) two group specific nested-PCR tests (Deng & Hiruki, 1991; Lee *et al.*, 1994; Schneider *et al.*, 1995; Clair *et al.*, 2003); (3) two real-time PCR tests for specific detection of 16SrV group phytoplasmas (Angelini *et al.*, 2007; Hren *et al.*, 2007); (4) two real-time PCR tests for co-detection of 16SrV and 16SrXII group phytoplasmas, and internal grapevine control (Pelletier *et al.*, 2009 and oligonucleotides under patent IPADLAB). The results obtained in the test performance study showed that real-time PCR tests developed by Hren *et al.* (2007) (Appendix 4), Pelletier *et al.* (2009) (Appendix 5) and under patent oligonucleotides (IPADLAB, Appendix 6) presented the best performance characteristics (Loiseau, 2015). Since some laboratories do not have the equipment to perform real-time PCR tests, the conventional PCR test that gave the

best performance characteristics in the test performance study is included in this protocol and described in Appendix 3.

Sequencing of PCR/nested PCR product can be performed to distinguish grapevine flavescencee doree *sensu stricto* from other phytoplasmas belonging to the same group (elm yellow group).

5. Reference material

Reference material can be obtained from: Institut national de la recherche agronomique, UMR GDPP Bordeaux, BP 81, 33883 Villenave D'Ornon Cedex, France or
Phytoplasmology Laboratory, Plant Pathology, DiSTA – *Alma Mater* Studiorum – University of Bologna, Italy (assunta.bertaccini@unibo.it). Phytoplasma Collection. International Phytoplasmologists Working Group. http://www.ipwgnet.org/index.php?option=com_content&view=article&id=29&Itemid=5

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 (1) *Documentation and reporting on a diagnosis*.

7. Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8. Further information

Further information on this organism can be obtained from:

L. Ferretti, CRA-PAV (Centro di ricerca per la Patologia Vegetale, Via C.G. Bertero 22, 00156 Rome, Italy.

X. Foissac, Institut national de la recherche agronomique, Equipe Mollicutes, UMR 1332 Biologie du Fruit et Pathologie, 33882 Villenave d'Ornon, France; foissac@bordeaux.inra.fr

9. Feedback on this diagnostic protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

11. Acknowledgements

This protocol was originally drafted for EPPO by: Dr Paola Del Serrone, Istituto Sperimentale per la Patologia Vegetale, Rome (IT). This revision was prepared by Dr Natasa Mehle, National Institute of biology, Vecna pot 111, 1000 Ljubljana, Slovenia (natasa.mehle@nib.si), and Marianne Loiseau, ANSES-LSV Plant Health Laboratory, 49044 Angers, France (marianne.loiseau@anses.fr).

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Appendix 1 – DNA extraction procedures

1. DNA extraction from plant material

Several methods have been developed and compared (Palmano, 2001; Boudon-Padieu *et al.*, 2003). The method described below is an optimization of a method described by Doyle & Doyle (1990) for DNA extraction from grapevine and woody plants.

CTAB procedure for Nucleic acids extraction (Boudon-Padieu *et al.*, 2003 modified from Doyle & Doyle (1990).

Nucleic acids can be extracted from fresh or frozen (20 or 80°C) grapevine tissues (preferably veins or petioles).

Grind 1 g of tissue in 10 mL of 3% CTAB buffer (3% CTAB or cethyl-trimethyl-ammonium bromide in 1 M Tris–HCl pH8, 10 mM EDTA, 1.4 M NaCl) at room temperature. Transfer into a 2 mL clean Eppendorf tube and centrifuge at 1000 *g* for 10 min. Transfer 1 mL of the suspension to an Eppendorf tube, add 2 µL of 2-marcaptoethanol (for a final concentration of 0.2%), vortex briefly and incubate for 20 min at 65°C. Then, add an equal volume of chloroform:isoamyl alcohol (24:1). Vortex and centrifuge at 10 000 *g* for 10 min. Recover the aqueous phase and precipitate the nucleic acids with an equal volume of cold isopropanol. Shake by inversion and centrifuge at 10 000 *g* for 15 min to recover the precipitate. Wash the pellet with 70% ethanol, dry and dissolve in 400 µL of TE buffer or nuclease-free water.

Alternative methods

Another DNA extraction method applicable to a large number of plant samples combines a simple and quick homogenization step of crude extracts with DNA extraction based on the binding of DNA to magnetic beads is described in

Appendix 2 and 4 since it has been validated for these two DNA amplification procedure only. Alternative commercial kits (e.g. DNeasy, Qiagen or NucleoSpin Plant, Macherey-Nagel) can also be used for DNA extraction. Those alternative methods not described in this Standard should be validated depending on the chosen DNA amplification procedure.

2. DNA extraction from vectors

DNA extraction applicable for insect vectors is described by Marzachı *et al.* (1998) and further modifications are reported in Trivellone *et al.* (2005).

Appendix 2 – Loop-mediated isothermal amplification (LAMP)

1. General information

1.1 The following LAMP test is performed for the detection of 16SrV phytoplasmas including FD phytoplasma.
1.2 The test was developed by Kogovsek *et al.* (2015), and it is appropriate for on-site detection as well as for screening method in laboratories. The test can be applied to plant material after a DNA extraction, and to crude homogenates, without DNA extraction. In the last case it can be completed in 1 h.
1.3 The FD test was designed within the 23S rRNA sequence.
1.4 Primers are available in kit form from OptiGene Ltd (http://www.optigene.co.uk)
1.5 The test has been successfully performed on-site using GenieII (OptiGene) and in the laboratory using GenieII (OptiGene) or Roche LC480 instrument.
1.6 The software GenieII (Optigene) or LightCycler 480 (Roche) or any other software for fluorescence acquisition in real-time should be used. The specific instrument manual should be consulted. Note: software for cycling (qPCR devices) should be programmed to measure the fluorescence during the amplification and the melting curve analysis.

2. Methods

2.1. Nucleic acid extraction and purification

The test can be applied to plant material after a DNA extraction (see point 2.1.2), and to crude homogenates, without DNA extraction (see point 2.1.1).

2.1.1 Crude homogenates, without DNA extraction

This method is applicable when testing on-site and in the laboratory. 3-5 grapevine berries or two veins from each of five leaves or five small flower clusters are homogenized manually with vigorous shaking for 2 min in tubes filled with metallic beads (5 mm diameter), sand (0.2–0.8 mm) and buffer. 2–5 mL (for leaf veins or flowers) or 10 mL (for berries) of ELISA buffer (264 mM Tris, 236 mM Tris–HCl, 137 mM NaCl, 2% PVP K-25, 2 mM PEG 6000, 0.05% Tween 20, pH 8.2) is used. An automated approach for homogenization, using the Ultra-Turrax Tube Drive (IKA) device, is also applicable. With this device, samples are homogenized for 1 min at maximum settings. Tenfold diluted plant homogenate is suitable for testing.

2.1.2 DNA extraction from plants

One gram of grapevine leaf mid-vein tissue is homogenized in 2 mL of ELISA (see above) or lysis buffer (from QuickPick™ SML Plant DNA kit, Bio-Nobile) using tissue homogenizer, e.g., FastPrep^{R}-24 with TN 12 × 15-TeenPrep™ Adapter (MP Biochemicals). Alternative grinding procedures: with liquid nitrogen using mortar and pestle or homogenization in extraction bags using Homex 6 homogenizer (Bioreba). Total DNA can be reliably extracted using QuickPick™ SML Plant DNA kit (Bio-Nobile) and a magnetic particle processor (e.g. KingFisher^{R} mL, Thermo Scientific) (Mehle *et al.*, 2013a) or CTAB extraction method (Appendix 1). Other extraction methods can be used but should be validated against the QuickPick™ SML Plant DNA kit + KingFisher or CTAB extraction methods to ensure compatibility. Total DNA extract is eluted in 200 µL of elution buffer (QuickPick™ SML Plant DNA kit + KingFisher) or in 50 µL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) (CTAB extraction method). For grapevine leaf mid-vein tissue undiluted DNA is suitable for testing. Extracted total DNA can be kept at −20°C.

2.2 LAMP

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	5	N.A.
Isothermal master mix (*Optigene*)	2×	12.5	1×
Primer mix (*Optigene*)	10×	2.5	1×**
Subtotal		20	
DNA or tenfold diluted plant homogenate		5	
Total		25	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.
**Final concentration of primers: 0.2 µM F3 and B3 primers, 2 µM FIP and BIP primers and 1 µM F-loop and B-loop primers.

2.2.2. LAMP amplification conditions: 62°C for 40 min; melting curve analysis: 98–80°C, 0.05°C per second.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.

Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).

Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.

Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene).

Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract/plant homogenate. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results

Verification of the controls

NAC (and if relevant NIC) should produce no fluorescence.

The PAC (and if relevant PIC, IC) amplification curve should be exponential. The Tm (melting temperature) should be between 84.0 and 85.0°C when samples are analysed on GenieII and between 84.9 and 86.9°C when samples are analysed on the Roche LC480 device. Similar Tm range is expected when analysed on any other device, but needs to be verified.

When these conditions are met

A test will be considered positive if it produces a positive reaction as defined for PAC (see above).

A test will be considered negative, if it produces no fluorescence.

Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Validation data available from the National Institute of Biology, SI.

Further validation is in progress in the framework of the Euphresco project GRAFDEPI2. Another validation project is also in progress to compare the performance of the LAMP test performed on crude homogenate and the real-time PCR.

These validation data will be made available through the EPPO database on diagnostic expertise when available.

4.1. Analytical sensitivity data

Testing of crude homogenates: maximum dilution of FD phytoplasma infected homogenate that was detected had 9–27 copies of FD phytoplasma DNA.

Testing of extracted DNA: maximum dilution of FD phytoplasma DNA that was detected was up to 9–27 copies of FD phytoplasma DNA.

Maximum dilution of FD phytoplasma DNA that was detected using real-time PCR (Hren *et al.*, 2007) was up to 1–3 copies of FD phytoplasma DNA.

4.2. Analytical specificity data

LAMP is specific to Elm yellows group (16SrV) (includes FD phytoplasma). *In silico* analysis indicated no significant sequence homology with non-targets.

Number of strains of targets tested: 65 FD isolates/infected samples (FD70, FD-C, FD-D) and 2 EY-phytoplasma isolates. Additionally, 15 samples with targets

(11 samples positive for FD phytoplasma, and 4 samples infected by other phytoplasmas belonging to 16SrV group) from the test performance study (Euphresco: Grafdepi) were analysed.
Number of non-targets tested: 132 (phytoplasma DNA from other 16Sr groups, bacterial and fungal isolates and healthy hosts; 9 samples were from the test performance study (Euphresco: Grafdepi))
Percentage of accurate results: 99%.
False negative results: 0%; false positive results: 1.5% (one healthy grapevine sample and a DNA sample of '*Ca. P. fraxini*' (16SrVII; isolate: ASHY 2; origin: USA) from the test performance study (Euphresco: Grafdepi) were positive with LAMP.

4.3. Data on repeatability

High FD phytoplasma conc. (5 samples with more than 729 copies of FD phytoplasma DNA): 100% (29 pos/29 repeats)
Medium FD phytoplasma conc. (4 samples with 81–729 copies of FD phytoplasma DNA): 100% (12 pos/12 repeats)
Low FD phytoplasma conc. (8 samples with <81 copies of FD phytoplasma DNA): 81% (22 pos/27 repeats).

4.4. Data on reproducibility

Testing was done on 2–9 different days, with two different operators and with two different devices. Percentage of identical results:
High FD phytoplasma conc. (3 samples with more than 729 copies of FD phytoplasma DNA): 100% (3 pos/3 repeats)
Medium FD phytoplasma conc. (2 samples with 81–729 copies of FD phytoplasma DNA): 100% (2 pos/2 repeats)
Low FD phytoplasma conc. (8 samples with <81 copies of FD phytoplasma DNA): 100% (8 pos/8 repeats).

4.5. Other performance criteria available

Diagnostic sensitivity:
Proportion of infected samples tested positive compared to results from the real-time PCR (Hren *et al.*, 2007):
Testing of extracted DNA: 100% (no. of targets analysed: 52 FDp infected samples (38 grapevine leaf vein, 8 *Clematis vitalba*, 3 *Alnus glutinosa*, 1 *Scaphoideus titanus* and 2 *Orientus ishidae* samples)).
Testing of crude homogenates: 100% (no. of targets analysed: 27 FDp infected grapevine samples).
See also validation data deposited with the EPPO database on Diagnostic Expertise: http://dc.eppo.int/validationlist.php.

Appendix 3 – Direct generic PCR followed by nested group-specific PCR with primers R16(V)F1/R1

1. General information

1.1 The following nested PCR protocol is performed for the detection and identification of 16SrV phytoplasmas including FD phytoplasma.
1.2 Two sets of primers are used: P1/P7 primers (Deng & Hiruki, 1991; Schneider *et al.*, 1995) for first PCR, and R16(V)F1/R1 (Lee *et al.*, 1994) for second PCR (nested PCR). The first step confirms the presence of a phytoplasma, while the second step (nested PCR) is specific for 16SrV group phytoplasma.
1.3 The P1/P7 primers amplify the whole length of 16S and intergenic 16S-23S and a small part of 23S rRNA gene (1850 bp).
1.4 The amplicon size of nested PCR (R16(V)F1/R1) is around 1100 bp.
1.5 Oligonucleotides:
P1: 5′-AAGAGTTTGATCCTGGCTCAGGATT-3′
P7: 5′-CGTCCTTCATCGGCTCTT-3′
R16(V)F1: 5′-TTAAAAGACCTTCTTCGG-3′
R16(V)R1: 5′-TTCAATCCGTACTGAGACTACC-3′
1.6 The test performance study (Euphresco: Grafdepi) was performed with a GoTaq DNA polymerase from Promega, Ready mix from Sigma, Taq DNA Polymerase from RBC Bioscience and Taq Platinum from Invitrogen.

2. Methods

2.1 Nucleic Acid Extraction and Purification.
2.1.1 DNA extraction methods that are described in Appendix 1 may be used.
2.2 Conventional PCR, followed by nested PCR
2.2.1 Master Mix for PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	16.375	N.A.
Green GoTaq Reaction buffer (*Promega*)	5×	5	1×
dNTPs (*Promega*)	10 mM	0.5	0.2 mM
Forward primer (P1)	10 µM	1	0.4 µM
Reverse primer (P7)	10 µM	1	0.4 µM
GoTaq DNA polymerase (*Promega*)	5 U/µL	0.125	0.625 U
Subtotal		24	
Genomic DNA extract		1	
Total		25	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.

2.2.2 PCR conditions: initial denaturation step at 94°C for 2 min; 35 cycles consisting of 1 min at 94°C,

1 min at 55°C, and 2 min at 72°C; final extension at 72°C for 10 min.

2.2.3 Master Mix for nested PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	16.375	N.A.
Green GoTaq Reaction buffer (*Promega*)	5×	5	1×
dNTPs (*Promega*)	10 mM	0.5	0.2 mM
Forward primer (R16(V)F1)	10 µM	1	0.4 µM
Reverse primer (R16(V)R1)	10 µM	1	0.4 µM
GoTaq DNA polymerase (*Promega*)	5 U/µL	0.125	0.625 U
Subtotal		24	
1/40 diluted P1/P7 PCR product		1	
Total		25	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.

2.2.4 Nested PCR conditions: initial denaturation step at 94°C for 2 min; 35 cycles consisting of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C; final extension at 72°C for 10 min.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.

Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).

Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.

Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the external positive control PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample.

Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results

Verification of the controls

NIC and NAC should produce no amplicons.

PIC, PAC (and if relevant IC) should produce amplicons of the expected size (depending if the target, endogenous or exogenous nucleic acid is used).

When these conditions are met:

A test will be considered positive if amplicons of nested PCR around 1100 bp are produced

A test will be considered negative, if it produces no band or a band of a different size.

Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Validation data available from the test performance study in 2013 (Euphresco: Grafdepi), where the 13 participant laboratories analysed a total of 24 blind samples including 4 grapevine phytoplasma-free samples and 20 single or mixed infected samples. In particular: 11 samples positive for FD phytoplasma, 4 samples infected by other phytoplasmas belonging to 16SrV group, 9 samples positive for Stolbur phytoplasma and 3 samples infected by phytoplasmas belonging to 16SrI, 16SrIII and 16SrVII groups.. Repeatability, reproducibility and last level of positive results were evaluated with dilution series of 3 positive DNA extracts in 5 laboratories.

3.1 Analytical sensitivity data

91.4%

Last level at 100% positive results: <1/10 (levels tested between 1/10 and 1/2700 for 3 different positive DNA extract diluted in healthy DNA extract)
Last level with positive result(s): 1/2700

3.2 Analytical specificity data
88.3%

3.3 Data on Repeatability
Between 57 to 100%

3.4 Data on Reproducibility
73.8%

Appendix 4 – Real-time PCR (Hren *et al.*, 2007)

1. General information

1.1 The following real-time PCR protocol is performed for the detection and identification of 16SrV phytoplasmas including FD phytoplasma.

1.2 The test was developed by Hren *et al.* (2007), and the testing protocol was published by Mehle *et al.* (2013b). Therein described real-time PCR detection system includes also a test for group-specific detection of BN, and an universal phytoplasma test as a tool to detect other phytoplasma types.

1.3 The FDgen amplicon was designed within the SecY gene using nucleotide sequences of several FD isolates (with their GenBank accession no. in parenthesis) including FD-C (AF458382, AY197688), FD-D (AY197685), FD70 (AF458383, AY197686), FD92 (AF458384), FD2000 (AY093581) and HD1 (AF458381); and other strains from the elm yellows group (16SrV) (AY197684, AY197687, AY197689, AY197690, AY197691, AY197692, AY197693, AY197694, AY197695, AY197696, AY093580).

1.4 The FDgen amplicon is situated within the primer pairs FD9f/r (Daire *et al.*, 1997) and FD9f3b/r2 (Clair *et al.*, 2003), which are used for detection of FD by nested conventional PCR.

1.5 The FDgen amplicon covers a 85 bp region of the SecY gene of FD, corresponding to nucleotides 428–512 in isolate FD-D (accession number AY197685).

1.6 Oligonucleotides for FDgen: forward primer 5′ TTA TGC CTT ATG TTA CTG CTT CTA TTG TTA 3′; reverse primer 5′ TCT CCT TGT TCT TGC CAT TCT TT 3′; probe 5′ FAM-ACC TTT TGA CTC AAT TGA-MGB 3′

1.7 Test has been successfully performed using reagents from different manufacturers including TaqMan Universal PCR Master Mix (Applied Biosystems) and Maxima Probe qPCR master mix (Fermentas); and on a range of different real-time PCR systems including ABI (7900, 7900HT Fast, ViiA™7) and Roche (LightCycler 480).

1.8 Software (e.g. SDS 2.4, Applied Biosystems) for fluorescence acquisition and calculation of threshold cycles (Ct) should be used. The transformation of the fluorescence signal into Ct data, as well as methods for baseline and threshold settings, vary between instrument models. The specific instrument manual should be consulted. When analyzing the raw data it is important to adjust the cycle threshold (Ct) of the amplification plot to within the geometric (exponential) phase of amplification, preferably at the beginning of the geometric phase. At the log view this is the linear part of the amplification plot. It has been experimentally determined that automatic baseline and threshold at 0.065 are usually suitable when using Applied Biosystems thermal cyclers and TaqMan Universal PCR Master Mix (Applied Biosystems).

2. Methods

2.1 Nucleic Acid Extraction and Purification

1 g of grapevine leaf mid-vein tissue is homogenized in 2 mL of ELISA (264 mM Tris, 236 mM Tris–HCl, 137 mM NaCl, 2% PVP K-25, 2 mM PEG 6000, 0.05% Tween 20, pH 8.2) or lysis buffer (from QuickPick™ SML Plant DNA kit, Bio-Nobile) using tissue homogenizer, e.g., FastPrep^{R}-24 with TN 12 × 15-TeenPrep™ Adapter (MP Biochemicals). Alternative grinding procedures: with liquid nitrogen using mortar and pestle or homogenization in extraction bags using Homex 6 homogenizer (Bioreba).

Total DNA can be reliably extracted using QuickPick™ SML Plant DNA kit (Bio-Nobile) and a magnetic particle processor (e.g. KingFisher^{R} mL, Thermo Scientific) (Mehle *et al.*, 2013a) or CTAB extraction method (Appendix 1). Other extraction methods can be used but should be validated against the QuickPick™ SML Plant DNA kit + KingFisher or CTAB extraction methods to ensure compatibility.

Total DNA extract is eluted in 200 µL of elution buffer (QuickPick™ SML Plant DNA kit + KingFisher) or in 50 µL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) (CTAB extraction method). For grapevine leaf mid-vein tissue tenfold diluted DNA is suitable for testing.

Extracted total DNA can be kept at −20°C.

2.2 Real-time PCR

2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (µL)**	Final concentration
Molecular grade water *	N.A.	0.2	N.A.
	2×	5.0	1×

(continued)

Table *(continued)*

Reagent	Working concentration	Volume per reaction (µL)**	Final concentration
TaqMan Universal PCR Master Mix (*ABI*)			
Forward Primer	10 µM	0.9	0.9 µM
Reverse Primer	10 µM	0.9	0.9 µM
Probe	2.5 µM	1.0	0.25 µM
Subtotal		8.0	
DNA dilution		2.0	
Total		10.0	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.
**If 25 µL reaction volume is used, multiply each component by 2.5.

2.2.2 Real-time PCR conditions: Uracil N-glycosylase (UNG) activation step at 50°C for 2 min; initial denaturation at 95°C for 10 min; 45 cycles consisting of 15 s at 95°C and 1 min at 60°C.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.

Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).

Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.

Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the external positive controls PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).

Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results

Verification of the controls

The PIC and PAC (as well as IC and IPC) amplification curves should be exponential.

NIC and NAC should give no amplification.

When these conditions are met:

A test will be considered positive if it produces an exponential amplification curve.

A test will be considered negative, if it produces no exponential amplification curve or if it produces a curve which is not exponential.

Tests should be repeated if any contradictory or unclear results are obtained.

Note: The cycle cut-off value is set at 38.5, and was obtained using the equipment and chemicals as described in this appendix. The cycle cut-off value was determined by a procedure described in Mehle *et al.* (2013b). It is the highest Ct values plus 0.5 observed after analyzing dilutions of positive samples. When using extraction procedure, equipment and chemicals as described in this appendix, Ct values above 38.5 are obtained exceptionally. In such cases, repeated DNA extraction or analysing of undiluted DNA sample resulted usually in Ct values lower than 38.5. As a Ct cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

4. Performance criteria available

4.1. Analytical sensitivity data

Validation data available from the National Institute of Biology, SI:

- Determined using nonlinear model (programming environment R) based on the Ct value of the FD dilutions in a positive grapevine sample.

- The following dilutions of the sample DNA was tested (the average Ct value at 10^0 was 24.0): non-diluted up to $24.3 \times 10^5 \times$ diluted
- Analytical sensitivity represented as Ct values with 95% probability of detection: 35.7 (the dilutions of the sample DNA at calculated Ct value: $10^3 < \times < 10^4$)

Validation data available from the test performance study in 2013 (Euphresco: Grafdepi): 97.3%*

Last level at 100% positive results: <1/10 (levels tested between 1/10 and 1/2700 for 3 different positive DNA extract diluted in healthy DNA extract)**.

Last level with positive result(s): 1/2700**.

4.2. Analytical specificity data

Validation data available from the National Institute of Biology, SI: Real-time PCR is specific to Elm yellows group (16SrV) (comprises FD phytoplasma). *In silico* analysis indicated no significant sequence homology with non-targets.

- Number of strains of targets tested: 10 (9 FD isolates/infected samples and one EY-phytoplasma isolate)
- Number of non-targets tested: 169 (various grapevine cultivars showing no disease symptoms, BN infected grapevine, 39 bacterial isolates that could be present in grapevine as epiphytes or saprophytes, other phytoplasmas that are kept in *Catharathus roseus* (aster yellows phytoplasma (AY, 16SrI-B), Western X-disease (WX, 16SrIII-A), apple proliferation phytoplasma (AP, 16SrX-A), pear decline phytoplasma (PD,16SrX-C) European stonefruit yellows phytoplasma (ESFY, 16SrX-B) MA (Marguerite Daisy) phytoplasma (16SrIII-B), stolbur phytoplasma isolate SE (SE,16SrXII-A).
- Percentage of accurate results: 100%.

Validation data available from the test performance study in 2013 (Euphresco: Grafdepi): 94.1%*

4.3. Data on repeatability

Validation data available from the National Institute of Biology, SI:

- High FD phytoplasma conc. (two samples with average Ct 24 and 26): 100% (10 pos/10 repeats)
- Medium FD phytoplasma conc. (a sample with average Ct 29): 100% (5 pos/5 repeats)
- Low FD phytoplasma conc. (two samples with average Ct 33 and 37): 100% (10 pos/10 repeats)

Validation data available from the test performance study in 2013 (Euphresco: Grafdepi): 68 to 100%**

4.4. Data on reproducibility

Validation data available from the National Institute of Biology, SI: Testing was done on 29 different days, with two different operators and with two different devices. Percentage of identical results:

- Sample with medium FD phytoplasma conc. (average Ct 28.7): 100%
- Sample with low FD phytoplasma conc. (average Ct 35.5): 100%

Validation data available from the test performance study in 2013 (Euphresco: Grafdepi): 84.9%**

*The 10 participant laboratories analysed a total of 24 blind samples including 4 grapevine phytoplasma-free samples and 20 single or mixed infected samples. In particular: 11 samples positive for FD phytoplasma, 4 samples infected by other phytoplasmas belonging to 16SrV group, 9 samples positive for Stolbur phytoplasma and 3 samples infected by phytoplasmas belonging to 16SrI, 16SrIII and 16SrVII groups.

** Repeatability, reproducibility and last level of positive results were evaluated with dilution series of 3 positive DNA extracts in 5 laboratories.

4.5. Other performance criteria available

The full validation data (also for BN) and report on the critical points in the diagnostic process and relating to uncertainty of measurement are available from the National Institute of Biology, SI: see validation data deposited with the EPPO database on Diagnostic Expertise: http://dc.eppo.int/validationlist.php.

Appendix 5 – Multiplex real-time PCR (Pelletier *et al.*, 2009)

1. General information

1.1 The following real-time PCR protocol is performed to detect simultaneously the 16SrV phytoplasmas including FD and the 16SrXII phytoplasmas including BN as well as grapevine chloroplastic DNA with TaqMan minor groove binder probes.

1.2 The test was developed by Pelletier *et al.* (2009). Primers and probe sequences of the 16SrV group phytoplasmas were determined on the basis of the *map* gene sequence alignment of 19 reference strains (accession numbers AM384884 to AM384902). Amplified fragment was 71 bp long, from position 175 to 245 after the start codon of the *map* gene. Probe was 5′ labelled with FAM™ reporter dye.

1.3 Primers and probe sequences of the 16SrXII group phytoplasmas were determined on the basis of the alignment of their map gene sequences of 14 reference strains (accession AM990976 to AM990988 and AM422018). Amplified fragment was 72 bp long, from position 32 to 103 after the start codon of the map gene. Probe was 5′ labelled with VIC™ reporter dye.

1.4 Primers and probe sequences for the amplification of an endogenous control (EC) were determined on the sequences alignment of the chloroplast trnL-F spacer of different *Vitis* species already described by (Rossetto *et al.*, 2001, 2002; Soejima & Wen, 2006). Accession numbers are AF300295 and AB235073 to AB235084. Amplified fragment was 73 bp long. Probe was 5′ labelled with Cy5 reporter dye.

1.5 All probes were 3′ labelled with a non-fluorescent quencher.

1.6 Primers and probe:

Primers	Sequence	Specificity
mapFD-F	5′-TCA AGG CTT CGG BGG TTA TA-3′	16SrV group
mapFD-R	5′-TTG TTT TAG AAG GTA ATC CGT GAA CTA C-3′	
mapFD-FAM	FAM- TTG TAT TTC AGT GAA TGA AG –MGB	
mapBN-F	5′-ATT TGA TGA AAC ACG CTG GAT TAA-3′	16SrXII group
mapBN-R	5′-TCC CTG GAA CAA TAA AAG TYG CA-3′	
mapBN-VIC	VIC- AAA CCC ACA AAA TGC –MGB	
VITIS-F	5′-AAA TTC AGG GAA ACC CTG GAA-3′	Grapevine
VITIS-R	5′-CCC TTG GTT GTT TTC GGA AA-3′	
VITIS-Cy5	Cy5- C**t**G **ag**C **c**AA **atc** C –BHQ-2	

1.7 Test has been successfully performed using reagents from different manufacturers including QuantiTect Multiplex RT-PCR with or without Rox (Qiagen), rotor-Gene multiplex PCR kit (Qiagen) and Master mix TAYKON; and on a range of different real-time PCR systems including Stratagene, ABI 7500 and Rotor gene.

1.8 Software for fluorescence acquisition and calculation of threshold cycles (Ct) should be used. The transformation of the fluorescence signal into Ct data, as well as methods for baseline and threshold settings, varies between instrument models. The specific instrument manual should be consulted. When analyzing the raw data it is important to adjust the cycle threshold (Ct) of the amplification plot to within the geometric (exponential) phase of amplification, preferably at the beginning of the geometric phase. At the log view this is the linear part of the amplification plot.

2. Methods

2.1 Nucleic Acid Extraction and Purification

CTAB DNA extraction procedure described in Appendix 1 was used during the validation of this PCR method. Alternative commercial kits can also be used.

2.2 Real-time PCR

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (μL)	Final c oncentration
Molecular grade water *	N.A.	6.5	N.A.
QT Mix PCR	2×	12.5	1×
mapFD-F	10 μM	0.5	0.2 μM
mapFD-R	10 μM	0.5	0.2 μM
mapFD-FAM	10 μM	0.5	0.2 μM
mapBN-F	10 μM	0.5	0.2 μM
mapBN-R	10 μM	0.5	0.2 μM
mapBN-VIC	10 μM	0.5	0.2 μM
VITIS-F	10 μM	0.5	0.2 μM
VITIS-R	10 μM	0.5	0.2 μM
VITIS-Cy5	10 μM	0.5	0.2 μM
Subtotal		20	
DNA		5	
Total		25	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 μm filtered) and nuclease-free.

2.2.2 Real-time PCR conditions: initial denaturation at 95°C for 15 min; 45 cycles consisting of 1 min at 94°C and 1 min 30 s at 59°C.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.

Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue).

Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.

Positive amplification control for each target (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

Other recommended controls: positive amplification control to the limit of reproducibility (PAC_{LR}): it could be prepared with a series dilution of a positive DNA extract in "healthy" DNA extract and it corresponds to the highest dilution for which 6 repetitions on 6 are positive.

Note: The cycle value of grapevine target from which each DNA extract could be considered acceptable in quantity and quality ($Ct_{VITISref}$) needs to be determined for each laboratory when implementing the test (dependent on equipment, material and chemicals). $Ct_{VITISref}$ could be determined like this:

$Ct_{VITISref}$ = mean Ct_{VITIS} value obtained with at least 20 positive samples + 5 cycles.

3.2. Interpretation of results:

Verification of the controls

The PIC, PAC and PAC_{LR} amplification curves should be exponential.

NIC and NAC should give no amplification.

When these conditions are met this decision scheme should be applied:

FD and/or BN amplification	Grapevine amplification	
	$Ct_{VITIS} < Ct_{VITISref}$	$Ct_{VITIS} \geq Ct_{VITISref}$
No exponential amplification curve or a curve which is not exponential	*Negative FD* and/or *BN*	*Uninterpretable* If possible, extraction should be repeated
Ct FD ≤ Ct PAC_{LR}	*Positive FD*	
Ct FD > Ct PAC_{LR}	Amplification should be repeated	*Uninterpretable* If possible, extraction should be repeated
Ct BN < 45	*Positive BN*	

4. Performance criteria available

4.2. Analytical sensitivity data

Validation data available from Pelletier *et al.* (2009):

- For FD phytoplasma target: 100 times more sensitive than nested PCR (Clair *et al.*, 2003) and up to dilution of 5^7 (positive DNA extract in water).
- For BN target: 5 times more sensitive than nested PCR (Clair *et al.*, 2003) and up to dilution of 5^4 (positive DNA extract in water).

Validation data available from the test performance study in 2013 (Euphresco: Grafdepi):

- For FD phytoplasma target: 97.7%*
  Last level at 100% positive results: 1/100 to 1/2700 (levels tested between 1/10 and 1/2700 for 3 different positive DNA extract diluted in healthy DNA extract) **
  Last level with positive result(s): 1/2700**
- For BN phytoplasma target: 100%*
  Last level at 100% positive results: 1/100 (levels testes between 1/10 and 1/2700 for 1 positive DNA extract diluted in healthy DNA extract)***
  Last level with positive result(s): 1/2700***

4.3. Analytical specificity data

Validation data available from Pelletier *et al.*, 2009: mapFD primers and probe were specific to Elm yellows group (16SrV) (comprises FD phytoplasma).

- Number of strains of targets tested: 12 (4 FD isolates/infected samples and 8 phytoplasmas of 16SrV group). Two isolates of Elm yellows (not FD phytoplasma) were not detected during the evaluation.
- Number of non-targets tested: 29 (various healthy grapevine cultivars, BN infected grapevine and, other phytoplasmas that are kept in *C.roseus* (16SrI, II, III, VI, VII and X groups))
- Percentage of accurate results: 100%.

mapBN primers and probe were specific to Stolbur group (16SrXII).

Number of strains of targets tested: 10.

Number of non-targets tested: 30 (various healthy grapevine cultivars, FD infected grapevine and, other phytoplasmas that are kept in *C.roseus* (16SrI, II, III, VI, VII and X groups))

Percentage of accurate results: 100%.

Validation data available from the test performance study in 2013 (Euphresco: Grafdepi):

- For FD phytoplasma target: 93.3%*
- For BN phytoplasma target: 100%*

4.4. Data on repeatability

Validation data available from the test performance study in 2013 (Euphresco: Grafdepi):

- For FD phytoplasma target: Between 84 to 100%**
- For BN phytoplasma target: Between 52 to 100%***

4.5. Data on reproducibility

Validation data available from the test performance study in 2013 (Euphresco: Grafdepi):

- For FD phytoplasma target: 93.3%**
- For BN phytoplasma target: 94.5%***

* The 6 participant laboratories analysed a total 24 blind samples including 4 grapevine phytoplasma-free samples and 20 single or mixed infected samples. In particular: 11 samples positive for FD phytoplasma, 4 samples infected by other phytoplasmas belonging to 16SrV group, 9 samples positive for Stolbur phytoplasma and 3 samples

infected by phytoplasmas belonging to 16SrI, 16SrIII and 16SrVII groups.

**Repeatability, reproducibility and last level of positive results were evaluated with dilution series of 3 positive DNA extracts in 4 laboratories.

***Repeatability, reproducibility and last level of positive results were evaluated with dilution series of 1 positive DNA extracts in 4 laboratories.

Appendix 6 – Triplex real-time (Taq-Man) test based on *rpl*14 gene sequences (IPA-DLAB)

1. General information

1.1 The following protocol was originally developed by the University of Milan (Dipartimento di Scienze Agrarie e Ambientali, via Celoria, 2 – 2013 Milano) and the sequences and their use in diagnostic tests are patented by International Plant Analysis and Diagnostics s.r.l.(*) (htpp://www.ipadlab.eu). This triplex real-time PCR protocol is suitable to detect simultaneously 16SrV phytoplasmas including FD and the 16SrXII phytoplasmas including Stolbur phytoplasma as well as an endogenous control (IPC) (*COX* gene). The test for 16SrV was based on the *rpl*14 gene sequences.

1.2 A DNA extract from grapevine or other plant matrix is needed as a starting material. This material can be obtained by CTAB extraction or purification column.

1.3 The amplification of FD is performed with specific primers and a probe 5′ labelled with FAM. The amplification of Stolbur is performed with specific primers and a probe 5′ labelled with VIC. The amplification of IPC is performed with specific primers and a probe 5′ labelled with Cy5. The reaction can be performed in any real-time PCR instrument. In order to perform the amplification of IPC, the real-time PCR instrument should be calibrated for the fluorochrome Cy5.

(*) The Flavescence doree and Bois noir pathogen primers and probes sequences, and their use in diagnostic tests, are the subject of a PCT patent application (PCT/IB2010/053563) by International Plant Analysis and Diagnostics s.r.l.

A commercial kit, based on this protocol, is currently available. Further information on the Flavescence doree and Bois noir detection kit, including primers, probes and master mix, can be obtained from Qualiplante (htpp://www.qualiplante.eu) or from International Plant Analysis and Diagnostics s.r.l.

2. Methods

The Flavescence doree/Bois noir detection kit includes the ready-to-use reaction mix (primers and probes for Flavescence doree, Stolbur and IPC, master mix), a positive and a negative controls for the detection of both phytoplasma (Flavescence doree and Stolbur) and IPC.

3. Essential procedural information

3.1. Controls

In order to obtain reliable test results, the following controls should be included for each test and/or series of tests:

Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix.

Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).

Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of the only master mix provided by the kit.

Negative amplification control of the kit (NAC_{KIT}) to rule out false positives due to contamination during the preparation of the reaction mix: nucleic acid extracted from plants uninfected by the target organism (negative control provided by the kit).

Positive control (PAC) to monitor the efficiency of the amplification of nucleic acid from the target organism. This control includes nucleic acid extracted from plants infected by the target organism or a synthetic control (e.g. cloned PCR product) and it permits to verify that the amplification step has been correctly run. An extracted DNA from an infected grapevine plant is provided by the kit.

3.2. Interpretation of results

Verification of the control

NIC and NAC_{KIT} should not generate any amplification curves associated with the fluorochromes FAM and VIC, but only with Cy5.

NAC should not generate any amplification curves.

PIC and PAC (positive control of the kit) should generate exponatial curves for the fluorochromes FAM, VIC and Cy5.

The amplification curve associated to the IPC should be present in all the samples, NIC (negative control of the kit), PIC and PAC (positive control of the kit) controls.

When these conditions are met this decision scheme shoul be applied:

The cycle cut off value is set at 40 using procedures described in Mehle *et al.* (2013b)

	Ct IPC < 22	Ct IPC ≥ 22
Ct FD ≤ 40	Positive FD	Positive FD
Ct BN ≤ 40	Positive BN	Positive BN
40 < Ct FD < 45	*Uninterpretable*	*Unreliable*
40 < Ct BN < 45	*Uninterpretable*	*Unreliable*
No Ct	Negative FD and/or BN	*Unreliable*

Probe	Fluorochrome
FD	FAM
BN	VIC
IPC	Cy5

Tests should be repeated if any contradictory, unclear, unreliabe or uninterpretable results are obtained.

4. Performance criteria available

Validation data available from the test performance study in 2013 (Euphresco Grafdepi Project)

4.1. Analytical sensitivity data

For FD phytoplasma target: 100%*

Last level at 100% positive results: 1/10 to 1/300 (levels tested between 1/10 and 1/2700 for 4 different positive DNA extract diluted in healthy DNA extract)**

Last level with positive result(s): 1/2700**

For BN phytoplasma target: 85.25.%*

Last level at 100% positive results: 1/100 (levels tested between 1/10 and 1/2700 for 3 different positive DNA extract diluted in healthy DNA extract)***

Last level with positive result(s): 1/2700***

4.2. Analytical specificity data

For FD phytoplasma target: 100%*

For BN phytoplasma target: 100%*

4.3. Data on repeatibility

For FD phytoplasma target: between 63 and 100%**

For BN phytoplasma target: between 68 and 100%***

4.3. Data on reproducibility

For FD phytoplasma target: 86.73%**

For BN phytoplasma target: 96.2%***

*The 7 participant laboratories analysed a total of 24 blind samples including 4 grapevine phytoplasma-free samples and 20 single or mixed infected samples. In particular: 11 samples positive for FD phytoplasma, 4 samples infected by other phytoplasmas belonging to 16SrV group, 9 samples positive for Stolbur phytoplasma and 3 samples infected by phytoplasmas belonging to 16SrI, 16SrIII and 16SrVII groups.

**Repeatability, reproducibility and last level of positive results were evaluated with dilution series of 3 positive DNA extracts in 4 laboratories.

***Repeatability, reproducibility and last level of positive results were evaluated with dilution series of 1 positive DNA extracts in 4 laboratories.

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Corrigendum

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Since the publication of the EPPO Standard PM 7/079 (2) Grapevine flavescence dorée phytoplasma (EPPO, 2016) the authors have brought to our attention that the CTAB buffer used in routine analysis, and that was evaluated by the authors, is not the same as the one in the publication quoted. The authors considered an erratum should be prepared to describe the concentration that they used and evaluated.

Therefore the following paragraph in Appendix 1 should be replaced:

Old version

CTAB procedure for Nucleic acids extraction (Boudon-Padieu et al., 2003 modified from Doyle & Doyle (1990).

Nucleic acids can be extracted from fresh or frozen (−20 or −80°C) grapevine tissues (preferably veins or petioles). Grind 1 g of tissue in 10 mL of 3% CTAB buffer (3% CTAB or cethyl-trimethyl-ammonium bromide in **1 M** Tris–HCl pH8, **10 mM** EDTA, 1.4 M NaCl) at room temperature. Transfer into a 2 mL clean Eppendorf tube and centrifuge at 1000 g for 10 min. Transfer 1 mL of the suspension to an Eppendorf tube, add 2 μL of 2-marcaptoethanol (for a final concentration of 0.2%), vortex briefly and incubate for 20 min at 65°C. Then, add an equal volume of chloroform:isoamyl alcohol (24:1). Vortex and centrifuge at 10 000 g for 10 min. Recover the aqueous phase and precipitate the nucleic acids with an equal volume of cold isopropanol. Shake by inversion and centrifuge at 10 000 g for 15 min to recover the precipitate. Wash the pellet with 70% ethanol, dry and dissolve in 400 μL of TE buffer or nuclease-free water.

New version

*CTAB procedure for Nucleic acids extraction (***adapted from** *Boudon-Padieu et al., 2003 modified* **and** *Doyle & Doyle (1990).*

Nucleic acids can be extracted from fresh or frozen (−20 or −80°C) grapevine tissues (preferably veins or petioles). Grind 1 g of tissue in 10 mL of 3% CTAB buffer (3% CTAB or cethyl-trimethyl-ammonium bromide in **0.1 M** Tris–HCl pH8, **25 mM** EDTA, 1.4 M NaCl) at room temperature. Transfer into a 2 mL clean Eppendorf tube and centrifuge at 1000 g for 10 min. Transfer 1 mL of the suspension to an Eppendorf tube, add 2 μL of 2-marcaptoethanol (for a final concentration of 0.2%), vortex briefly and incubate for 20 min at 65°C. Then, add an equal volume of chloroform:isoamyl alcohol (24:1). Vortex and centrifuge at 10 000 g for 10 min. Recover the aqueous phase and precipitate the nucleic acids with an equal volume of cold isopropanol. Shake by inversion and centrifuge at 10 000 g for 15 min to recover the precipitate. Wash the pellet with 70% ethanol, dry and dissolve in 400 μL of TE buffer or nuclease-free water.

Reference

EPPO (2016) PM 7/079 (2) Grapevine flavescence dorée phytoplasma, *Bulletin OEPP/EPPO Bulletin*, **46**, 78–93.

