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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/133 (1)

Diagnostics
Diagnostic

PM 7/133 (1) Generic detection of phytoplasmas

Specific scope

This Standard describes a diagnostic protocol for the generic detection of phytoplasmas.[1]

This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*

Specific approval and amendment

Approved in 2018–09.

This diagnostic protocol was prepared on the basis of the IPPC diagnostic protocol adopted in 2016 (Appendix 12 to ISPM 27). Although this EPPO Diagnostic Standard differs in terms of format it is fully consistent with the content of the IPPC Standard.

1. Introduction

Phytoplasmas were first discovered by Doi *et al.* (1967) during their search for the causal agent of aster yellows. These unicellular organisms were called mycoplasma-like organisms because of their morphological similarity to animal mycoplasmas and their sensitivity to tetracycline antibiotics (Ishiie *et al.*, 1967). Phytoplasmas are obligate prokaryotic plant pathogens that do not possess cell walls; they are pleomorphic in profile, with a mean diameter of 200–800 nm. They inhabit the phloem sieve cells of their plant hosts. Phytoplasmas have genomes ranging in size from around 550–1500 kb – a relatively small genome compared with other prokaryotes – and they lack several biosynthetic functions (Marcone *et al.*, 1999; Davis *et al.*, 2005; Bai *et al.*, 2006; Oshima *et al.*, 2013).

Phytoplasmas are associated with a wide variety of symptoms in a diverse range of plant hosts (Lee *et al.*, 2000). Characteristic symptoms associated with phytoplasma infection include: virescence (the development of green flowers and the loss of normal floral pigments); phyllody (the development of floral parts into leafy structures); witches' broom (proliferation of auxiliary or axillary shoots) and other abnormal proliferation of shoots and roots; foliar yellowing, reddening and other discoloration; reduced leaf and fruit size; phloem necrosis; and overall decline and stunting (Davis & Sinclair, 1998). Some plant species are tolerant or resistant to phytoplasma infections; when infected, these plants may be asymptomatic or exhibit mild symptoms (Lee *et al.*, 2000).

Seemüller *et al.* (2002) estimated that about 1000 plant species are affected by phytoplasmas. Most phytoplasma host plants are dicotyledons. Fewer phytoplasmas have been detected in monocotyledons; such hosts are mainly from the families Palmae and Poaceae (Seemüller *et al.*, 2002).

Phytoplasmas occur worldwide. The geographical distribution and impact of phytoplasma diseases depends on the host range of the phytoplasma as well as on the presence and the feeding behaviour of the insect vector. Some phytoplasmas have a broad range of plant hosts and polyphagous vectors and therefore have a wide distribution. Other phytoplasmas have restricted host ranges and oligophagous or monophagous insect vectors, which restrict their geographical distribution. For a review of the geographical distribution of the main phytoplasma taxonomic groups see Foissac & Wilson (2010).

Phytoplasmas can be transmitted by insect vectors, dodders and grafting and can be spread by vegetative propagation of infected plant parts. Insect vectors of phytoplasmas, responsible for much of their natural spread, are restricted to phloem-feeding leafhoppers, plant hoppers and psyllids (Hemiptera, Auchenorrhyncha). They transmit the pathogen in a persistent manner. Weintraub & Beanland (2006) list more than 90 species that are known to be vectors, some of which are capable of vectoring more than one phytoplasma. Other methods of transmission of phytoplasmas include dodder and graft transmission. Dodders (*Cuscuta* and

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

Cassytha spp.) are parasitic vines that develop vascular connections with their hosts through haustoria. When a bridge is established between a healthy plant and a phytoplasma-infected plant, the phytoplasma will transfer to the healthy plant via the connecting phloem elements. Graft transmission and micropropagation of plants in tissue culture can be used to maintain phytoplasmas for reference purposes (IPWG, n.d.).

Further information on phytoplasmas, including photos showing disease symptoms, a list of insect vectors and a phytoplasma classification database, can be found at the following websites: COST Action FA0807 Integrated Management of Phytoplasma Epidemics in Different Crop Systems (http://www.costphytoplasma.ipwgnet.org/), Phytoplasma Resource Center (https://plantpathology.ba.ars.usda.gov/phytoplasma.html) and the EPPO Global Database (https://gd.eppo.int/).

2. Identity

Name: Phytoplasma
Synonyms: Mycoplasma-like organism (MLO), mycoplasma
Taxonomic position: Bacteria, Firmicutes, Mollicutes, Acholeplasmatales, Acholeplasmataceae, '*Candidatus* Phytoplasma'

The International Research Programme on Comparative Mycoplasmology (IRPCM) Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group has published guidelines for the description of '*Candidatus* (*Ca.*) Phytoplasma' species (IRPCM, 2004). Delineation of '*Ca.* Phytoplasma' species is based on 16S ribosomal (r)RNA gene sequences as well as on biological characteristics. In general, phytoplasmas within a species are ≥97.5% identical over ≥1200 nucleotides of their 16S rRNA gene. When a '*Ca.*' species includes phytoplasmas with different biological characteristics (vectors and host plants) they can be taxonomically distinguished following specific rules reported in The IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group (2004). Descriptions of '*Ca.* Phytoplasma' species are published in the *International Journal of Systematic and Evolutionary Microbiology* (The IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004), and as of January 2018, 44 '*Ca.* Phytoplasma' species including 3 provisional species have been described (Miyazaki *et al.*, 2018).

3. Detection

Polymerase chain reaction (PCR) techniques are the method of choice for phytoplasma detection. Successful molecular detection of phytoplasmas is dependent on appropriate sampling of plant tissue and reliable nucleic acid extraction methods (Palmano, 2001; Firrao *et al.*, 2007).

3.1 Sampling

Phytoplasmas can be distributed unevenly and in an uneven titre throughout a plant, particularly in woody hosts; therefore, symptomatic tissue is optimal for phytoplasma detection (Constable *et al.*, 2003; Garcia-Chapa *et al.*, 2003; Christensen *et al.*, 2004; Necas & Krska, 2006). Symptomless infection can occur in some plant hosts, and if this is suspected it is important to thoroughly sample different parts of the plant.

The phytoplasma titre in the plant host affects the reliability of the PCR test (Marzachì, 2004). Phytoplasma titre can be affected by phytoplasma strain or species, host plant species, timing of infection and climatic conditions. The timing for sampling plant tissues is important as location in the plant and titre of phytoplasmas may be affected by seasonal changes (Seemüller *et al.*, 1984; Jarausch *et al.*, 1999; Berges *et al.*, 2000; Constable *et al.*, 2003; Garcia-Chapa *et al.*, 2003; Prezelj *et al.*, 2012).

For most phytoplasma diseases, leaves with symptoms are the best sources of samples for diagnosis. Phytoplasmas reside in the phloem sieve elements of infected plants, and therefore the leaf petioles and midveins, stems or inner bark are often used for DNA extraction. In some cases (e.g. X-disease phytoplasma), fruit peduncles contain the highest phytoplasma titre (Kirkpatrick, 1991). Although phytoplasmas can be detected in roots and bark scrapings of dormant trees, generally it is best to test for phytoplasmas at the end of summer. Collected plant samples can be stored at −20°C for up to 6 months before testing. Longer term storage is at −80°C, or the plant material can be freeze-dried or dried over calcium chloride and stored at 4°C.

3.2 PCR tests

A number of universal PCR primers have been designed that allow amplification of the 16S rRNA gene of any known phytoplasma. The most commonly used primers are the P1/P7 (Deng & Hiruki, 1991; Schneider *et al.*, 1995) and R16F2n/R16R2 (Lee *et al.*, 1993; Gundersen & Lee, 1996) primer pairs, which can be used in a nested PCR protocol. The P1/P7 primer pair amplifies a PCR product that contains the entire 16S rRNA gene as well as the 16S/23S rRNA spacer region. Real-time PCR has been reported to have an analytical sensitivity equal to or higher than that of nested PCR, depending on the host–phytoplasma combination (Christensen *et al.*, 2004), and is more amenable to high-throughput analysis because post-amplification processing is not required. Real-time PCR using TaqMan probes also has better analytical specificity and there is less chance of cross-contamination than with nested PCR. False positives with bacteria (*Bacillus* spp.) can occur with the PCR tests recommended in this protocol – a necessary compromise for a universal test (Pilotti *et al.*, 2014). If the outcome of the test result is critical (e.g. post-entry quarantine samples, new host record, new distribution), PCR tests

which are more specific should be performed, or alternatively the conventional/nested PCR product should be sequenced. Another generic test based on fU5/rU3 (Lorenz *et al.,* 1995) is described in Appendix 6 of PM 7/62 (2) (EPPO, 2016a).

As well as amplification of the 16S rRNA gene, PCR methods have also been used to amplify other genome regions for phytoplasma detection and classification, including ribosomal protein genes (Lim & Sears, 1992; Jomantiene *et al.*, 1998; Lee *et al.*, 1998; Martini *et al.*, 2007), the *tuf* gene (Schneider *et al.*, 1997; Makarova *et al.*, 2012), the 23S rRNA gene (Guo *et al.*, 2003) and the *secY* gene (Lee *et al.*, 2010; Davis *et al.*, 2013; Quaglino *et al.*, 2013). These primers may be useful when a second independent region of the phytoplasma genome is required.

Samples may contain compounds that are inhibitory to PCR depending on the host species and type and age of the tissue. Therefore, it is important to check the PCR competency of the DNA extractions using internal control primers that amplify a gene from the plant host. Inhibitory effects of the host can be overcome by further purifying the DNA through a sephacryl spin column or by adding bovine serum albumin (BSA) to the PCR mixture to a final concentration of 0.5 mg mL^{-1} (Kreader, 1996).

The tests described in this protocol are:

- Appendix 2 Conventional nested PCR using the primers P1/P7 (Deng & Hiruki, 1991; Schneider *et al.*, 1995) and R16F2n/R16R2 (Lee *et al.*, 1993; Gundersen & Lee, 1996)
- Appendix 3 Real-time PCR for the generic detection of phytoplasmas (Christensen *et al.*, 2004)
- Appendix 4 Real-time PCR for the generic detection of phytoplasmas (Hodgetts *et al.*, 2009).

The real-time PCR test (Christensen *et al.*, 2004) was evaluated by testing phytoplasmas from 18 subgroups and was found to have an analytical sensitivity equal to or up to ten times higher than conventional nested PCR, depending on the host–phytoplasma combination (Christensen *et al.*, 2004). A test performance study for the detection of fruit tree phytoplasmas involving 22 laboratories suggested that the Christensen *et al.* (2004) and Hodgetts *et al.* (2009) tests are similar in terms of analytical sensitivity and analytical specificity (EUPHRESCO FruitPhytoInterlab Group, 2011).

4. Identification

For identification of a specific phytoplasma the following EPPO diagnostic protocols are available:

PM 7/61 'Candidatus *Phytoplasma aurantifoliae'* (EPPO, 2005)

PM 7/62 'Ca. *P. mali',* 'Ca. *P. pyri' and* 'Ca. *P. prunorum'* (EPPO, 2016a)

PM 7/79 *Grapevine flavescence dorée phytoplasmas* (EPPO, 2015)

General procedures for DNA barcoding of phytoplasmas are described in EPPO Standard PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2016b).

Sequence analysis

Detailed guidance for sequence analysis is also given in Appendices 7 and 8 of EPPO Standard PM 7/129 *DNA barcoding as an identification tool for selected regulated* (EPPO, 2016b).

Appendix 12 to ISPM 27 (FAO, 2016) includes that: PCR products should be sequenced either directly or by first cloning them into a PCR cloning vector. Sequence data can be analysed using the Basic Local Alignment Search Tool, BLASTN, available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). If the sequence shares less than 97.5% identity with its closest relative, the phytoplasma is considered to be a new '*Ca.* Phytoplasma' species. In this case, the entire 16S rRNA gene should be sequenced and phylogenetic analysis performed. Sequencing a separate region of the genome such as the 16S/23S rRNA spacer region, *secY* gene, ribosomal protein genes or the *tuf* gene is also desirable.

The Panel on Diagnostics in Virology and Phytoplasmology noted that high-throughput sequencing is a technology that may be used for obtaining (almost) complete genome sequences, which can be analysed for identification of a phytoplasma isolate.

5. Reference material

Phytobacteriology 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

Sequences for different strains are available in Q-bank (http://www.q-bank.eu/Phytoplasmas/).

INRA UMR Fruit Biology and Pathology, Bordeaux (FR) https://www6.bordeaux-aquitaine.inra.fr/bfp_eng/Resources/Phytoplasmas-collection

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *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 is 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 those organisms can be obtained from:

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Ms Constable, Department of Economic Development, Jobs, Transport and Resources, Victoria, AgriBio, 5 Ring Road, Bundoora, Vic. 3083, Australia (fiona.constable@ecodev.vic.gov.au).

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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.

Acknowledgements

This diagnostic protocol was drafted by Ms Liefting (Plant Health and Environment Laboratory, Ministry for Primary Industries, New Zealand; see preceding section), Mr Jones (Plant Pathogen Interactions Division, Rothamsted Research, United Kingdom), Ms Constable (Department of Economic Development, Jobs, Transport and Resources, Victoria, Australia; see preceding section), Ms Torres (Department of Territory and Sustainability, Barcelona, Spain; see preceding section), Mr Jelkmann (Federal Biological Research Centre for Agriculture and Forestry, Institute for Plant Protection and Fruit Crops, Germany; see preceding section) and Mr Verhoeven (NPPO NL NRC, Wageningen, the Netherlands).

The current formatted text was reviewed by the Panel on Diagnostics in Virology and Phytoplasmology.

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Appendix 1 – DNA extraction

Cetyl-trimethyl-ammonium bromide (CTAB) procedure (modified from Doyle & Doyle, 1990)

Several methods have been developed and compared (Palmano, 2001). The method described below is an optimization of a method described by Doyle & Doyle (1990) for extraction of DNA from woody plants.

Nucleic acids can be extracted from fresh or frozen (−20°C or −80°C) tissues or tissues freeze-dried or dried over calcium chloride [leaf veins, vascular tissue (phloem) from bark or roots]. For material that has been freeze-dried or dried over calcium chloride, the quantity needed for DNA extraction should be adapted depending on the weight loss during dry process.

Grind approximately 1 g of tissue in 10 mL of 3% CTAB buffer (3% CTAB in 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 1.4 M NaCl) at room temperature. Transfer 1 mL of the suspension to an Eppendorf tube, add 2 μL of 2-mercaptoethanol (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, air dry and dissolve in 100 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) 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. This extraction method has been validated in combination with the PCR tests described in Appendices 2–4. It has also been used with other molecular tests (nested PCR and loop-mediated isothermal amplification, LAMP) and performed well; however, validation data has not yet been published (Mehle *et al.*, 2017).

Preparation of crude extracts

Option 1: approximately 1 g of leaf mid-vein tissue or vascular tissue (phloem) from bark or roots is homogenized in 2 mL of extraction 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) or lysis buffer (from a QuickPick™ SML Plant DNA kit, Bio-Nobile) using tissue homogenizer (e.g. FastPrep®-24 with TN 12 × 15-TeenPrep™ Adapter, MP Biochemicals) or in extraction bags using a Homex 6 homogenizer (BIOREBA).

Option 2: the tissue is homogenized with liquid nitrogen using a mortar and pestle.

DNA extraction
Total DNA can be reliably extracted using a QuickPick™ SML Plant DNA kit (Bio-Nobile) and a magnetic particle processor (e.g. KingFisher® mL, Thermo Scientific) (Mehle *et al.*, 2013).

Total DNA extract is eluted in 200 µL of elution buffer (QuickPick™ SML Plant DNA kit + KingFisher). For leaf mid-vein tissue and bark/root phloem tissue tenfold diluted DNA is suitable for testing.

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

Other extraction methods may be used but should be validated in combination with the PCR test to be used.

Please note that testing for vectors is not included in this protocol.

Appendix 2 – Conventional nested PCR using the primers P1/P7 and R16F2n/R16R2

1. General Information

1.1 Detection of phytoplasmas in plant extracts
1.2 Nested PCR using two sets of primers, namely P1/P7 primers (Deng & Hiruki, 1991; Schneider *et al.*, 1995) for the first PCR and R16F2n/R16R2 (Lee *et al.*, 1993 and Gundersen & Lee, 1996) for the second PCR.
1.3 Oligonucleotides

	Primers	Target region	Amplicon size (bp)
P1 (forward)	5′-AAG AGT TTG ATC CTG GCT CAG GAT T-3′	16S, intergenic 16S–23S and a small part of the 23S rDNA gene	~1800
P7 (reverse)	5′-CGT CCT TCA TCG GCT CTT-3′		
R16F2n (forward)	5′-GAA ACG ACT GCT AAG ACT GG-3′	16S rDNA	~1250
R16R2 (reverse)	5′-TGA CGG GCG GTG TGT ACA AAC CCC G-3′		

2. Methods

2.1 Nucleic acid extraction and purification
2.1.1 See Appendix 1
2.1.2 DNA should preferably be stored at approximately −20°C.
2.2 Conventional PCR, followed by nested PCR
2.2.1 Master mix for the PCR.

Note: no specific sources are indicated for buffer and dNTPs (these are not available in the IPPC protocol). As no validation data is available, each laboratory will have to adapt the test.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	12.4	N.A.
PCR buffer	10×	2	1×
$MgCl_2$	25 mM	1.2	1.5 mM
dNTPs	20 mM	0.2	0.2 mM
Forward primer (P1)	10 µM	1	0.5 µM
Reverse primer (P7)	10 µM	1	0.5 µM
Polymerase	5 U μL^{-1}	0.2	1 U
Subtotal		18	
Genomic DNA extract		2	
Total		20	

*Molecular grade water should preferably be used, or prepared, purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free water.

2.2.2 PCR conditions
Initial denaturation step of 94°C for 2 min followed by 40 cycles of 94°C for 30 s, 53°C (P1/P7 primers) for 30 s and 72°C for 1 min, and a final extension step of 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.	12.4	N.A.
PCR buffer	10×	2	1×
$MgCl_2$	25 mM	1.2	1.5 mM
dNTPs	20 mM	0.2	0.2 mM
Forward primer (R16F2n)	10 µM	1	0.5 µM
Reverse primer (R16R2)	10 µM	1	0.5 µM
Polymerase	5 U μL^{-1}	0.2	1 U
Subtotal		18	
Non-diluted to 1/30 diluted P1/P7 PCR product		2	
Total		20	

*Molecular-grade water should preferably be used, or prepared, purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free water.

2.2.4 PCR conditions
Initial denaturation step of 94°C for 2 min followed by 40 cycles of 94°C for 30 s, 50°C for 30 s and 72 °C for 1 min, and a final extension step of 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 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 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 to (or in addition to) the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. IPCs can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative IPCs 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. the universal eukaryotic 28S rRNA gene primers of Werren *et al.* (1995))
- 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.

Laboratories should take additional care to prevent risks of cross-contamination when using high-concentration positive controls (e.g. cloned products, gBlocks and whole genome amplicons).

3.2 Interpretation of results

Verification of the controls

- NIC and NAC should produce no amplicons
- PIC, PAC (as well as IC and IPC as applicable) should produce amplicons of the expected size (depending on whether the target, endogenous or exogenous nucleic acid is used).

When these conditions are met:

- A test will be considered positive if amplicons of 1250 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

None.

Appendix 3 – Real-time PCR for the generic detection of phytoplasmas (Christensen *et al.*, 2004)

1. General information

1.1 The following real-time PCR protocol is performed for the detection of phytoplasmas.

1.2 The test was developed by Christensen *et al.* (2004) and the test description was published by Christensen *et al.* (2013).

1.3 Primers

	Primers	Target region
Forward primer	5′-CGT ACG CAA GTA TGA AAC TTA AAG GA-3′	16S rDNA
Reverse primer	5′-TCT TCG AAT TAA ACA ACA TGA TCC A-3′	16S rDNA
Probe	5′-FAM-TGA CGG GAC TCC GCA CAA GCG-TAMRA-3′	16S rDNA

Probe and primers were based on alignments of 16S rDNA obtained from GenBank from a range of phytoplasma strains (one of each phytoplasma 16Sr group), bacteria and mycoplasmas. This test is considered as generic, although validation data is not available for all phytoplasmas.

1.4 A test performance study (Euphresco, FruitPhytoInterlab) was performed with a TaqMan Universal PCR Master Mix from Applied Biosystems.

1.5 Validation data has been generated using software (e.g. SDS 2.4, Applied Biosystems) for fluorescence acquisition and calculation of threshold cycles (Ct). 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 analysing the raw data, it is important to adjust the 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 increase of fluorescence in the amplification plot.

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 Real-time PCR

2.2.1 Master mix

Reagents	Working concentration	Volume per reaction (μL)*	Final concentration
Molecular-grade water†	N.A.	1.4	N.A.
TaqMan Universal PCR Master Mix (Applied Biosystems, containing UNG‡)	2×	5.0	1×
Forward primer	10 μM	0.3	0.3 μM
Reverse primer	10 μM	0.9	0.9 μM§
Probe¶	2.5 μM	0.4	0.1 μM
Subtotal		8.0	
DNA		2.0	
Total		10	

*If a 25 μL reaction volume is used, multiply each component by 2.5.
†Molecular-grade water should preferably be used, or prepared, purified (deionized or distilled), sterile (autoclaved or 0.22 μm filtered) and nuclease-free water.
‡UNG or UDG (uracil-DNA glycosylase).
§The real-time PCR of Christensen *et al.* (2004) uses 0.9 μM of the reverse primer, and this was updated to 0.3 μM in a later report (Christensen *et al.*, 2013).
¶Modified protocol is used in New Zealand with primers of Christensen *et al.* (2004) with probes from Malandraki *et al.* (2015) to avoid cross-reaction, as the authors of the protocol have no experience with this modified protocol the probe has not been changed.

2.2.2 Real-time PCR conditions: 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 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 (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product[2]). The PAC should preferably be near the limit of detection.

In addition to the external positive controls (PIC), the IPPC diagnostic protocol recommends using internal positive controls (IPC) to monitor each individual sample separately. IPCs can either be genes present in the matrix DNA or added to the DNA solutions. IPCs 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, for example a plant cytochrome oxidase gene (Weller *et al.*, 2000; Papayiannis *et al.*, 2011) or eukaryotic 18S rDNA (AB Kit, cat. no. 4319413E)
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation to the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Laboratories should take additional care to prevent risks of cross-contamination when using high-concentration positive controls (e.g. cloned products, gBlocks and whole-genome amplicons)

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.

[2]Laboratories should take additional care to prevent risks of cross-contamination when using cloned PCR products.

4. Performance criteria available

Validation data is available from the test performance study in 2011 (Euphresco, FruitPhytoInterlab), in which the 10 participating laboratories analysed a total of 30 blind samples. This consisted of samples from nine healthy fruit trees, six bacteria (*Bacillus subtilis*, *Erwinia chrysanthemi*, *Fructobacillus fructosus*, *Paenibacillus alvei*, *Pseudomonas syringae* pv. *cersicola*, *Ralstonia solanacearum*), five samples infected by '*Ca*. P. mali', five samples infected by '*Ca*. P. prunorum' and five samples infected by '*Ca*. P. pyri'. In five participating laboratories analytical sensitivity was also tested using a serial dilution of cloned P1/P7 fragments from '*Ca*. P. mali' and '*Ca*. P. pyri' at concentration of 10^7 to 10^1 copy numbers per mL.

4.1 Analytical sensitivity
Analytical sensitivity for '*Ca*. P. mali': as low as 10^1 copy numbers per mL.
Analytical sensitivity for '*Ca*. P. pyri': as low as 10^1–10^2 copy numbers per mL.
4.2 Diagnostic sensitivity
100%
4.2 Diagnostic specificity
96%
4.3 Repeatability
Not available.
4.4 Reproducibility
Agreement between laboratories measured by calculation of the Kappa coefficient (Fleiss *et al*., 2003) is 0.926.[3]

Appendix 4 – Real-time PCR for the generic detection of phytoplasmas (Hodgetts *et al.*, 2009)

1. General information

1.1 The test was developed by Hodgetts *et al.* (2009).
1.2 Primers and probes.

	Primers	Target region
Forward primer JH-F1	5′-GGT CTC CGA ATG GGA AAA CC-3′	23S rDNA
Forward primer JH-F all	5′-ATT TCC GAA TGG GGC AAC C-3′	23S rDNA
Reverse primer JH-R	5′-CTC GTC ACT ACT ACC RGA ATC GTT ATT AC-3′	23S rDNA
Probe JH-P uni	5′-FAM-AAC TGA AAT ATC TAA GTA AC-MGB-3′	23S rDNA

1.3 The test performance study (Euphresco, FruitPhytoInterlab) was performed with a TaqMan Universal PCR Master Mix from Applied Biosystems.
1.4 Validation data has been generated using software (e.g. SDS 2.4, Applied Biosystems) for fluorescence acquisition and calculation of threshold cycles (Ct). 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 analysing the raw data it is important to adjust the 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 increase of fluorescence in the amplification plot.

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 Real-time PCR
2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (μL)*	Final concentration
Molecular-grade water†	N.A.	1.7	N.A.
TaqMan Universal PCR Master Mix (Applied Biosystems), containing UNG‡	2×	5.0	1×
Forward primer (JH-F1)	10 μM	0.3	0.3 μM
Forward primer (JH-F all)	10 μM	0.3	0.3 μM
Reverse primer (JH-R)	10 μM	0.3	0.3 μM
Probe (JH-P uni)	2.5 μM	0.4	0.1 μM
Subtotal		8.0	
DNA		2.0	
Total		10.0	

*If a 25 μL reaction volume is used, multiply each component by 2.5.
†Molecular-grade water should preferably be used, or prepared, purified (deionized or distilled), sterile (autoclaved or 0.22 μm filtered) and nuclease-free water.
‡UNG or UDG (uracil-DNA glycosylase).

2.2.2 Real-time PCR conditions: uracil *N*-glycosylase activation step at 50°C for 2 min; initial denaturation at 95°C for 10 min; 40 cycles consisting of 15 s at 95°C and 1 min at 60°C.

[3]Interpretation of Kappa values: <0, poor agreement; 0.00–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; 0.81–1.00, almost perfect agreement (Landis & Koch, 1977).

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 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 (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product[1]). The PAC should preferably be near to the limit of detection.

In addition to the external positive controls (PIC), the IPPC DP recommends using internal positive controls (IPC) to monitor each individual sample separately. IPCs can be either genes present in the matrix DNA or added to the DNA solutions.

IPCs 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, for example a plant cytochrome oxidase gene (e.g. Weller *et al.*, 2000; Papayiannis *et al.*, 2011) or eukaryotic 18S rDNA (AB Kit, cat. no. 4319413E)
- 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.

Laboratories should take additional care to prevent risks of cross-contamination when using high-concentration positive controls (e.g. cloned products, gBlocks and whole-genome amplicons)

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.

4 Performance criteria available

Validation data is available from the test performance study in 2011 (Euphresco, FruitPhytoInterlab), where the 12 participating laboratories analysed a total of 30 blind samples This consisted of samples from nine healthy fruit trees, six bacteria (*Bacillus subtilis*, *Erwinia chrysanthemi*, *Fructobacillus fructosus*, *Paenibacillus alvei*, *Pseudomonas syringae* pv. *cersicola*, *Ralstonia solanacearum*), five samples infected by '*Ca.* P. mali', five samples infected by '*Ca.* P. prunorum' and five samples infected by '*Ca.* P. pyri'.

This test is considered as generic, although validation data is not available for all phytoplasmas.

4.1 Diagnostic sensitivity

99.4%

4.2 Diagnostic specificity

97.2%

4.3 Data on repeatability

Not available.

4.4 Data on reproducibility

Agreement between laboratories measured by calculation of the Kappa coefficient (Fleiss *et al.*, 2003) is 0.945.[3]