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Diagnostics
Diagnostic

PM 7/20 (2)* *Erwinia amylovora*

Specific scope

This standard describes a diagnostic protocol for *Erwinia amylovora*[1].

Specific approval and amendment

This standard was developed under the EU DIAGPRO Project (SMT 4-CT98-2252) and EUPHRESCO Pilot project (ERWINDECT) by partnership of contractor laboratories. Test performance studies were performed with different laboratories in 2002, 2009 and 2010.

Approved as an EPPO Standard in 2003-09. Revised in 2012-09.

Introduction

Erwinia amylovora is the causal agent of fire blight in most species of the subfamily *Maloideae* of the family *Rosaceae*. The most economically important hosts are *Pyrus* spp., *Malus* spp., *Cydonia* spp., *Eriobotrya japonica*, *Cotoneaster* spp., *Crataegus* spp., *Pyracantha* spp. and *Sorbus* spp. Other hosts include *Chaenomeles*, *Mespilus* and *Photinia*. A *forma specialis* was described from *Rubus* spp. (Starr *et al.*, 1951; Bradbury, 1986). An exhaustive list of affected plants, including those susceptible only after inoculation, was reported by van der Zwet & Keil (1979). It includes more than 180 species from 39 genera of the *Rosaceae*. *Erwinia amylovora* was the first bacterium described as a causal agent of a plant disease by Burrill (1883). It was reported in North America and was later detected in New Zealand in 1920. In Europe, fire blight was reported in 1957 in the United Kingdom and has since been identified in most areas where susceptible hosts are cultivated. *Erwinia amylovora* is now present in more than 40 countries (van der Zwet, 2002; CABI/EPPO, 2007), but it has not been recorded in South America, Asia or sub-Saharan African countries. It has been recorded in some North African countries and only once in Australia (Bonn & van der Zwet, 2000). It represents a threat to the pome fruit industry of all the countries. Details on geographical distribution can be found in the EPPO Plant Quarantine Data Retrieval system (PQR, 2012).

[1]Use of names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.
*This protocol number was corrected online on 25th April 2013.

Fire blight is probably the most serious disease affecting *Pyrus* spp. (pear) and *Malus* spp. (apple) cultivars in many countries. Although the life cycle of the bacterium is still not fully understood, it is known that it can survive as endophyte or epiphyte for variable periods depending on environmental factors (Thomson, 2000). The development of fire blight symptoms follows the seasonal growth development of the host plant. It begins in the spring with production of primary inoculum and infection of flowers, continues in summer with infection of shoots and fruits, and ends in autumn with the development of cankers. The pathogen is apparently quiescent through the dormant period of the host (van der Zwet & Beer, 1995).

Flow diagrams describing the diagnostic procedure for *E. amylovora* in symptomatic and asymptomatic material are presented in Figs 1 and 2.

Identity

Name: *Erwinia amylovora* (Burrill) Winslow *et al.*
Synonyms: *Micrococcus amylovorus* Burrill
Bacillus amylovorus (Burrill) Trevisan
Bacterium amylovorus (Burrill) Chester
Erwinia amylovora f. sp. *rubi* Starr, Cardona & Falson
Taxonomic position: Bacteria, Proteobacteria, γ Subdivision, *Enterobacteriales*, *Enterobacteriaceae*
EPPO code: ERWIAM
Phytosanitary categorization: EPPO A2 list no. 52, EU Annex designation II/A2

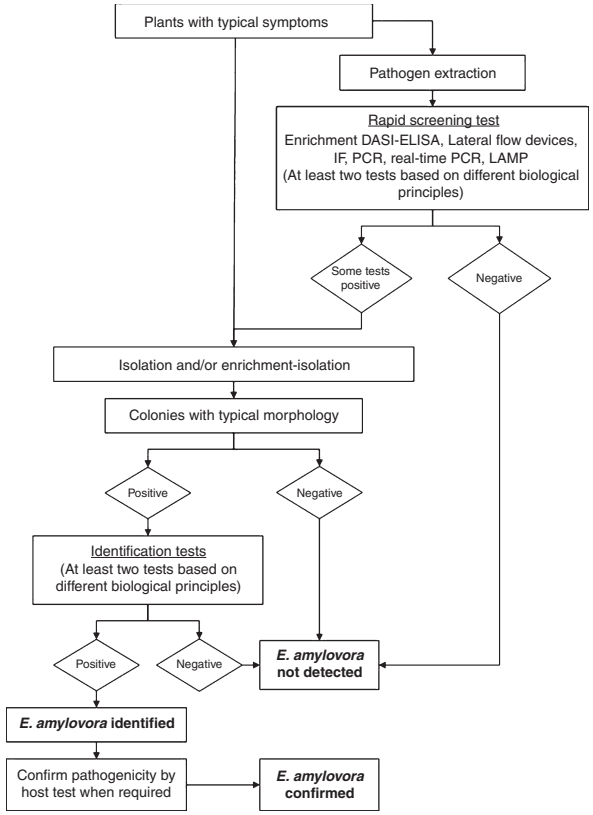

Fig. 1 Flow diagram for diagnosis of fire blight in plants with symptoms.

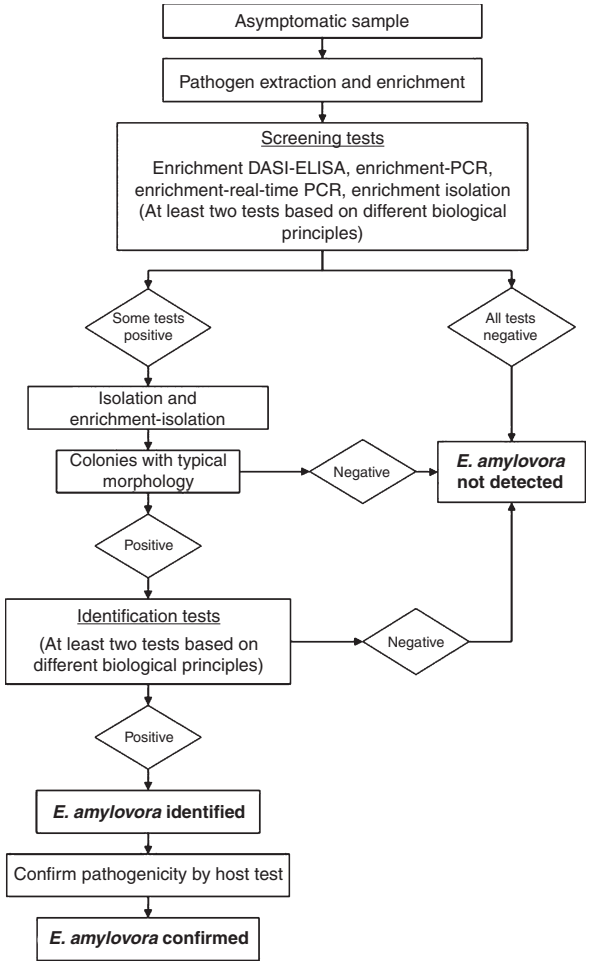

Fig. 2 Flow diagram for analysis of *Erwinia amylovora* in asymptomatic samples.

Detection

Disease symptoms

Symptoms of fire blight on the principal hosts are relatively similar and easily recognized (Figs 3–5). The name of the disease is descriptive of its major characteristic: the brownish appearance of twigs, flowers and leaves as though burned by fire. Typical symptoms on pome fruit trees are the brown to black colour of leaves on affected branches, the production of exudates under humid conditions, and the typical 'shepherd's crook' in the shoots. Depending on the affected plant part, the disease causes blossom blight, shoot or twig blight, leaf blight, fruit blight, limb and trunk blight, collar or rootstock blight (van der Zwet & Keil, 1979; van der Zwet & Beer, 1995).

In apple and pear, the first symptoms usually appear in early spring during warm and humid weather, and can progress very quickly under favourable conditions. Flowers appear to be water-soaked, then wilt, shrivel and turn pale brown to black. Peduncles may also appear water-soaked, become dark green, and finally brown or black, sometimes oozing droplets of sticky bacterial exudates. Leaves wilt and shrivel, and entire spurs turn brown in most hosts, or dark brown to black in pear, but remain attached to the tree for some time. Immature fruits (or less frequently mature fruits) have infected parts that appear oily or water-soaked, becoming brown to black and often exuding droplets of bacterial ooze. They also remain attached to the tree. Characteristic reddish-brown streaks are often found in the subcortical tissues when bark is peeled from infected twigs, branches or trunks (van der Zwet & Keil, 1979). Brown to black, slightly depressed cankers can develop in the bark of twigs or branches, or even the trunk, in autumn and winter. These cankers may later become defined by cracks near the margin of diseased and healthy tissue (Dye, 1983).

Confusion between fire blight and blight- or blast-like symptoms, especially in blossoms and shoots, may occur with diseases/disorders caused by other bacteria, fungi, insect damage and physiological disorders, and consequently laboratory analysis is always necessary. Other bacteria can cause blight-like symptoms, including *Erwinia pyrifoliae*, causal agent of bacterial shoot blight of *Pyrus pyrifolia* (Asian pear) (Kim *et al.*, 1999); *Erwinia piriflorinigrans*, isolated from necrotic pear blossoms in Spain (López *et al.*, 2011); *Erwinia* sp. and *Erwinia uzenensis*, causing different types of pear

Fig. 3 Symptoms of fire blight on pear trees. (A) necrotic flowers; (B) necrosis on leaves and typical shepherd's crook; (C) mummified immature fruits with small ooze drops; (D) canker after removing bark showing necrotic inner tissues.

Fig. 4 Typical symptoms of fire blight on: (A) pear branches; (B) apple shoot; (C) quince shoot; (D) loquat shoot.

symptoms in Japan (Tanii *et al.*, 1981; Matsuura *et al.*, 2011); and *Pseudomonas syringae* pv. *syringae*, the causal agent of blossom blast.

Detection from symptomatic samples

Sampling

Symptomatic samples can be processed individually or in small batches combining material from several samples (see Appendix 1). Precautions to avoid cross-contamination should be taken when collecting samples and during the extraction process. Samples with symptoms for diagnosis of fire blight should preferably be composed of flowers, shoots or twigs, leaves, fruitlets (all with necrosis and with exudates if possible), or the discoloured subcortical tissues (after peeling bark from cankers in twigs, branches, trunk or collar). Samples should be processed as soon as possible after collection and stored at 4–8°C before analysis. Samples may be cold stored after processing for up to 2 weeks in case further testing is required.

Isolation

Fresh sample extracts are necessary for successful isolation. Details on the extraction procedure from plant material are given in Appendix 1. Details on isolation are provided in Appendix 5. Isolating *E. amylovora* from symptomatic samples is relatively easy because the number of culturable

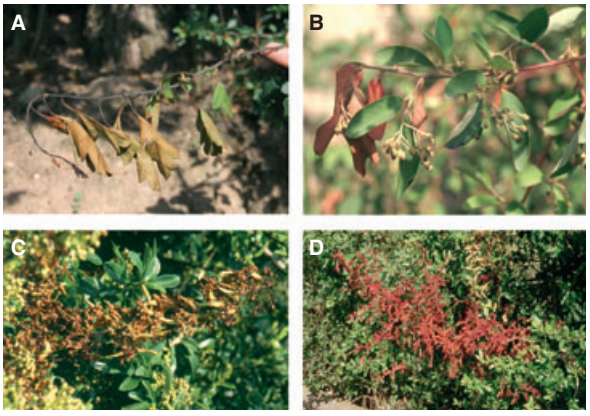

Fig. 5 Typical symptoms of fire blight on: (A) *Crataegus* sp. shoot; (B) *Cotoneaster* sp. shoot; (C,D) *Pyracantha* sp. branches.

bacteria in such samples is usually high. However, when symptoms are advanced or when environmental conditions are not favourable for fire blight symptom expression, the number of *E. amylovora* culturable cells can be very low. When plates are overcrowded by plant microbiota, the sample should be retested and enrichment according to Appendix 4 performed before isolation, as described in Appendix 5. Enrichment is also recommended when the presence of antagonistic bacteria in the sample is suspected.

Fig. 6 Typical colony morphology of *E. amylovora* on: (A) King's medium; (B) levan (NSA) medium and (C) CCT medium.

For direct isolation, plating on three media is advised for maximum recovery of *E. amylovora*, in particular when samples are in poor condition. The efficiency of the different media depends on the number and composition of microbiota in the sample. Three media: King's B, levan and CCT (Appendix 2) have been validated in a test performance study. Figure 6 shows the typical appearance of *E. amylovora* bacterial cultures in the three media.

Rapid screening tests

These tests facilitate presumptive diagnosis on plants with symptoms, in samples with more than 10^5–10^6 cfu g^{-1} (the minimum concentrations usually present in symptomatic samples). Several tests are described in Appendices 3–14. At least two tests, based on different biological principles, should be performed: one may be a serological test, preferably using specific monoclonal antibodies; the other a PCR-based test. Test performance studies were organized and the results are indicated. As differences in analytical sensitivity were observed in such studies, the decision on the tests selected should be based on a comparative analysis of the sensitivity and specificity of the different techniques in each laboratory, the number of samples to analyse, etc.

In areas where the disease is endemic, these tests can be used without further confirmation in routine analyses of samples.

- Serological tests

Indirect immunofluorescence (IF), enrichment DASI-ELISA and lateral flow devices are described for analyses of organs with symptoms. Quality of the antibodies is critical for performance of the tests. In test performance studies, several commercial antisera and monoclonal antibodies were compared for IF [polyclonal antiserum from Loewe, Biochemica GmbH (Sauerlach, Germany) and monoclonal antibodies from Plant Print Diagnostics S. L. (Faura, Spain)]. For ELISA, a complete kit based on a combination of specific monoclonal antibodies, from Plant Print Diagnostics S.L., was also evaluated.

Two lateral flow devices commercialized by Bioreba, Reinach, Switzerland (Ea AgriStrip) and Forsite Diagnostics, York, UK (Pocket Diagnostics) are available for the rapid analysis of symptomatic plant material (Braun-Kiewnick *et al.*, 2011).

Details of the tests are given in Appendix 3.

- Molecular tests

Conventional PCR, real-time PCR and loop-mediated isothermal amplification (LAMP) were evaluated in a test performance study in 2010 and are also recommended for the analyses of organs with symptoms after a DNA extraction step. The DNA extraction protocols that were evaluated in a test performance study in 2009 (Dreo *et al.*, 2009) are indicated in Appendix 6. Some other commercial kits for extracting DNA are available, but have not yet been validated. Amplification protocols for PCR and real-time PCR are indicated in Appendices 7–13 and a LAMP protocol is included in Appendix 14.

Detection from asymptomatic samples

Sampling and sample preparation

Warning: detection of *E. amylovora* in asymptomatic plants has been shown to be difficult.

The analyses of asymptomatic plants should be performed in summer or early autumn to increase the likelihood of detecting *E. amylovora*. Asymptomatic samples may be processed individually, or bulked (see Appendix 1). Precautions to avoid cross-contamination should be taken when collecting the samples and during the extraction process. Sampling and sample preparation can be performed following one of the methods for asymptomatic samples described in Appendix 1.

Direct analysis of asymptomatic samples is usually negative for *E. amylovora* due to the low bacterial population. Consequently, an enrichment step is advised (Appendix 4).

Screening tests

Enrichment-isolation, enrichment-DASI ELISA, and enrichment followed by conventional PCR or real-time PCR can be used as screening tests and are described in Appendices 4–13. At least two screening tests should be performed.

Confirmation of positive results of screening tests

If these screening tests are positive, an attempt should be made to isolate the pathogen directly from the extract of non-enriched samples (Appendices 1–3), or from the enriched samples (Appendices 4 and 5). As little is usually known about the microbiota present in the samples, the three media (CCT, King's B, levan) indicated in Appendix 2 should be used to maximize the likelihood of successful direct isolation of *E. amylovora*. However, plating only

on CCT medium is advised after enrichment of the samples in King's B or in CCT. If the isolation is still negative, and cross-reactions or non-desired amplifications can be disregarded as the controls were correct, it is reasonable to consider *E. amylovora* presumptively detected in the sample. Confirmation requires isolation and identification of the bacterium. If necessary, the extract conserved at −80°C under glycerol (Appendix 2) can also be plated on the three media.

Identification

Pure cultures of presumptive *E. amylovora* isolates should be identified with at least two tests based on different characteristics of the pathogen (e.g. combinations of biochemical, serological or molecular tests) and, when necessary, a pathogenicity test. Two molecular tests may be used if they are based on different DNA sequence targets in the genome and provided that the specificity of the primers has been evaluated. Known *E. amylovora* reference strains should be included for each test performed (see section on Reference material).

Biochemical tests

The genus *Erwinia* has been defined as Gram-negative bacteria, facultative anaerobes, motile by peritrichous flagella, rod-shaped, acid produced from glucose, fructose, galactose and sucrose. The phenotypic properties listed in Table 1 (Paulin, 2000), which are universally present or absent in *E. amylovora*, should be determined according to the methods of Jones & Geider (2001). The tests in Table 2, based mainly on results in API 50 CH strips, allow differentiation of *E. amylovora* from *E. pyrifoliae*, causal agent of Asian pear blight on *Pyrus pyrifolia* (Kim *et al.*, 1999, 2001) and a new *Erwinia* species, *E. piriflorinigrans*, isolated from necrotic pear blossoms in Spain (López *et al.*, 2011). However, certain physiological and biochemical characteristics can vary for some strains. For API 50 CH, a suspension of OD = 1.0 should be prepared in PBS (Appendix 2), and 1 mL added to 20 mL Ayers' medium (Appendix 2). The manufacturer's instructions should be followed for inoculation of the strip. After incubation at 25–26°C in aerobiosis, the strip should be read after 24 and 48 h. Utilization of the different carbohydrates is indicated by a yellow colour in the wells.

Table 1 Biochemical tests for identification

Test	Result
Gram staining	−
Levan production*	+
Fluorescent pigment production in King's B (under UV)	−
Oxidation/fermentation (O/F) test	O+/*F*+
Kovac's oxidase test	−
Reduction of nitrate	−
Utilization of citrate	+
Growth at 39°C	−
Gelatine liquefaction	+
Urease	−
Indole	−
Reducing substances from sucrose	+
Acetoin	+

*Spontaneous mutants found in nature can be levan-negative.

Table 2 Differences between *Erwinia amylovora*, *Erwinia pyrifoliae* and *Erwinia piriflorinigrans*

Microbiological tests	*Erwinia amylovora*	*Erwinia pyrifoliae*	*Erwinia piriflorinigrans*
Gelatine hydrolysis	+	−	−
Inositol*	−	ND	+
Sorbitol*	+	+	−
Esculin*	V	−	+
Melibiose*	−	−	+
D-Raffinose*	−	−	+
β-Gentibiose*	+	−	+

ND, not determined; V, variable.
*Oxidation of substrates in API 50 CH (BioMérieux) with a modified protocol from Roselló *et al.* (2003). More than 90% of strains give the results indicated here.

Table 3 Typical results of *Erwinia amylovora* in API 20E tests after 48 h

Test*	Reaction (48 h)†
ONPG	Variable
ADH	− (or weak +)
LDC	−
ODC	−
CIT	−
SH2	−
URE	−
TDA	−
IND	−
VP	+ (or variable)
GEL	Variable
GLU	+
MAN	Variable
INO	Variable
SOR	Variable
RHA	−
SAC	+
MEL	− (or weak +)
AMY	−
ARA	Variable

*Abbreviations used in API 20 E strips.
†More than 90% of the strains give the results indicated here.

Biochemical characterization by API system (BioMérieux, France)

Biochemical identification of *E. amylovora* can be obtained by specific profile in API 20 E and API 50 CH strips. For API 20 E, the manufacturer's instructions should be fol-

lowed for preparing the suspension and inoculating the strip. After incubation at 25–26°C, the strips should be read after 24 and 48 h (Table 3).

Automated Biolog identification system
The new version (third generation) Biolog GENIII 96 microplate allows rapid identification of isolated bacteria, both Gram-negative and Gram-positive, using the same microplate. The identification system is based on 94 phenotypic tests: 71 carbon-source utilization tests and 23 tests for biochemical and physiological properties including pH, salt, lactic acid tolerance and antibiotics. Every species tested creates a unique 'phenotypic fingerprint' which is automatically compared with 1200 aerobic species in the database.

The microplate and the program are commercially available (Biolog, Omnilog, US). The manufacturer's instructions should be followed for automatic identification of suspected strains of *E. amylovora*.

Fatty acid profiling

Erwinia amylovora-like colonies should be grown on trypticase soy agar for 48 h at 28°C, and an appropriate fatty acid profiling (FAP) procedure applied. A positive FAP test is achieved if the profile of the presumptive culture is identical to that of the positive control (Sasser, 1990). Commercial software from the MIDI system (Newark, DE, USA) allows rapid identification of *E. amylovora*-like colonies. The manufacturer's instructions should be followed for automatic identification. Fatty acid composition can be affected by growth medium, physiological age of cells and chromatograph sensitivity, but in general *E. amylovora* strains have a similarity index between 0.6 and 0.9 in this system.

Serological tests

Performing only two serological tests is not adequate for identification; at least two tests based on different biological principles are needed. Different sources of antibodies should be used for detection (or diagnosis) and identification to reduce the risk of false positives.

Agglutination test
Suspected *E. amylovora* colonies can be tested for agglutination by mixing them in a drop of PBS (Appendix 2) with a drop of *E. amylovora*-specific antiserum (not diluted, or five- or tenfold dilution) on a slide. Monoclonal antibodies can be used only if they agglutinate with the reference strains.

Immunofluorescence test
The IF test is described in PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria*. For identification, IF can be performed using specific monoclonal antibodies from Plant Print Diagnostics S.L. or antiserum from Loewe, Biochemica GmbH.

ELISA tests
ELISA tests are described in PM 7/101 *ELISA tests for plant pathogenic bacteria* (EPPO, 2010).

DASI-ELISA for isolate identification can be performed using the same specific monoclonal antibodies as used for analysis of plant samples (kit from Plant Print Diagnostics S.L.). For DASI-ELISA, a suspension of approximately 10^8 cells mL^{-1} from suspected colonies is prepared in PBS (Appendix 2). The DASI-ELISA procedure (Appendix 3) can be followed without prior enrichment for isolate identification.

Lateral flow immunoassays
A suspension of approximately 10^8 cells mL^{-1} prepared in PBS (Appendix 2) from suspected colonies should be used following the manufacturers' instructions. The two kits evaluated in a test performance study (Agri-strip and Pocket Diagnostic) and recommended for analyses of symptomatic plants can be used for identification of isolates.

Molecular tests

Conventional and/or real-time PCR and LAMP are the recommended molecular tests for rapid identification, but other available techniques are also indicated.

Conventional PCR
A suspension of approximately 10^6 cells mL^{-1} in molecular-grade water should be prepared from *E. amylovora*-like colonies. Appropriate PCR procedures should be applied, following Appendices 7–11, without DNA extraction, just after treatment at 100°C for 10 min.

Real-time PCR
The first protocol for detection of *E. amylovora* by real-time PCR was described by Salm & Geider (2004) and used primers based on sequences of the pEA29 plasmid. However, the sensitivity and specificity of this test were similar to those of conventional PCR tests. Since then, two real-time PCR tests have been published, described in Appendices 12 and 13. Colonies can be prepared as for conventional PCR.

*Macrorestriction with Xba*I and pulse field gel electrophoresis
Pulse field gel electrophoresis (PFGE) analysis of genomic DNA after *Xba*I digestion according to Jock *et al.* (2002) shows six patterns for *E. amylovora* European strains. This method can provide information useful for strain differentiation and has been used to analyse the spread of fire blight in Europe.

DNA sequencing methods
Comparisons of commercially sequenced PCR products amplified from selected housekeeping genes allow differentiation of *E. amylovora* isolates from other members of the Enterobacteriaceae (see EPPO Standard PM 7/XXX on *DNA barcoding as an identification tool for plant pests*,

in preparation). For example, all isolates of *E. amylovora* tested so far are clonally related according to partial *recA* gene sequence using the method described by Parkinson *et al.* (2009).

Hypersensitivity and pathogenicity tests

When necessary, suspected *E. amylovora* colonies from the isolation and/or enrichment plates may be inoculated to test plants to confirm their pathogenicity.

The hypersensitive reaction in tobacco leaves can give an indication of the presence of the *hrp* pathogenicity genes, but is also positive for many other plant pathogenic bacteria. Tobacco plants of cv. Xanthi or Samsun with more than 5–6 leaves are used. Bacterial suspensions of 10^8–10^9 cfu mL^{-1} (OD at 620 nm = 1.0) are injected into the intercellular space of adult leaves with a 25 GA 5/8 0.5 × 16 needle and syringe. Complete collapse of the infiltrated tissue after 24 h at room temperature is recorded as positive.

To verify the pathogenicity of suspected *E. amylovora* colonies, a fire blight host should be inoculated (Appendix 15).

Reference material

The following *E. amylovora* isolates are recommended for use as positive controls: NCPPB683 (type strain) and CFBP 1430. The following collections can provide different *E. amylovora* reference strains: (i) National Collection of Plant Pathogenic Bacteria (NCPPB), Fera, Sand Hutton, York (GB); (ii) Culture Collection of the Plant Protection Service (PD), Wageningen (NL); (iii) Collection Française de Bactéries Phytopathogènes (CFBP), EmerSys – IRHS – INRA Beaucouzé (FR). Authenticity of the strains can be guaranteed only if obtained directly from the culture collections.

Reporting and documentation

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

Performance criteria

If available, performance criteria are provided with the test description. 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 (on analytical specificity, full validation reports, etc.).

Further information

Further information on this organism can be obtained from:

M M López, Bacteriología, Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera Moncada-Náquera km 5, 46113 Moncada, Valencia, Spain; e-mail: mlopez@ivia.es.

T Dreo, National Institute of Biology, Vecna pot 111, SL-1000, Ljubljana, Slovenia; e-mail: tanja.dreo@nib.si.

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.

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

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References

Ayers SH, Rupp P & Johnson WT (1919) *A study of alkali forming in milk*. USDA Bulletin no. 782. USDA, Washington (US).

Bereswill S, Jock S, Aldridge P, Janse JD & Geider K (1997) Molecular characterization of natural *Erwinia amylovora* strains deficient in levan synthesis. *Physiological and Molecular Plant Pathology* **51**, 215–225.

Bereswill S, Pahl A, Bellemann P, Zeller W & Geider K (1992) Sensitive and species-specific detection of *Erwinia amylovora* by polymerase chain reaction analysis. *Applied and Environmental Microbiology* **58**, 3522–3526.

Bonn WG & van der Zwet T (2000) Distribution and economic importance of fire blight. In: *Fire Blight, The Disease and its Causative Agent Erwinia amylovora* (Ed. Vanneste J), pp. 37–53. CAB International, Wallingford (GB).

Bradbury JF (1986) *Guide to Plant Pathogenic Bacteria*. CAB International, Wallingford (GB).

Braun-Kiewnick A, Altenbach A, Oberhansli T, Bitterlin W & Duffy B (2011) A rapid lateral-flow immunoassay for phytosanitary detection of *Erwinia amylovora* and on-site fire blight diagnosis. *Journal of Microbiological Methods* **987**, 1–9.

Burrill TJ (1883) New species of *Micrococcus*. *American Naturalist* **17**, 319.

CABI/EPPO (2007) *Erwinia amylovora*. *Distribution Maps of Plant Diseases No. 2*. CABI/EPPO, Wallingford (GB).

Dreo T, Duffy B, López M, Paulin JP, Poliakoff F & Reisenzein H (2009) Development and validation of innovative diagnostic tools for the detection of fire blight (*Erwinia amylovora*). EUPHRESCO report.

Dye DW (1983) *Erwinia*: the '*amylovora*' and '*herbicola'* groups. In: *Plant Bacterial Diseases, A Diagnosis Guide* (Ed. Fahy PC & Persley GJ), pp. 67–86. Academic Press, Sydney (AU).

EPPO (2009) PM 7/097 (1) indirect immunofluorescence test for plant pathogenic bacteria. *Bulletin OEPP/EPPO Bulletin* **39**, 413–416.

EPPO (2010) PM 7/101 (1) ELISA tests for plant pathogenic bacteria. *Bulletin OEPP/EPPO Bulletin* **40**, 369–372.

EPPO (2012) EPPO plant quarantine data retrieval system. http://www.eppo.org/DATABASES/pqr/pqr.htm (accessed on 20 January 2012).

Gorris MT, Cambra E, Paulin JP, Chartier R, Cambra M & López MM (1996a) Production and characterization of monoclonal antibodies specific for *Erwinia amylovora* and their use in different serological techniques. *Acta Horticulturae* **411**, 47–51.

Gorris MT, Cambra M, Llop P, Lecomte P, Chartier R, Paulin JP & López MM (1996b) A sensitive and specific detection of *Erwinia amylovora* based on the ELISA-DASI enrichment method with monoclonal antibodies. *Acta Horticulturae* **411**, 41–45.

Gottsberger RA (2010) Development and evaluation of a real-time PCR assay targeting chromosomal DNA of *Erwinia amylovora*. *Letters in Applied Microbiology* **51**, 285–292.

Guilford PJ, Taylor RK, Clark RG, Hale CN, Forster RLS & Bonn WG (1996) PCR-based techniques for the detection of *Erwinia amylovora*. *Acta Horticulturae* **411**, 53–56.

Ishimaru ES & Klos EJ (1984) New medium for detection of *Erwinia amylovora* and its use in epidemiological studies. *Phytopathology* **74**, 1342–1345.

Jock S, Donat V, López MM, Bazzi C & Geider K (2002) Following spread of fire blight in Western, Central and Southern Europe by molecular differentiation of *Erwinia amylovora* strains with PFGE analysis. *Environmental Microbiology* **4**, 106–114.

Jones A & Geider K (2001) II Gram negative bacteria. B. *Erwinia* and *Pantoea*. In: *Guide for Identification of Plant Pathogenic Bacteria*, 2nd edn (Ed. Schaad NW, Jones JB & Chum W), pp. 40–55. APS Press, St Paul (US).

Kim WS, Gardan L, Rhim SL & Geider K (1999) *Erwinia pyrifoliae* sp., a novel pathogen that affects Asian pear trees (*Pyrus pyrifolia*). *International Journal of Systematic Bacteriology* **49**, 899–906.

Kim WS, Jock S, Rhim SL & Geider K (2001) Molecular detection and differentiation of *Erwinia pyrifoliae* and host range analysis of the Asian pear pathogen. *Plant Disease* **85**, 1183–1188.

King EO, Ward M & Raney DE (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine* **44**, 301–307.

Lecomte P, Manceau C, Paulin JP & Keck M (1997) Identification by PCR analysis on plasmid pEA29 of isolates of *Erwinia amylovora* responsible of an outbreak in Central Europe. *European Journal of Plant Pathology* **103**, 91–98.

López MM, Llop P, Gorris MT, Keck M, Peñalver J, Donat V & Cambra M (2006) European protocol for diagnosis of *Erwinia amylovora*. *Acta Horticulturae* **704**, 99–103.

López MM, Roselló MM, Llop P, Ferrer S, Christen R & Gardan L (2011) *Erwinia piriflorinigrans* sp. nov. a novel pathogen that causes necrosis of pear blossoms. *International Journal of Systematic and Evolutionary Microbiology* **61**, 561–567.

Llop P, Bonaterra A, Peñalver J & López MM (2000) Development of a highly sensitive nested-PCR procedure using a single closed tube for detection of *Erwinia amylovora* in asymptomatic plant material. *Applied and Environmental Microbiology* **66**, 2071–2078.

Llop P, Caruso P, Cubero J, Morente C & López MM (1999) A simple extraction procedure for efficient routine detection of pathogenic bacteria in plant material by polymerase chain reaction. *Journal of Microbiological Methods* **37**, 23–31.

Llop P, Cabrefiga J, Smits THM, Dreo T, Barbé S, *et al*. (2011) *Erwinia amylovora* Novel Plasmid pEI70: Complete Sequence, Biogeography, and Role in Aggressiveness in the Fire Blight Phytopathogen. PLoS ONE 6(12): e28651. doi:10.1371/journal.pone.0028651.

Llop P, Donat V, Rodríguez M, Cabrefiga J, Ruz L, Palomo JL, Montesinos E & López MM (2006) An indigenous virulent strain of *Erwinia amylovora* lacking the ubiquitous plasmid pEA29. *Phytopathology* **96**, 900–907.

Maes M, Garbeva P & Crepel C (1996) Identification and sensitive endophytic detection of the fire blight pathogen *Erwinia amylovora* with 23S ribosomal DNA sequences and the polymerase chain reaction. *Plant Pathology* **45**, 1139–1149.

Matsuura T, Mizuno A, Tsukamoto T, Shimizu Y, Saito N, Sato S, Kikuchi S, Uzuki T, Azegami K & Sawada H (2011) *Erwinia uzenensis* sp. nov., a novel pathogen that affects European pear trees (*Pyrus communis* L.). *International Journal of Systematic and Evolutionary Microbiology* **62**, 1799–1803. doi: 10.1099/ijs.1090.032011-032010.

McManus PS & Jones AL (1995) Detection of *Erwinia amylovora* by nested PCR and PCR-dot-blot and reverse blot hybridisations. *Phytopathology* **85**, 618–623.

Obradovic D, Balaz J & Kevresan S (2007) Detection of *Erwinia amylovora* by novel chromosomal polymerase chain reaction primers. *Mikrobiologiia* **76**, 844–852.

Parkinson N, Stead D, Bew J, Heeney J, Tsror L & Elphinstone JG (2009) *Dickeya* species relatedness and clade structure determined by comparison of recA sequences. *International Journal of Systematic and Evolutionary Microbiology* **59**, 2388–2393.

Paulin JP (2000) *Erwinia amylovora*: general characteristics, biochemistry and serology. In: *Fire Blight, The Disease and its Causative Agent, Erwinia amylovora* (Ed. Vanneste J), pp. 87–116. CAB International, Wallingford (GB).

Persen U, Gottsberger RA & Reisenzein H (2011) Spread of *Erwinia amylovora* in apple and pear trees of different cultivars after artificial inoculation. *Acta Horticulturae* **896**, 319–330.

Pirc M, Ravnikar M, Tomlinson J & Dreo T (2009) Improved fire blight diagnostics using quantitative real-time PCR detection of *Erwinia amylovora* chromosomal DNA. *Plant Pathology* **58**, 872–881.

Powney R, Plummer K, Luck JE, Beer S & Rodoni B (2007) Evaluation of PCR-based Protocols for the Detection of Erwinia amylovora. *XI International Workshop on Fire Blight Conference Proceedings published in Acta Horticulturae*.

Salm H & Geider K (2004) Real-time PCR for detection and quantification of *Erwinia amylovora*, the causal agent of fire blight. *Plant Pathology* **53**, 602–610.

Sasser M (1990) Identification of bacteria through fatty acid analysis. In: *Methods in Phytobacteriology* (Ed. Klement F, Rudolf K & Sands DC), pp. 199–204. Akademiai Kiadó, Budapest (HU).

Starr MP, Cardona C & Folsom D (1951) Bacterial fire blight of raspberry. *Phytopathology* **41**, 951–959.

Stöger A, Schaffer J & Ruppitsch W (2006) A rapid and sensitive method for direct detection of *Erwinia amylovora* in symptomatic and asymptomatic plant tissues by polymerase chain reaction. *Journal of Phytopathology* **154**, 469–473.

Tanii A, Tamura O & Ozaki M (1981) The causal agent of a fire blight-like disease. *Annals of Phytopathological Society of Japan* **47**, 102.

Taylor RK, Guilford P, Clark RG, Hal CN & Forster RLS (2001) Detection of *Erwinia amylovora* in plant material using novel

polymerase chain reaction (PCR) primers. *New Zealand Journal of Crop and Horticultural Science* **29**, 35–43.

Temple TN & Johnson KB (2011) Evaluation of loop-mediated isothermal amplification for rapid detection of *Erwinia amylovora* on pear and apple fruit flowers. *Plant Disease* **95**, 423–430.

Temple TN, Stockwell VO & Johnson K (2008) Development of a rapid detection method for *Erwinia amylovora* by loop-mediated isothermal amplification (LAMP). *Acta Horticulturae* **793**, 497–504.

Thomson SV (2000) Epidemiology of fire blight. In: *Fire Blight, The Disease and Its Causative Agent, Erwinia amylovora* (Ed. Vanneste J), pp. 9–36. CAB International, Wallingford (GB).

van der Zwet T (2002) Present worldwide distribution of fire blight. *Acta Horticulturae* **590**, 33–34.

van der Zwet T & Beer S (1995) Fire blight – its nature, prevention and control. *A Practical Guide to Integrated Disease Management*. USDA Agricultural Information Bulletin no. 631. USDA, Washington (US).

van der Zwet T & Keil HL (1979) *Fire Blight: A Bacterial Disease of Rosaceous Plants*. USDA Handbook no. 510. USDA, Washington (US).

Appendix 1 – Extraction procedures

Samples from symptomatic material

The samples may be processed in different buffers according to the tests to be performed. The use of freshly prepared antioxidant maceration buffer (Appendix 2) is required for successful enrichment of *E. amylovora* in plant material (Gorris *et al.*, 1996b). This buffer has been evaluated in a test performance study. Sterile phosphate-buffered saline, pH 7.2 10 mM (PBS) (Appendix 2) or sterile water can also be used for direct isolation, immunofluorescence or PCR.

Carefully select the plant parts showing the freshest symptoms, with exudates if possible. The leading edge of lesions on each organ should be selected for analysis. The exudates can be processed separately, in 1–4.5 mL sterile water or buffer. For shoots, take pieces of symptomatic shoots, including leaves, at the margin between the necrotic and healthy tissue. Take one or several flowers, with peduncles. Take one or several leaves and petioles, preferably select leaves with vein necrosis, but not fully necrosed. Take one or several fruits. For stems or trunk, peel off the external bark of stems showing symptoms using a sterile scalpel and take pieces underneath with typical subcortical discoloration symptoms.

The protocol evaluated in a test performance study was as follows: cut 0.1 g shoots, flowers, leaves, stems, trunks or fruits into pieces and place in plastic bags. Add to each bag 4.5 mL of the antioxidant maceration buffer described by Gorris *et al.* (1996a) (Appendix 2). Allow the samples to macerate for at least 5 min. Crush the plant material slightly in the plastic bag with a rubber hammer, or with a Bioreba homogenizer or similar equipment, avoiding droplets splashing out of the bag. Hold the samples on ice for a few minutes and decant approximately 2, 1 and 1 mL of each macerate into three sterile Eppendorf tubes. Use the tube containing 2 mL for the analysis. Store one tube with 1 mL of each sample at −20°C for subsequent analysis or confirmation; add 30% glycerol (Difco) to the other tube and store it at −80°C.

The isolation should be done on the same day as the maceration of the samples, as well as the enrichment and the fixation of the slides for immunofluorescence. PCR analysis can be performed at earliest convenience, using the 1 mL stored at −20°C.

Samples from asymptomatic material

After favourable conditions for multiplication of the causal agent of fire blight have been confirmed, or at least when the average temperature is higher than 18°C (van der Zwet & Beer, 1995), collect flowers, shoots, fruitlets or stem segments in sterile bags or containers. For nursery plants: cut young shoots approximately 20 cm long from the most susceptible hosts available, disinfecting scissors or pruning shears between plants. For plants growing in the field, cut flowers when available, and/or young shoots about 20 cm long, disinfecting between plants. Take flowers or peduncle and the base of the limb of mature leaves or stem segments of selected plants. If analyses need to be performed in winter, collect 5–10 buds per plant.

Direct analysis of asymptomatic samples is usually negative for *E. amylovora* due to the low bacterial population. Consequently it is recommended to enrich the samples (Appendix 4) in antioxidant buffer (Gorris *et al.*, 1996a) (Appendix 2). When analysing asymptomatic material, enrichment should be done for 72 h at approximately 25°C.

Weigh 0.1–1 g plant material and use for maceration in antioxidant buffer (Appendix 2) (not in PBS or water), in the same amount as for symptomatic material (above). It is not advised to analyse larger amounts of plant material in one sample. Process the samples immediately by enrichment, followed by DASI-ELISA and/or PCR and/or isolation, according to the protocols described in the Appendices 3–13. At the same time, direct isolation can also be performed using the extract, or later with the sample kept at −20°C with glycerol for a short time.

A sampling procedure for the analysis of twigs of asymptomatic woody material in nurseries is presented. A sample consists of 100 twigs, approximately 10 cm long, from 100 plants. If there are several plant genera in the lot, these should be represented equally in the sample (with a maximum of three genera per sample). From each sample, individual twigs are tested or 30 cut twigs are randomly taken and cut into four pieces (120 stem pieces). Place these for 1.5 h in a rotary shaker at room temperature in sterile PBS (Appendix 2) with 0.1% Tween 20 in Erlenmeyer flasks. Filter with a paper held in a sintered glass filter ($n2$ = 40–100 μm) using a vacuum pump and collect the filtrate. Use the filtrate directly for analysis, or

centrifuge it for 20 min at 10 000 *g*. Suspend the pellet in 4.5 mL sterile PBS (Appendix 2). Depending on the season, a similar procedure may be applied for leaves, shoots, flowers or buds.

Depending on the season of survey, the expected recovery of *E. amylovora* will vary, being high in summer (provided weather conditions are favourable to the pathogen) and low in winter.

Whichever procedure is followed, prepare 3 Eppendorf tubes for each sample with about 2, 1 and 1 mL macerate, and use them as for symptomatic material (see above).

Appendix 2 – Preparation of media and buffers

Buffers

Phosphate buffered saline 10 mM, pH 7.2 (PBS)

NaCl	8.0 g
KCl	0.2 g
	2.9 g
$Na_2HPO_4 \cdot 12H_2O$	
KH_2PO_4	0.2 g
Distilled water to	1 L

Sterilize by filtration.

Antioxidant maceration buffer (Gorris *et al.*, 1996a)

Polyvinylpyrrolidone (PVP-10)	20.0 g
Mannitol	10.0 g
Ascorbic acid	1.76 g
Reduced glutathion	3.0 g
PBS 10 mm pH 7.2	1 L

Adjust pH to 7. Sterilize by filtration. This buffer should be prepared immediately before use.

Extraction buffer (Llop *et al.*, 1999)

Tris HCl	31.52 g
NaCl	14.6 g
EDTA	9.3 g
SDS	5.0 g
Polyvinylpyrrolidone (PVP-10)	20.0 g
Distilled water to	1 L

Adjust pH to 7.5. Sterilize by filtration.

Media

Media are sterilized by autoclaving at 120°C for 15 min unless stated otherwise.

Ayers' medium (Ayers *et al.*, 1919)

$NH_4H_2PO_4$	1.0 g
KCl	0.2 g
$MgSO_4$	0.2 g
Bromothymol blue (solution 0.2%)	75 mL
Distilled water to	1 L

Adjust pH to 7.

CCT medium (Ishimaru & Klos, 1984)

Sucrose	100 g
Sorbitol	10.0 g
Niaproof	1.2 mL
Crystal violet (sol. 0.1% ethanol)	2 mL
Nutrient agar	23.0 g
Distilled water to	1 L

Adjust pH to 7.0–7.2; sterilize by autoclaving at 115°C for 10 min. Then prepare: thallium nitrate 2 mL (1% w/v aqueous solution); 0.05 g cycloheximide. Sterilize by filtration (0.45 μm). Add to 1 L sterile medium (at about 45°C).

Enrichment media: use CCT medium and King's B medium prepared in liquid form, without agar, for enrichment as described in Appendix 4. Tubes of at least 5 mL should be used and 0.9 mL medium added.

King's B medium (King *et al.*, 1954)

Proteose peptone No. 3	20 g
Glycerol	10 mL
K_2HPO_4	1.5 g
	1.5 g
$MgSO_4 \cdot 7H_2O$	
Agar	15 g
Distilled water to	1 L

Adjust pH to 7.0–7.2.

Levan medium

Yeast extract	2 g
Bactopeptone	5 g
NaCl	5 g
Sucrose	50 g
Agar	20 g
Distilled water to	1 L

Adjust pH to 7–7.2.

Appendix 3 – Rapid serological screening tests

1. Immunofluorescence

Follow the standard instructions described in PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria*.

Antibodies to *E. amylovora* currently used in detection and identification tests:

- *E. amylovora*, polyclonal antibodies, for detection using IF test (validated in test performance studies), Loewe Biochemica GmbH.
- IVIA EPS 1430, polyclonal antibodies, for detection using IF test (validated in test performance studies), Plant Print Diagnostics, S.L.
- IVIA Mab 7 A, monoclonal antibodies, for detection using IF test (validated in test performance studies), Plant Print Diagnostics, S.L.

Use undiluted macerates and 1:10 and 1:100 dilutions in PBS (Appendix 2) to spot windows of IF slides. Prepare one slide for each sample and its dilutions. Use the monoclonal or polyclonal antibodies at the appropriate dilutions in PBS (Appendix 2). Determination of the contamination level is usually not required. Immunofluorescence is not recommended after enrichment of samples.

Performance criteria available

1.1 Analytical sensitivity data
10^3–10^4 cfu mL^{-1} plant extract

1.2 Analytical specificity data
Not tested for polyclonal antibodies.
For monoclonal antibody 7A
Target organisms tested: 50 *E. amylovora* strains. All positive in the test conditions.
Non-target organisms tested: 123 unidentified strains from *E. amylovora* hosts, 121 negative and two *Erwinia*-related bacteria positive (*Erwinia persicina* and *Dickeya* sp.).

1.3 Data on repeatability
In IVIA: 100%

1.4 Data on reproducibility
In IVIA: 60%

2. Enrichment DASI-ELISA

After the enrichment step, the use of validated specific monoclonal antibodies is recommended to avoid cross-reactions. A complete kit based on polyclonal and monoclonal antibodies (3B + 5H IVIA), including extraction buffer, semi-selective media, ELISA plates and reagents, is available from Plant Print Diagnostics S.L. This commercial kit for Enrichment DASI-ELISA (Gorris *et al.*, 1996b) has been validated in two test performance studies. It is based on the monoclonal antibodies and technique described in Gorris *et al.* (1996a,b). As positive controls, use aliquots of a sample extract that previously gave a negative result on testing, mixed with 10^8 cells of *E. amylovora* per mL. As negative controls, include a sample extract that has previously given a negative result for *E. amylovora* and a suspension of a non-*E. amylovora* strain in PBS (Appendix 2).

Before ELISA, treat the necessary amount of enriched extracts and controls in a water bath (or in a thermoblock) at 100°C for 10 min, ensuring the tubes are not opened. Keep the remaining enriched samples for isolation and/or PCR. Process the boiled samples (once at room temperature) by ELISA on the same day or store them at −20°C for subsequent analysis. This heat treatment is necessary for optimum sensitivity and specificity using the monoclonal antibodies obtained by Gorris *et al.* (1996a). Then follow the instructions for DASI-ELISA given in PM 7/101 (1) *ELISA tests for plant pathogenic bacteria* (EPPO, 2010) and those of the manufacturers of the commercial kit.

Positive ELISA readings in negative control wells indicate cross-contaminations or non-specific antibody binding. In either case, the test should be repeated or a second test based on a different biological principle should be performed.

Performance criteria available

2.1 Analytical sensitivity data
10 cfu mL^{-1} plant extract in King's B and in CCT (Gorris *et al.*, 1996b).
10–10^2 cfu mL^{-1} plant extract in King's B and 10^3–10^4 cfu mL^{-1} plant extract in CCT (in the performance study in 2010).

2.2 Analytical specificity data
For monoclonal antibodies 3B + 5H
Target organisms tested: 250 *E. amylovora* strains. All positive in the test conditions (Gorris *et al.*, 1996a,b; and IVIA tests).
Non-target organisms tested: 258 unidentified strains from *E. amylovora* hosts and 45 strains of other plant pathogenic bacteria. They were all negative (Gorris *et al.*, 1996a,b).

2.3 Data on repeatability
In IVIA: 100%

2.4 Data on reproducibility
In IVIA: 98%

3. Lateral flow devices

Two lateral flow devices were evaluated in performance studies in 2009 and 2010 and showed similar results. They were appropriate for the analysis of symptomatic plants only, and are based on *E. amylovora* polyclonal antibodies that are non-specific. Follow the manufacturer's instructions when performing the analysis.

Performance criteria available for Ea Agri-strip (Bioreba)

3.1 Analytical sensitivity data (in a test performance study performed in 2010)
10^5–10^6 cfu mL^{-1} plant extract

3.2 Analytical specificity data
Target organisms tested: 39 strains all positive
Non-target organisms tested: 61 strains (all negative except *E. pirifoliae*, *E. tasmaniensis* and *E. piriflorinigrans*). False positive results with *E. pirifoliae*, *E. tasmaniensis* and *E. piriflorinigrans* are also reported in AGES (AT) and Braun-Kiewnick *et al.* (2011).

3.3 Data on repeatability
In IVIA: 94%

3.4 Data on reproducibility
In IVIA: 96%

Performance criteria available for Pocket Diagnostics (Forsite Diagnostics, York, UK)

3.5 Analytical sensitivity data (in the performance study performed in 2010)
10^5–10^6 cfu mL^{-1} plant extract

3.6 Analytical specificity data
Non-target organisms tested: false positive results with *E. pirifoliae*, *E. tasmaniensis* and *E. piriflorinigrans* are reported in AGES (AT).

3.7 Data on repeatability
In IVIA: 94%

3.8 Data on reproducibility
In IVIA: 96%

Appendix 4 – Enrichment

Enrichment is used to multiply the initial population of culturable *E. amylovora* in the sample. It is needed before detection by ELISA because of the low level of sensitivity of this technique when using specific monoclonal antibodies. It should also be used before isolation or before PCR (even in symptomatic samples) when a low number of culturable *E. amylovora* is expected (copper-treated samples, old symptoms, unfavourable weather conditions for fire blight, winter, etc.) or when a high level of inhibitory organisms are expected. After preparation of the samples in the freshly prepared antioxidant buffer, use of two validated media is advised [one non-selective (King's B) and one semi-selective (CCT) (Appendix 2)] because the composition and number of microbiota is unknown.

As soon as the macerates have been made (Appendix 1), dispense at least 0.9 mL of each sample into two sterile 5 mL tubes prepared in advance with the same volume of each enrichment medium. Do not use Eppendorf tubes, for maximum aeration. As additional negative controls, prepare three tubes with 0.9 mL maceration buffer (Appendix 2) and add the same volume of the same buffer and of each enrichment medium (Appendix 2). Incubate at 25°C for 48 h without shaking. Incubate for 72 h when very low numbers of *E. amylovora* are expected, as indicated above for asymptomatic samples.

Appendix 5 – Isolation

1. Direct isolation

Use CCT, King's B and levan (or nutrient agar sucrose, NAS or NSA) media (Appendix 2). Plating on three media is advised for maximum recovery of *E. amylovora* particularly when samples are in poor condition. Prepare 1:10 and 1:100 dilutions of each macerate (Appendix 1) in PBS (Appendix 2). Pipette 50 µL of the diluted and undiluted macerates onto separate plates of each medium. Start with the 1:100 dilution and proceed to the undiluted macerate. Use sterile loops or dip a glass spreader in denatured ethanol, flame and allow to cool. Carefully spread the pipetted volumes by triple streaking. Plate a 10^3, 10^4 and 10^5 cfu mL^{-1} dilution of a pure culture of *E. amylovora* as a quality control of the media. Incubate the plates at approximately 25°C for 48–72 h. Final reading is at 72–96 h.

Colonies of *E. amylovora* on CCT appear at about 48 h and are pale violet, circular, highly convex to domed, smooth and mucoid after 72 h, showing slower growth than on King's B or levan. CCT medium inhibits most pseudomonads but not *Pantoea agglomerans*. Colonies of *E. amylovora* on King's B appear at 24 h and are creamy white, circular, tending to spread and non-fluorescent under UV light at 366 nm after 48 h. This allows distinction from fluorescent pseudomonads. Colonies of *E. amylovora* on levan medium appear at 24 h and are whitish, circular, domed, smooth and mucoid after 48 h. Levan-negative colonies of *E. amylovora* have also been reported (Bereswill *et al.*, 1997). Figure 3 shows the appearance of cultures in the three media.

Obtain pure cultures from individual suspect colonies of each sample by plating on King's B medium. Identify presumptive colonies of *E. amylovora* as indicated in the Identification section. Store cultures on nutrient agar slants covered with vaseline oil at 10°C or for long-term storage in 30% glycerol at −80°C or lyophilized.

The isolation is negative if no bacterial colonies with morphology similar to *E. amylovora* are observed after 96 h in any of the three media (provided no inhibition is suspected due to competition or antagonism) and that typical *E. amylovora* colonies are found in the positive controls. The isolation is positive if presumptive *E. amylovora* colonies are isolated in at least one of the media used and the identification is confirmed by one of the methods indicated.

Performance criteria available

1.1 Analytical sensitivity data (in a performance study in 2010):
10^3 cfu mL^{-1} in King's B; 10–10^2 cfu mL^{-1} in levan and CCT

1.2 Analytical specificity data
Not evaluated

1.3 Data on repeatability
In IVIA: 100%

1.4 Data on reproducibility
In IVIA: 100%

2. Enrichment isolation

Plate the enrichments only on CCT plates (Appendix 2). Spread 50 µL of each enriched extract and of the 1:10, 1:100 and 1:1000 dilutions prepared in PBS (Appendix 2) by triple streaking (as for isolations) to obtain isolated colonies. Incubate at approximately 25°C for 72–96 h. The use of only this semi-selective medium and dilutions is advised because of the possible abundant multiplication of different bacteria during the enrichment step.

Performance criteria available

2.1 Analytical sensitivity data (in a performance study in 2010)
10 cfu mL^{-1} after enrichment in CCT
10–10^2 cfu mL^{-1} after enrichment in King's B

2.2 Analytical specificity data
Not evaluated

2.3 Data on repeatability
In IVIA: 100%

2.4 Data on reproducibility
In IVIA: 100%

Appendix 6 – DNA extraction and conventional PCR

DNA extraction

Two protocols for DNA extraction from plant samples (Llop *et al.*, 1999; Taylor *et al.* 2001) and one commercial kit have been validated in the test performance studies. Other commercial kits for extracting DNA are available, but they have not been evaluated.

1. DNA extraction according to Llop *et al.* (1999)

Use 1 mL of each macerate and/or 1 mL of the enriched macerates prepared according to Appendices 1 and 4. Centrifuge the macerates at 10 000 *g* for 5 min at room temperature. Discard the supernatant, resuspend the pellet in 500 µL extraction buffer (Llop *et al.*, 1999 Appendix 2) and shake for 1 h at room temperature. Centrifuge at 4000 *g* for 5 min. Take 450 µL of the supernatant and add the same volume of isopropanol, invert and leave for 30 min–1 h at room temperature. Centrifuge at 10 000 *g* for 5 min, discard the supernatant and dry. If there is still a coloured precipitate (brown or green) at the bottom of the tubes, carefully take it while discarding the supernatant to obtain a cleaner DNA. Resuspend the pellet in 200 µL of water. Use for PCR reaction or store at −20°C.

2. DNA extraction based on the procedure described by Taylor *et al.* (2001) but with minor modifications (elimination of Gene Releaser which was considered unnecessary).

Add 200 µL of each macerate and/or of the enriched macerates in 500 µL of buffer [140 mM NaCl; 50 mM KCl; 0.05% Tween 20; 2% polyvinylpyrrolidone (PVP) 10; 0.4% BSA, distilled water 1 L] for 15 min at room temperature. The resulting suspension can be used for PCR reaction or stored at −20°C.

3. DNA extraction using RED-Extract N-Amp T Plant kit (Sigma-Aldrich, USA)

Take 100 µL of each macerate and/or the enriched macerates into an Eppendorf tube. Add 150 µL extraction solution (kit) supplemented with 0.1% (v/v) Triton X-100 and 0.05% (v/v) Nonidet NP-40 Igepal. Incubate at 95°C for 30 min on a heating block. Transfer 50 µL of extract to a new tube and dilute it with 50 µL of the dilution buffer (kit). Use for PCR reaction or store at −20°C.

The three DNA extraction protocols were validated in test performance studies in 2009 and 2010, with four PCR protocols (Appendices 8–11), and showed comparable results. Their efficiency was not improved after diluting the extracts 1:10, suggesting that no, or few, inhibitors were present. The PCR protocols are detailed in the following appendices.

Performance criteria

Performance criteria are provided together with the different PCR tests.

Conventional PCR protocols

Many PCR primers and tests for conventional PCR exist for *E. amylovora* diagnosis, detection and identification. Some can be used reliably (Guilford *et al.*, 1996; Taylor *et al.*, 2001) but others have shown specificity problems. This is the case for the test described by Maes *et al.* (1996), which also amplifies *Erwinia piriflorinigrans* isolated from necrotic pear blossoms (López *et al.*, 2011). Tests described by Bereswill *et al.* (1992), McManus & Jones (1995) and Llop *et al.* (2000) are based in sequences of the plasmid pEA29 that is not universal in *E. amylovora* strains (Llop *et al.*, 2006; Llop *et al.*, 2011).

Two protocols for conventional PCR were validated in a test performance study in 2002 and four in test performance studies conducted in 2009 and 2010. The primers and protocols validated in 2002 were those of Bereswill *et al.* (1992), Llop *et al.* (2000), with or without previous enrichment. The primers and protocols validated in 2009 and 2010 were those of Llop *et al.* (2000), Taylor *et al.* (2001), Stöger *et al.* (2006) and Obradovic *et al.* (2007). Taking into account the discovery of fully virulent *E. amylovora* strains without pEA29 (Llop *et al.*, 2006) and the experi-

ence from different countries (Powney *et al.*, 2007), it is necessary to use two PCR tests, one with primers based on pEA29 sequences and the second based on chromosomal sequences. Conventional PCR can be applied using the primers and conditions validated in test performance studies. Precautions should be taken to avoid contamination of samples. Prepare positive controls in a laboratory separate from the one where the samples will be tested.

Appendix 7 – PCR according to Bereswill *et al.* (1992)

1. General information

1.1 This test was widely used for many years. The sequences of the primers are based in the plasmid pEA29, and it has been discovered that it is not universal for all *E. amylovora* strains (Llop *et al.*, 2006; Llop *et al.*, 2011). In addition, it frequently shows non-specific banding (see below).

1.2 The test can be applied to any kind of plant material or bacterial colonies.

1.3 The targeted sequences are in the plasmid pEA29.

1.4 Oligonucleotides:
A: 5′-CGG TTT TTA ACG CTG GG-3′
B: 5′-GGG CAA ATA CTC GGA TT-3′

1.5 The amplicon size is 900 bp. (Bereswill *et al.*, 1992). However, variations can occur between 900 and 1100 bp (Lecomte *et al.*, 1997), due to the number of 8 bp repeat sequences within the fragment (Jones & Geider, 2001).

1.6 Enzyme: the test performance study was performed with a DNA polymerase from Biotools.

2. Methods

2.1 Nucleic acid extraction and purification:
For plant material, three DNA extraction methods are described in Appendix 6. These DNA extraction methods were evaluated in a test performance study. The sensitivity of the tests increases after enrichment of the samples in King's B and CCT (López *et al.*, 2006).

2.2 Polymerase chain reaction

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		34.80	
PCR buffer	10×	5.00	1×
$MgCl_2$	50 mM	3.00	3 mM
dNTPs	10 mM	1.00	0.2 mM of each dNTP
Primer A	10 µM	0.50	0.1 µM
Primer B	10 µM	0.50	0.1 µM
Taq polymerase	5 U μL^{-1}	0.20	1 U
Subtotal		45.00	
DNA		5.00	
Total reaction volume of a single PCR reaction		50.00	

2.3 PCR cycling conditions: 5 min at 93°C, 40 cycles of 30 s at 93°C, 30 s at 52°C and 1 min 15 s at 72°C and a final step of 10 min at 72°C.

2.4 Observations: if the expected target concentration is high, i.e. in enriched samples, it is highly recommended to carry out a tenfold dilution of the purified DNA solution in water or TE buffer before amplification in order to dilute inhibitor compounds. Amplification is performed on stock solution and the dilution.

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 isolation and amplification of the target organism and target nucleic acid.

- 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 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 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). For PCR tests not performed on bacterial colonies, the PAC should preferably be near 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 may 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 to the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

3.2 Interpretation of results

Verification of controls

- NIC and NAC should produce no amplicons
- PIC and PAC should produce amplicons of 900 bp size.
- If IPCs are used, the amplicons should be of the expected size.

When these conditions are met:

- A test will be considered positive if amplicons of 900 bp are produced
- A test will be considered negative if it produces no band or a band of a different size.
- If the test gives a value >900 and $\leq$1100 bp, confirmation with another test is recommended.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

When available, performance criteria are provided for the PCR test after enrichment.

4.1 Analytical sensitivity data (in the performance study in 2002, after DNA extraction according to Llop *et al.*, 1999)
10^5–10^6 cfu mL^{-1} plant extract
10^2–10^3 cfu mL^{-1} plant extract after enrichment of the samples in King's B or CCT.

4.2 Analytical specificity data
According to Bereswill *et al.* (1992)
Target organisms tested: 5 strains all positive
Non-target organisms tested: 5 strains all negative

4.3 Data on repeatability
In IVIA: 92%

4.4 Data on reproducibility
In IVIA: 84%

Appendix 8 – PCR according to Taylor *et al.* (2001)

1. General information

1.1 This test is universal for all known *E. amylovora* strains to date. The protocol was validated in a test performance study in 2010.

1.2 The test can be applied to any kind of plant material and to bacterial colonies.

1.3 The targeted sequences are chromosomal (Taylor *et al.*, 2001).

1.4 Oligonucleotides:
G1-F: 5′-CCT GCA TAA ATC ACC GCT GAC AGC TCA ATG-3′
G2-R: 5′-GCT ACC ACT GAT CGC TCG AAT CAA ATC GGC-3′

1.5 The amplicon size is 187 bp.

1.6 Enzyme: the test performance study was performed with a DNA polymerase from Biotools.

2. Methods

2.1 Nucleic acid extraction and purification: for plant material, three DNA extraction methods are described in Appendix 6. These DNA extraction methods were evaluated in the test performance study.

2.2 Polymerase chain reaction

	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water		14.3	
PCR buffer	10×	2.5	1×
$MgCl_2$	50 mM	0.75	1.5 mM
dNTPs	10 mM	0.25	0.1 mM of each dNTP
G1-F primer	10 μM	1.00	0.4 μM
G2-F primer	10 μM	1.00	0.4 μM
Taq polymerase	5 U μL^{-1}	0.2	1 U
Subtotal		20.00	
DNA		5.00	
Total reaction volume of a single PCR reaction		25.00	

2.3 PCR cycling conditions: 3 min at 95°C, 40 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, a final step of 5 min at 72°C and cooling at 15°C.

2.4 Observations: if the expected target concentration is high, i.e. in enriched samples, it is highly recommended to carry out a tenfold dilution of the purified DNA solution in water or TE buffer before amplification in order to dilute inhibitor compounds. Amplification is performed on stock solution and the dilution.

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 isolation and amplification of the target organism and target nucleic acid.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer.
- Positive isolation control (PIC) to ensure 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 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). For PCR tests not performed on bacterial colonies, the PAC should preferably be near 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 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.

3.2 Interpretation of results

Verification of controls

- NIC and NAC should produce no amplicons
- PIC and PAC should produce amplicons of 187 bp size.
- If IPC are used, the amplicons should be of the expected size.

When these conditions are met:

- A test will be considered positive if amplicons of 187 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

Performance criteria are provided for the PCR test without enrichment

4.1 Analytical sensitivity data (according to a test performance study in 2010)

10^3–10^4 cfu mL^{-1} plant extract after DNA extraction following Llop *et al.* (1999)

10^4–10^5 cfu mL^{-1} plant extract after DNA extraction modified after Taylor *et al.* (2001)

10^3–10^4 cfu mL^{-1} plant extract after DNA extraction using RED-Extract N-Amp T Plant kit (Sigma-Aldrich)

4.2 Analytical specificity data (according to Taylor *et al.*, 2001)

Target organisms tested: 69 strains all positive. Negative reaction with strains from *Rubus* sp.

Non-target organisms tested: 49 strains all negative

4.3 Data on repeatability

In IVIA: 100%

4.4 Data on reproducibility

In IVIA: 100%

Appendix 9 – PCR according to Stöger *et al.* (2006)

1. General information

1.1 This method uses the same primers as the nested PCR from Llop *et al.* (2000), see below.

1.2 The test can be applied to any kind of plant material or bacterial colonies.

1.3 The target sequences are located in the plasmid pEA29.

1.4 Oligonucleotides:

PEANT 1: 5′-TAT CCC TAA AAA CCT CAG TGC-3′

PEANT 2: 5′-GCA ACC TTG TGC CCT TTA-3′

1.5 The amplicon size is 391 bp.

1.6 Enzyme: included in the RED-Extract-N-Amp PCR Ready+ mix (Sigma-Aldrich).

2. Methods

2.1 Nucleic acid extraction and purification. Stöger *et al.* (2006) recommended that this method should be used with DNA extracted with the RED-Extract N-Amp T Plant kit (Sigma-Aldrich); details are provided in Appendix 6.

2.2 Polymerase chain reaction

	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water		5.00	
RED-Extract-N-Amp PCR Ready Mix (Sigma)		10.00	
$MgCl_2$	Included in master mix		Included in master mix
dNTPs	Included in master mix		Included in master mix
PEANT 1	10 μM	0.5	0.25 μM
PEANT 2	10 μM	0.5	0.25 μM
Taq polymerase	Included in master mix		Included in master mix
Subtotal		16.00	
DNA		4.00	
Total reaction volume of a single PCR reaction		20.00	

2.3 PCR cycling conditions: 95°C for 5 min; 35 cycles of: 95°C for 15 s, 58°C for 30 s, and 72°C for 45 s; and a final step of 72°C, 5 min, and cooling at 15°C.

2.4 Observations: if the expected target concentration is high, i.e. in enriched samples, it is highly recommended to carry out a tenfold dilution of the purified DNA solution in water or TE buffer before amplification, to dilute inhibitor compounds. Amplification is performed on stock solution and the dilution.

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 isolation and amplification of the target organism and target nucleic acid.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer.
- Positive isolation control (PIC) to ensure 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 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 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). For PCRs not performed on bacterial colonies, the PAC should preferably be near 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 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.

3.2 Interpretation of results:

Verification of controls

- NIC and NAC should produce no amplicons
- PIC and PAC should produce amplicons of 391 bp
- If IPC are used, the amplicons should be of the expected size

When these conditions are met:

- A test will be considered positive if amplicons of 391 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

Performance criteria are provided for the PCR test without enrichment

4.1 Analytical sensitivity data (in the performance study in 2010

10^4–10^6 cfu mL^{-1} plant extract after DNA extraction using RED-Extract N-Amp T Plant kit (Sigma-Aldrich)

4.2 Analytical specificity data

Not evaluated

4.3 Data on repeatability

In IVIA: 92%

4.4 Data on reproducibility

In IVIA: 80%

Appendix 10 – PCR according to Gottsberger adapted from Obradovic *et al.* (2007)

1. General information

1.1 The original protocol and primers from Obradovic *et al.* (2007) were modified by Gottsberger for optimized specificity and maximum sensitivity in plant samples. The protocol was validated in the 2010 test performance study.

1.2 The test can be applied to any kind of plant material or bacterial colonies.

1.3 The targeted sequences are chromosomal.

1.4 Oligonucleotides:
FER1-F: 5′-AGC AGC AAT TAA TGG CAA GTA TAG TCA-3′
rgER2R: 5′-AAA AGA GAC ATC TGG ATT CAG ACA AT-3′

1.5 The amplicon size is 458 bp.

1.6 Enzyme: the test performance study was performed with a DNA polymerase from Biotools.

2. Methods

2.1 Nucleic acid extraction and purification: for plant material, three DNA extraction methods are described in Appendix 6. These DNA extraction methods were evaluated in a test performance study.

2.2 Polymerase chain reaction

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		14.3	
1× PCR buffer	10×	2.5	1×
$MgCl_2$ (or alternatives, specify)	50 mM	0.75	1.5 mM
dNTPs	10 mM	0.25	0.1 mM of each of the dNTP
FER1-F	10 µM	1.00	0.4 µM
rgER2R	10 µM	1.00	0.4 µM
Taq polymerase	5 U μL^{-1}	0.2	1 U
Subtotal		20.00	
DNA		5.00	
Total reaction volume of a single PCR reaction		25.00	

2.3 PCR cycling conditions: 3 min at 94°C, 41 cycles of 10 s at 94°C, 10 s at 60°C and 30 s at 72°C, a final step for 5 min at 72°C and cooling at 15°C.

2.4 Observations: if the expected target concentration is high, i.e. in enriched samples, it is highly recommended to carry out a tenfold dilution of the purified DNA solution in water or TE buffer before amplification, to dilute inhibitor compounds. Amplification is performed on the stock solution and the dilution.

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 isolation and amplification of the target organism and target nucleic acid.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer
- Positive isolation control (PIC) to ensure 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 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). For PCRs not performed on bacterial colonies, the PAC should preferably be near 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 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.

3.2 Interpretation of results

Verification of controls

- NIC and NAC should produce no amplicons
- PIC and PAC should produce amplicons of 458 bp size.
- If IPC are used, the amplicons should be of the expected size.

When these conditions are met:

- A test will be considered positive if amplicons of 458 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

Performance criteria are provided for the PCR test without enrichment

4.1 Analytical sensitivity data (according to the test performance study in 2010)
10^3–10^4 cfu mL^{-1} plant extract after DNA extraction following Llop *et al.* (1999)
10^4–10^5 cfu mL^{-1} plant extract after DNA extraction following Taylor *et al.* (2001) modified and RED-Extract N-Amp T Plant kit (Sigma-Aldrich)

4.2 Analytical specificity data
According to Obradovic *et al.* (2007)
Target organisms tested: 44 strains all positive
Non-target organisms tested: 30 strains all negative

4.3 Data on repeatability
In IVIA: 92%

4.4 Data on reproducibility
In IVIA: 90%

Appendix 11 – Nested PCR (Llop *et al.*, 2000)

1. General information

1.1 Nested PCR in a single tube (Llop *et al.*, 2000) uses two sets of primers placed at the same time. Due to the different annealing temperatures, the two PCR reactions are performed consecutively. The external primers were designed by McManus & Jones (1995); the internal primers are those described by Llop *et al.* (2000). Both are based on sequences from pEA29.

1.2 The test can be applied to any kind of plant material or bacterial colonies.

1.3 The targeted sequences are in the plasmid pEA29.

1.4 Oligonucleotides:
External primers AJ75: 5′-CGT ATT CAC GGC TTC GCA GAT-3′ and AJ76: 5′-ACC CGC CAG GAT AGT CGC ATA-3′
Internal primers PEANT1: 5′-TAT CCC TAA AAA CCT CAG TGC-3′ and PEANT2: 5′-GCA ACC TTG TGC CCT TTA-3′

1.5 The amplicon size is 391 bp.

1.6 Enzyme: the test performance study was performed with a DNA polymerase from Biotools.

2. Methods

2.1 Nucleic acid extraction and purification: for plant material three DNA extraction methods are described in Appendix 6. These DNA extraction methods were evaluated in a test performance study.

2.2 Polymerase chain reaction

	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water		36.25	
PCR buffer	10×	5.00	1×
$MgCl_2$	50 mM	3.00	3 mM
dNTPs	10 mM	0.50	0.1 mM of each dNTP
PEANT1	10 μM	1.00	0.2 μM
PEANT2	10 μM	1.00	0.2 μM
AJ75	0.1 μM	0.32	0.00064 μM
AJ76	0.1 μM	0.32	0.00064 μM
Taq polymerase	5 U μL^{-1}	0.60	3 U
Subtotal		48.00	
DNA		2.00	
Total reaction volume of a single PCR reaction		50.00	

2.3 PCR conditions: 94°C for 4 min followed by 25 cycles of 94°C for 60 s and 72°C for 90 s. This first round PCR is followed in the same thermocycler by a second denaturation step of 94°C for 4 min and 40 cycles of 94°C for 60 s, 56°C for 60 s, and 72°C for 60 s, a final step of 72°C for 10 min and cooling at 15°C.

2.4 Observations: if the expected target concentration is high, i.e. in enriched samples, it is highly recommended to carry out a tenfold dilution of the purified DNA solution in water or TE buffer before amplification, in order to dilute inhibitor compounds. Amplification is performed on stock solution and the dilution.

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 isolation 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 of a sample of uninfected host tissue or clean extraction buffer.
- Positive isolation control (PIC) to ensure 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 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). For PCRs not performed on bacterial colonies, the PAC should preferably be near 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 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.

(3.2) Interpretation of results

Verification of controls

- NIC and NAC should produce no amplicons
- PIC and PAC should produce amplicons of 391 bp size.
- If IPC are used, the amplicons should be of the expected size.

When these conditions are met:

- A test will be considered positive if amplicons of 391 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

Performance criteria are provided for the PCR test without enrichment

4.1 Analytical sensitivity data (according to the performance study in 2010)

10^3–10^4 cfu mL^{-1} plant extract after DNA extraction following Llop *et al.* (1999) and following Taylor *et al.* (2001) modified

4.2 Analytical specificity data

According to Llop *et al.* (2000)

Target organisms tested: 71 strains all positive

Non-target organisms tested: 40 strains all negative

4.3 Data on repeatability

In IVIA: 98%

4.4 Data on reproducibility

In IVIA: 96%

Appendix 12 – Real-time PCR (Pirc *et al.*, 2009)

1. General information

1.1 Real-time PCR tests designed by Pirc *et al.* (2009) are based on chromosomal sequences.

1.2 The test can be applied to any kind of plant material or bacterial colonies.

1.3 The targeted genes are *ams*C (Ams assay) and 16S-23S rRNA intergenic spacer region (ITS assay). Only primers from *ams*C gene were evaluated in the test performance studies in 2009 and 2010.

1.4 Oligonucleotides:

Ams116F: 5′-TCC CAC ATA CTG TGA ATC ATC CA-3′

Ams189R: 5′-GGG TAT TTG CGC TAA TTT TAT TCG-3′

Ams141T: FAM-CCA GAA TCT GGC CCG CGT ATA CCG-TAMRA

ITS15F: 5′-TGA GTA ATG AGC GAG CTA AGT GAA G-3′

ITS93R: 5′-CGC AAT GCT CAT GGA CTC AA-3′

ITS43T: FAM-AGG CGT CAG CGC GCA GCA AC-TAMRA

1.5 Amplicon size in base pairs (including primer sequences): Ams primers 74 bp; ITS primers 79 bp.

1.6 Enzyme: Included in the TaqMan Universal master mix (Applied Biosystems, USA).

1.7 Real-time PCR system (ABI PRISM 7900 HT Sequence Detection System, Applied Biosystems)

using the universal cycling conditions for all amplicons.

2. Methods

2.1 Nucleic acid extraction and purification: three DNA extraction methods were used: (i) the silica-column based DNeasy Plant Mini Kit (Qiagen); (ii) the magnetic bead based QuickPick™ SML Plant DNA Kit (Bio-Nobile, Turku, Finland) with KingFisherR mL system (Thermo Labsystem); and (iii) a simple extraction method (Llop *et al.*, 1999). The DNeasy Plant Mini Kit was used according to the manufacturer's protocol for purification of total DNA from plant tissue with final DNA elution into 2 × 50 µL AE buffer. The protocol for extraction using the QuickPick™ SML Plant DNA Kit was as follows: 100 µL sample was mixed with 400 µL lysis buffer and 25 µL of proteinase K, incubated for 30 min at 65°C and centrifuged at 6000 *g* for 1 min. Lysate (300 µL) was transferred to tube 1 of a KingFisher mL tube strip. Strips contained 20 µL of MagaZorb™ Magnetic Particles and 500 µL binding buffer (tube 1), 800 µL wash buffer (tubes 2 and 3), 100 µL elution buffer (tube 4) and 100 µL water (tube 5). The instrument program Total_RNA_mL_1 in KingFisherR mL was used with minor modification: binding time in well A, 3 × 1 min release plus 2 min binding; wash in well B 15 s; wash in well C, 15 s; elution in well D, 10 min. The simple extraction procedure was performed according to the protocol given by Llop *et al.* (1999), explained in Appendix 6, except that only 100 µL aliquots of crude sample extract were used (Pirc *et al.*, 2009).

2.2 Polymerase chain reaction (for protocol using Ams primers)

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		1.00	
TaqMan universal master mix (Applied Biosystems)	2×	5.00	1×
Ams116F	10 µM	0.90	0.9 µM
Ams189R	10 µM	0.90	0.9 µM
Ams141T	10 µM	0.20	0.2 µM
Subtotal		8.00	
DNA		2.00	
Total reaction volume of a single PCR reaction		10.00	

2.3 PCR cycling conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C.

2.4 Note: if the expected target concentration is high, i.e. in enriched samples, it is highly recommended to carry out a tenfold dilution of the purified DNA solution in water or TE buffer before amplification, in order to dilute inhibitor compounds. Amplification is performed on stock solution and the dilution.

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 isolation and amplification of the target organism and target nucleic acid.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer.
- Positive isolation control (PIC) to ensure 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 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). For PCRs not performed on bacterial colonies, the PAC should preferably be near the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls 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 rela-

tion with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

3.2 Interpretation of results

The cycle cut-off value for this test is set at 38, and was obtained using the equipment/materials and chemistry used as described here. When necessary, the C_t cut-off value should be determined for the required control (e.g. when an internal positive control is used). The cycle cut-off value needs to be verified in each laboratory when implementing the test for the first time.

Verification of controls

- The PIC and PAC amplification curves should be exponential.
- NIC and NAC should be negative (Ct $\geq$ 40)
- PIC and PAC (and if relevant IPC) should have a Ct value below the relevant cut-off value.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve, a Ct value <38
- A test will be considered negative if it produces no exponential amplification curve and a Ct $\geq$ 40.
- The test should be repeated if any contradictory or unclear results are obtained, or if the Ct value is between 38 and 40.

4. Performance criteria available

Performance criteria are provided for the PCR test without enrichment

4.1 Analytical sensitivity data

Validation data available from the test performance study in 2010

10^3–10^4 cfu mL^{-1} plant extract after DNA extraction following Llop *et al.* (1999), Taylor *et al.* (2001) modified and RED-Extract-N-AmpTkit.

Validation data available from the National Biology Institute, SL

2×10^3 cfu mL^{-1} DNeasy Plant Mini Kit (Qiagen), 9×10^2 cfu mL^{-1} QuickPick™ SML Plant DNA Kit (Bio-Nobile), (data National Biology Institute, SL)

1×10^4 cfu mL^{-1} following Llop *et al.* (1999) (data National Biology Institute, SL)

4.2 Analytical specificity data

Target organisms tested: 423 strains all positive

Non-target organisms tested: 97 strains all negative

4.3 Data on repeatability

In IVIA: 98%

4.4 Data on reproducibility

In IVIA: 94%

Appendix 13 – Real-time PCR (Gottsberger, 2010)

1. General information

1.1 Real-time PCR targeting a hypothetical protein-coding gene was designed (Gottsberger, 2010). The accuracy in the 2010 test performance study could not be tested with this real-time PCR; however, it was tested by one laboratory in parallel with the real-time PCR described in Pirc *et al.* (2009) and gave the same qualitative results with the DNA extraction from Llop *et al.* (1999) protocol.

1.2 The test can be applied to any kind of plant material or bacterial colonies.

1.3 The target sequences are located in the chromosome.

1.4 Oligonucleotides:

hpEaF: 5′-CCG TGG AGA CCG ATC TTT TA-3′

hpEaR: 5′-AAG TTT CTC CGC CCT ACG AT-3′

hpEaP: FAM-TCG TCG AAT GCT GCC TCT CT-MGB

1.5 Amplicon size in base pairs (including primer sequences): 138 bp

1.6 Enzyme: included in the TaqMan Universal master mix (Applied Biosystems).

1.7 Real-time PCR system (Eppendorf Realplex Mastercycler Epgradient S, Eppendorf, Hamburg, Germany).

2. Methods

2.1 Nucleic acid extraction and purification: several DNA extraction methods were tested: (i) the silica-column based DNeasy Plant Mini Kit (Qiagen); (ii) the magnetic bead based QuickPick™ SML Plant DNA Kit (Bio-Nobile) and (iii) a simple extraction method (Llop *et al.*, 1999). The DNeasy Plant Mini Kit was used according to the manufacturer's protocol for purification of total DNA from plant tissue with final DNA elution into 1 × 100 µL of AE buffer. The protocol for extraction using QuickPick™ SML Plant DNA Kit was performed according to the manufacturer. The simple extraction procedure was performed according to the protocol given by Llop *et al.* (1999). Further protocols used are described in Stöger *et al.* (2006), Persen *et al.* (2011).

2.2 Polymerase chain reaction

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		6.00	
	2×	10.00	1×

(continued)

Table *(continued)*

	Working concentration	Volume per reaction (µL)	Final concentration
TaqMan Universal master mix (Applied Biosystems)			
$MgCl_2$	Included in master mix		Included in master mix
dNTPs	Included in master mix		Included in master mix
hpEaF	10 µM	1.00	0.5 µM
hpEaR	10 µM	1.00	0.5 µM
hpEaP	1 µM	1.00	0.05 µM
Taq polymerase	Included in master mix		Included in master mix
Subtotal		19.00	
DNA		1.00	
Total reaction volume of a single PCR reaction		20.00	

2.3 PCR cycling conditions: 2 min at 50°C, 10 min at 95°C, 50 cycles of 15 s at 95°C and 1 min at 60°C).

2.4 Observations: if the expected target concentration is high, i.e. in enriched samples, it is highly recommended to carry out a tenfold dilution of the purified DNA solution in water or TE buffer before amplification, in order to dilute inhibitor compounds. Amplification is performed on stock solution and the dilution.

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 isolation and amplification of the target organism and target nucleic acid.

–Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or 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 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). For PCRs not performed on bacterial colonies, the PAC should preferably be near 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 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.

3.2 Interpretation of results

The cycle cut-off value for this test is set at 48, and was obtained using the equipment/materials and chemistry used as described here. When necessary the Ct cut-off value should be determined for the required control (e.g. internal positive control). The cycle cut-off value needs to be verified in each laboratory when implementing the test for the first time.

Verification of controls

- The PIC and PAC amplification curves should be exponential.
- NIC and NAC should be negative ($Ct \geq 50$)
- PIC and PAC (and if relevant IPC) should have a Ct value below the relevant cut-off value.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve, a Ct value <48
- A test will be considered negative if it produces no exponential amplification curve and a $Ct \geq 50$.
- The test should be repeated if any contradictory or unclear results are obtained, or if the Ct value is between 48 and 50.

4. Performance criteria available (from AGES, AT, 2010)

This test was not evaluated in the test performance studies

4.1 Analytical sensitivity data (according to Gottsberger, 2010)
2×10^3 cfu mL^{-1}

4.2 Analytical specificity data
According to Gottsberger (2010)
Target organisms tested: 71 strains all positive
Non-target organisms tested: 41 strains all negative

4.3 Data on repeatability
In AGES: 100%

4.4 Data on reproducibility
In AGES: 100%

Appendix 14 – Loop-mediated isothermal amplification (LAMP)

1. General information

The test was developed by Temple *et al.* (2008), Temple & Johnson (2011), and was evaluated in a test performance study in 2010 because it was considered appropriate for laboratories that do not have PCR equipment, and is simple and easy to perform for analysis of symptomatic plants as well as for bacterial identification. However, the sequences are based on those of the pEA29 plasmid and this test lacks the appropriate sensitivity for the analysis of samples with low bacterial populations below 10^5 cfu mL^{-1} plant extract.

The test can be applied to any kind of plant material after a DNA extraction, as indicated in Appendix 6, and to bacterial colonies, without DNA extraction.

LAMP primers to detect *amsL B*:

ALB Fip: 5′-CTG CCT GAG TAC GCA GCT GAT TGC ACG TTT TAC AGC TCG CT-3′;

ALB Bip: 5′-TCG TCG GTA AAG TGA TGG GTG CCC AGC TTA AGG GGC TGA AG-3′;

ALB F: 5′-GCC CAC ATT CGA ATT TGA CC-3′;

ALB B: 5′-CGG TTA ATC ACC GGT GTC A-3′.

2. Methods

Melting temperatures for primers were between 58 and 60°C.

LAMP reaction mix:

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		24.60	
10× ThermoPol buffer	10×	5.00	1×
dNTPs	10 mM	5.00	1.0 mM
$MgSO_4$	100 mM	2.00	4.0 mM
BSA	10 mg mL^{-1}	2.00	0.4 mg mL^{-1}
ALB FIP	100 µM	(1.2)	2.4 µM
ALB BIP	100 µM	(1.2)	2.4 µM
ALB F	10 µM	1.00	0.2 µM
ALB B	10 µM	1.00	0.2 µM
Bst DNA polymerase	8 U μL^{-1}	2.00	16 U per reaction
Subtotal		45.0	
DNA		5.00	
Total reaction volume of a single PCR reaction		50.0	

Prior to starting the LAMP reaction, set a water bath at 65°C or a thermal cycler at 65°C for 55 min. Prepare the mix and pipette 24.6 µL molecular-grade water into each individual 0.2 mL PCR reaction tube, then pipette 18.4 µL of the master mix (see table above) into each individual PCR reaction tube, then pipette 2 µL *Bst* DNA polymerase into each individual PCR reaction tube. Finally, pipette 5 µL template DNA. Spin down tubes for just 30 s. Place tubes in water bath (65°C) in a holder so the reaction end is submerged in water bath, or place in a thermocycler (set at 65°C) for 55 min. Remove tubes and allow them to cool for approximately 10 s at room temperature. Observe tubes for the presence of visual precipitate, a cloudy tube or a solid white precipitate at the bottom of the tube (indicating a positive reaction). A clear solution is a negative reaction.

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 isolation and amplification of the target organism and target nucleic acid.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue (when working with plant material) or clean extraction buffer (when working with pure culture); 1 per DNA extraction series.
- Positive isolation control (PIC) to ensure 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); 1 per DNA extraction series.

- 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; 1 per LAMP run.
- Positive amplification control (PAC) to monitor the efficiency of 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); 1 per LAMP run. For PCRs not performed on bacterial colonies, the PAC should preferably be near 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 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.

3.2 Interpretation of results

Verification of controls

- NIC and NAC should produce no turbidity.
- PIC and PAC (and if relevant IPC) should produce the expected turbidity.

When these conditions are met:

- A test will be considered positive if it produces turbidity.
- A test will be considered negative if it produces no turbidity.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data (according to the results obtained in a performance study in 2010):

10^5–10^6 cfu mL^{-1} plant extract after DNA extraction following Taylor *et al.* (2001).

4.2 Analytical specificity data

According to Temple *et al.* (2011)

Target organisms tested: 10 strains all positive (except pEA29 free strains)

Non-target organisms tested: 30 strains all negative

4.3 Data on repeatability

In IVIA: 96%

4.4 Data on reproducibility

In IVIA: 90%

Appendix 15 – Pathogenicity tests

Inoculation of fruitlets (of susceptible cultivars of pear, apple or loquat) can be performed on whole disinfected immature fruits or on slices of them, using 10 µL of 10^9 cfu mL^{-1} suspensions of colonies in PBS (Appendix 2). Include a positive and negative control. Incubate in a humid chamber at 25°C for 3–7 days. A positive test on fruit is shown by browning around the wounding site and oozing of bacteria in 3–7 days (provided the negative control gives no lesion or only a necrotic lesion).

For whole plant inoculation, use susceptible cultivars of pear, apple or loquat, or susceptible species of *Crataegus*, *Cotoneaster* or *Pyracantha*. To inoculate a potted plant, cut a young leaf from a young shoot to the main vein with scissors dipped into a 10^9 cfu mL^{-1} suspension of each test colony prepared in PBS (Appendix 1).

Detached young shoots from glasshouse-grown plants can also be inoculated in the same way, after disinfection for 30 s with 70% ethanol and three washings with sterile distilled water, and kept in tubes with sterile 1% agar. Maintain the plants or tubes at 20–25°C at 80–100% relative humidity with 16 h light. Read results after 3, 7 and 15 days. Typical *E. amylovora* symptoms include wilting, discoloration, necrotic tissue and ooze.

E. amylovora-like colonies should be re-isolated from inoculated fruitlets, plants or shoots showing typical symptoms and their identity confirmed.