

For final adoption

Diagnostics

Diagnostic

***Erwinia amylovora*¹**

Specific scope

This standard describes a diagnostic protocol for *Erwinia amylovora*.

Specific approval and amendment

This Standard was developed under the EU DIAGPRO Project (SMT 4-CT98-2252) and EUPHRESKO 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 20xx-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*. *E. 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. *E. amylovora* is now present in more than forty countries (van der Zwet, 2002; CABI/EPPO, 2007), but it has not been recorded either in South America, Asia or in 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).

Fire blight is probably the most serious disease affecting pear or 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 of time depending of environmental

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.

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 Fig. 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

Con formato: Italiano (Italia)

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Detection

Disease symptoms

Symptoms of fire blight on the principal hosts are relatively similar and easily recognized (Figs. 3, 4 and 5). The name of the disease is descriptive of its major characteristic: the brownish aspect of twigs, flowers and leaves as though burned by fire. The 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 fast 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, 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 which 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 the bark is peeled from the 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 *E. pyrifoliae*, causal agent of bacterial shoot blight of Asian pear (*Pyrus pyrifolia*) (Kim *et al.*, 1999), *Erwinia piriflorinigrans* isolated from necrotic

pear blossoms in Spain (López *et al.*, 2011), *Erwinia* sp. and *E. uzenensis* that causes different types of pear symptoms in Japan (Tanii *et al.*, 1981; Matsuura *et al.*, 2012) 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 the samples and during the extraction process. The 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 the bark from cankers in twig, branches, trunk or collar). The samples should be processed as soon as possible after being collected and stored at 4–8 °C before analysis. The samples can be cold-stored after processing for up to two weeks in case further testing is required.

Isolation

Fresh sample extracts are necessary for successful isolation. Details on extraction procedure from plant material are given in Appendix 1. Isolating *E. amylovora* from symptomatic samples is relatively easy because the number of culturable bacteria in such samples is usually high. However, when symptoms are very advanced or when the environmental conditions are not favourable for fire blight symptoms expression, the number of *E. amylovora* culturable cells can be very low. When plates are overcrowded by plant microbiota, the sample should be re-tested 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.

For direct isolation, plating on three media is advised for maximum recovery of *E. amylovora* in particular when samples are in poor conditions. The efficiency of the different media depends on the number and composition of the microbiota of the sample. Three media: King's B, Levana and CCT (Appendix 2) have been validated in a test performance study. Fig 6 shows the typical aspect 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, (these are the minimum concentrations that are usually present in symptomatic samples). Several tests are described in Appendix 3 to 14 and at least two tests, based on different biological principles, should be performed. One test can be a serological test preferably using specific monoclonal antibodies and another PCR-based. Test performance studies were organized and results are indicated. As differences in analytical sensitivity were observed in such studies the decision on the tests selected should be based in a comparative analysis of the sensitivity and specificity of the different techniques in each laboratory, the number of samples to analyze, 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 DAS-ELISA, and lateral flow devices are described for analyses of organs with symptoms. Quality of the antibodies is critical for the

performance of the tests. 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., Spain). For ELISA a complete kit based on a combination of specific monoclonal antibodies from Plant Print Diagnostics S. L., Spain, was also evaluated.

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

Details on the tests are given in Appendix 3.

- Molecular tests

Conventional PCR, real-time PCR and LAMP (Loop-mediated isothermal amplification) were evaluated in a test performance study in 2010 and are also advised 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 3. 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 Appendix 6 to 13 and a LAMP protocol is included in Appendix 14.

Detection from asymptomatic samples

Sampling and sample preparation

Perform the analyses of asymptomatic plants in summer or early autumn in order to increase the likelihood of detecting *E. amylovora*. Asymptomatic samples can be processed preferably individually, or in groups of up to 100 samples (OEPP/EPPO, 1992). 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 Appendix 4 to 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 (Appendix 1, 2 and 3), or from the enriched samples (Appendix 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 (Appendix 2).

Biochemical tests

The genus *Erwinia* was defined for Gram-negative bacteria, facultative anaerobes, motile by peritrichous flagella, rod-shaped and acid produced from glucose, fructose, galactose and sucrose. The phenotypic properties of Table 1 (Paulin, 2000), that 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; Kim *et al.*, 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 of 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 ¹	+
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	+

¹=Spontaneous mutants found in nature can be levan negative.

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

Microbiological tests	<i>Erwinia amylovora</i>	<i>Erwinia pyrifoliae</i>	<i>Erwinia piriflorinigrans</i>
Gelatine hydrolysis	+	-	-
Inositol ¹	-	ND ³	+
Sorbitol ¹	+	+	-
Esculin ¹	V ⁴	-	+
Melibiose ¹	-	-	+
D-Raffinose ¹	-	-	+
β -Gentibiose ¹	+	-	+

1 = 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 in the Table.

2 = ND: Not determined.

3 = V: Variable.

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 followed 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).

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

Test ¹	Reaction (48 h) ²
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

¹= Abbreviations used in API 20 E strips

²= More than 90% of the strains give the results indicated in the Table.

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 including biochemical and physiological properties such as pH, salt, lactic acid tolerance and antibiotics. Every species tested creates unique "phenotypic fingerprint" which is automatically compared to 1200 aerobic species in the database.

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

Fatty acid profiling (FAP)

E. amylovora-like colonies should be grown on trypticase soy agar for 48 h at 28 °C, and an appropriate 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, 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 two serological tests only 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 positive.

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 5 or 10 fold dilution) on a slide. Monoclonal antibodies can be used only if they agglutinate with the reference strains.

IF test

The immunofluorescence 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. (Spain) or antiserum from Loewe, Biochemica GmbH, Sauerlach, Germany.

ELISA tests

ELISA tests are described in PM 7/101(1) [*ELISA tests for plant pathogenic bacteria*](#)

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., Spain). For DASI-ELISA, a suspension of approximately 10^8 cells per mL from suspected colonies is

prepared in PBS (Appendix 2). The DASI-ELISA procedure (Appendix 3) can be followed without prior enrichment for isolate's identification.

Lateral flow immunoassays.

A suspension of approximately 10^8 cells per mL prepared in PBS (Appendix 2) from suspected colonies should be used following the instructions of the manufacturers. The two kits evaluated in a test performance study (Agri-strip, from Bioreba, Reinach, Switzerland and Pocket Diagnostic, York, UK) 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 per mL in molecular-grade sterile water should be prepared from *E. amylovora*-like colonies. Appropriate PCR procedures should be applied, following Appendix 7 to 11, without DNA extraction, just after treatment at 100°C for 10 min.

Real-time PCR

Two real-time PCR tests have been selected and are described in Appendix 12 and 13. Colonies can be prepared as for conventional PCR.

Macrorestriction with Xba I and Pulse Field Gel Electrophoresis (PFGE)

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 *Erwinia amylovora* isolates from other members of the Enterobacteriaceae (see EPPO standard on DNA barcoding). For example, all isolates of *E. amylovora* so far tested 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: (1) National Collection of Plant Pathogenic Bacteria (NCPPB), Fera, Sand Hutton, York (GB); (2) Culture Collection of the Plant Protection Service (PD), Wageningen (NL); (3) Collection Française de Bactéries Phytopathogènes (CFBP), EmerSys - IRHS - INRA Beaucauzé, France. Authenticity of the strains can be guaranteed only if directly obtained from the culture collections.

Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM7/77 (1) *Documentation and reporting on a diagnosis*.

Further information

Further information on this organism can be obtained from:

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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 diagnostics@epo.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

This protocol was originally drafted by:

M. M. López, M. Keck, P. Llop, M. T. Gorris, J. Peñalver, V. Donat and M. Cambra., IVIA, Moncada (Valencia) (ES) and the revised version was prepared by M.M. López (IVIA, ES). T. Dreó (NIB, SL) and R. Gottesberger (AGES, AT) contributed by preparing the description of two PCR tests.

The DNA polymerase used in the test performance study for the PCR tests described in Appendix 7, 8, 10 and 11 was provided to all laboratories participating by Biotools B&M Labs, S.A, Madrid, Spain.

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Appendix 1

Extraction procedures

Samples from symptomatic material

The samples may be processed in different buffers according to the techniques used. Sample processing in an antioxidant maceration buffer (Appendix 2), that should be prepared immediately before the analysis, and that is advised for all techniques and required for optimal 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 should be selected on each organ to analyse. The exudates can be processed separately, in 1 to 4.5 mL of 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 with symptoms using a sterile scalpel and take pieces underneath with typical subcortical discoloration symptoms.

The protocol evaluated in a test performance study was the following: cut 0.1g of shoots, flowers, leaves, stems, trunks or fruits into pieces and put into plastic bags with a heavy net. Add to each bag 4.5 mL of the antioxidant maceration buffer described by Gorris *et al.* (1996a) (Appendix 2). Let the samples macerate for at least 5 min. Slightly crush the plant material 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 few minutes and transfer approximately 2 mL, 1 mL and 1 mL of each macerate into three sterile Eppendorf tubes by decantation. 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 and add 30% glycerol (Difco) to the other 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 1mL stored at -20°C .

Samples from asymptomatic material

Collect flowers, shoots, fruitlets or stem segments in sterile bags or containers, 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). For nursery plants: cut young shoots about 20 cm long from the most susceptible hosts available, disinfect scissors or pruning shears between plants. For plants growing in the field, cut flowers when available and/or young shoots about 20 cm long, disinfect between plants. Take flowers or peduncle and the base of the limb of mature leaves, or stem segments, of the selected plants. If analyses need to be performed in winter, collect 5 to 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, the enrichment should be done for 72 h at approximately 25°C .

Weigh 0.1 to 1 g of 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, following the protocols described in the Appendix 3 to 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 short time.

The EPPO phytosanitary procedure (OEPP/ EPPO, 1992) includes a sampling procedure for the analysis of twigs of asymptomatic woody material in nurseries. A sample consisted of 100 twigs about 10 cm in length from 100 plants. If there are several plant genera in the lot, these should be equally represented in the sample (with a maximum of three genera per sample). From each sample, randomly take 30 cut twigs and cut them into four pieces (120 stem pieces). Place them 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 (n°2 = 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). 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 *E. amylovora*) and low in winter.

Whichever procedure is followed, prepare for each sample 3 Eppendorf tubes with about 2 mL, 1 mL and 1 mL of 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 g; KCl 0.2 g; Na₂HPO₄·12H₂O 2.9 g; KH₂PO₄ 0.2 g; distilled water to 1 L. Sterilize by filtration.

Antioxidant maceration buffer (Gorris *et al.*, 1996a): polyvinylpyrrolidone (PVP-10) 20 g; mannitol 10 g; ascorbic acid 1.76 g; reduced glutathion 3 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 g; polyvinylpyrrolidone (PVP-10) 20 g; distilled water to 1 L. Adjust pH to 7.5. Sterilize by filtration.

50X TAE buffer: Tris 242 g; 0.5 m Na₂EDTA pH 8.0 100 mL; glacial acetic acid 57.1 mL; distilled water to 1 L. Adjust pH to 7.5-7.8.

Loading buffer: Bromophenol blue 0.025 g; glycerol 3 g; distilled water 10 mL.

Media

Ayers Medium (Ayers *et al.*, 1919): NH₄H₂PO₄ 1 g; KCl 0.2 g; MgSO₄ 0.2 g; bromothymol blue 75 mL (solution 0.2%); distilled water to 1 L. Adjust pH to 7. Sterilize by autoclaving at 120 °C for 15 min.

CCT medium (Ishimaru & Klos, 1984): sucrose 100 g; sorbitol 10 g; Niaproof 1.2 mL; crystal violet 2 mL (sol. 0.1% ethanol); nutrient agar 23 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); cycloheximide 0.05 g. Sterilize by filtration (0.45 µm). Add to 1 L of the sterile medium (at about 45 °C).

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

King's B medium (King *et al.*, 1954): Proteose peptone N°3 20 g; glycerol 10 mL; K₂HPO₄ 1.5 g; MgSO₄·7H₂O 1.5 g; agar 15 g; distilled water to 1 L. Adjust pH to 7.0–7.2. Sterilize by autoclaving at 120 °C for 15 min.

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. Sterilize by autoclaving at 120 °C for 15 min.

Appendix 3

Rapid serological screening tests

1- Immunofluorescence (IF)

Follow the standard instructions described in PM7/97 *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, (Sauerlach, Germany).
- IVIA EPS 1430, polyclonal antibodies, for detection using IF test (validated in test performance studies), Plant Print Diagnostics, S.L., (Spain).
- IVIA Mab 7 A, monoclonal antibodies, for detection using IF test (validated in test performance studies), Plant Print Diagnostics S.L. (Spain).

Use undiluted macerates and 1: 10 and 1: 100 dilutions in PBS (Appendix 2) to spot windows of the 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. IF is not recommended after enrichment of the samples.

Performance criteria

- 1 Analytical sensitivity data (in the performance study in 2002)
10³-10⁴ CFU/mL plant extract

- 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* & *Dickeya* sp.).

- 3 Data on Repeatability

In IVIA: 100%

- 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 commercially available from Plant Print Diagnostics S.L. (Faura, Spain). 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).

Treat the ELISA necessary amount of the enriched extracts and controls in a water bath (or in a thermoblock) at 100°C for 10 min before ELISA, making sure that 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

1.1 Analytical sensitivity data (in the performance study in 2002)

- 10 CFU/mL plant extract in King's B and in CCT (Gorris *et al.*, 1996b).
- 10^{-10^2} CFU/mL plant extract in King's B and 10^3 - 10^4 CFU/mL plant extract in CCT (in the performance study in 2010).

1.2 Analytical specificity data

For monoclonal antibodies 3B+5H

- Target organisms tested: 250 *E. amylovora* strains. All positive in the test conditions (Gorris *et al.*, 1996 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.*, 1996).

1.3 Data on Repeatability

In IVIA: 100%

1.4. 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 *Erwinia amylovora* polyclonal antibodies that are non-specific. Follow the manufacturers' instructions when performing the analysis.

Performance criteria for Ea Agri-strip (Bioreba, Reinach, Switzerland)

1 Analytical sensitivity data (in the performance study performed in 2010):

10^5 - 10^6 CFU/mL plant extract

2 Analytical specificity data

According to Braun-Kiewnick *et al.* (2011)

- 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 reported in AGES (AT) as well.

3 Data on Repeatability

In IVIA: 94%

4 Data on Reproducibility

In IVIA: 96%

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

1 Analytical sensitivity data (in the performance study performed in 2010):

- 10^5 - 10^6 CFU/mL plant extract

2 Analytical specificity data

Not evaluated

3 Data on Repeatability

In IVIA: 94%

4 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 amount of inhibitor organisms is expected. After preparation of the samples in the freshly prepared antioxidant buffer, use of two validated media, one non-selective (King's B) and one semi-selective (CCT) (Appendix 2), is advised because the composition and number of the 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. Prepare, as additional negative controls, three tubes with 0.9 mL of 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

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* in particular when samples are in poor conditions. 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 spreader or dip a glass spreader in denatured ethanol, flame and allow cooling. 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 about 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. but levan-negative colonies of *E. amylovora* have also been reported (Bereswill *et al.*, 1997). Figure 3 shows the aspect 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 in Nutrient agar slants covered with vaseline oil at 10 °C or for long-term 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 that 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

1 Analytical sensitivity data (in the performance study in 2010):
 10^3 CFU/mL in King's B ; 10 - 10^2 CFU/mL in Levan and CCT

2 Analytical specificity data
Not evaluated

3 Data on Repeatability
In IVIA: 100%

4 Data on Reproducibility
In IVIA: 100%

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 about 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

1 Analytical sensitivity data (in the performance study in 2010)

- 10 CFU/mL after enrichment in CCT
- 10-10² CFU/mL after enrichment in King's B

2 Analytical specificity data

Not evaluated

3 Data on Repeatability

In IVIA: 100%

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 Appendix 1 and 4. Centrifuge the macerates at 10000 g for 5 min at room temperature. Discard the supernatant, and resuspend the pellet in 500 µL of extraction buffer (Tris HCl, 31.52 g; NaCl 14.6 g; EDTA 9.3 g; SDS 5 g; polyvinylpyrrolidone (PVP-10) 20g; distilled water 1 L, pH 7.5; sterilised by filtration) 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 10000 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 litre) for 15 minutes at room temperature. The resultant 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 of 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 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 the 2009 and 2010 test performance studies with four PCR protocols, showing 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 of them can reliably be used (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), Mc Manus and 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) and 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 experience from different countries (Powney *et al.*, 2007) using two PCR tests (one with primers based on pEA29 sequences and the second based on chromosomal sequences) is necessary. 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 utilised 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 shows frequently non-specific banding (see below).

1.2. The test can be applied to any kind of plant material after a DNA extraction as indicated in Appendix 6 and to bacterial colonies.

1.3. The targeted sequences are in 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 about 900 bp. according to Bereswill *et al.* (2002) although variations can occur between 900 and 1100 bp (Lecomte *et al.*, 1997), due to the number of 8 bp repeats sequences within the fragment (Jones and Geider, 2001).

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

Con formato: Inglés (Reino Unido)

2. Methods

Nucleic acid extraction and purification: the three DNA extraction methods were evaluated in a test performance study. The sensitivity of the tests increases after the enrichment of the samples in King's B and CCT, respectively (López *et al.*, 2006).

2.1. Polymerase Chain Reaction – PCR

	Final concentration	Working concentration	Volume per reaction (µL)
Total reaction volume of a single PCR reaction in µL			50
PCR buffer	1X	10X	5
MgCl ₂	3mM	50mM	3
dNTPs	0.2 mM of each dNTP	10 mM	1
Taq polymerase	1U	5U/ µL	0.2
primer A	0.1 µM	10 µM	0.5
primer B	0.1 µM	10 µM	0.5
DNA			5
PCR grade water			34.8

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

2.3. 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 cross-reactions with the host tissue and/or 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 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 PCR grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

As alternative (or in addition to) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or bacterial 18S rDNA) amplification of samples spiked with exogenous nucleic 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: in order to assigning results from PCR-based test the following criteria should be followed:

- A sample will be considered positive if it produces the amplicon of 900 bp and provided that the NIC and NAC are negative.
- A sample will be considered negative, if it produces no band or a band of a different size and provided that PIC and PAC are positive,
- 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 plant extract
- 10^2 - 10^3 CFU/mL 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 after a DNA extraction as indicated in Appendix 6 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 ATG3';

G2-R 5'GCT ACC ACT GAT CGC TCG AAT CAA ATC GGC3'

1.5. The amplicon size is about 187 bp.

1.6. Enzyme: The test performance study was performed with a DNA polymerase from Biotoools.

2. Methods

Nucleic acid extraction and purification: The three DNA extraction methods were evaluated in a test performance study.

2.1.Polymerase Chain Reaction – PCR

	Final concentration	Working concentration	Volume per reaction (µL)
Total reaction volume of a single PCR reaction in µL			25
PCR buffer	1X	10X	2.5
MgCl ₂	1.5 mM	50 mM	0.75
dNTPs	0.1 mM of each dNTP	10 mM	0.25
Taq polymerase	1U	5U/ µL	0.2
G1-F primer	0.4 µM	10 µM	1
G2-F primer	0.4 µM	10 µM	1
DNA			5
PCR grade water			14.3

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

2.3. 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 cross-reactions with the host tissue and/or 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 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 PCR grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

As alternative (or in addition to) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or bacterial 18S rDNA) amplification of samples spiked with exogenous nucleic 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: in order to assigning results from PCR-based test the following criteria should be followed:

- A sample will be considered positive if it produces the amplicon of 187 bp and provided that the NIC and NAC negative.
- A sample will be considered negative, if it produces no band or a band of a different size and provided that the PIC and PAC are positive,
- 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)

- 10^3 - 10^4 CFU/mL plant extract after DNA extraction following Llop *et al.* (1999)
- 10^4 - 10^5 CFU/mL plant extract after DNA extraction modified after Taylor *et al.* (2001) and 10^3 - 10^4 CFU/mL plant extract after DNA extraction using REDExtract-N-AmpT kit (Sigma-Aldrich, USA)

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 utilises the same primers of the Nested PCR from Llop *et al.* (2000), see below:

1.2. 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.

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 about 391 bp.

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

2. Methods

Nucleic acid extraction and purification. Stöger *et al.* (2006) recommended that this method should be used with DNA extracted with the RED-Extract-N-AmpT kit.

2.1. Polymerase Chain Reaction – PCR

	Final concentration	Working concentration	Volume per reaction (µL)
Total reaction volume of a single PCR reaction in µL			20
RED-Extract-N-Amp PCR Ready Mix (Sigma)			10
MgCl ₂	Included in Master Mix	Included in Master Mix	
dNTPs	Included in Master Mix	Included in Master Mix	
Taq polymerase	Included in Master Mix	Included in Master Mix	
PEANT 1	0.25 µM	10 µM	0.5
PEANT 2	0.25 µM	10 µM	0.5
DNA			4
PCR grade water			5

2.2. 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.

2.3. 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 cross-reactions with the host tissue and/or 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 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 PCR grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

As alternative (or in addition to) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or bacterial 18S rDNA) amplification of samples spiked with exogenous nucleic 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: in order to assigning results from PCR-based test the following criteria should be followed:

- A sample will be considered positive if it produces the amplicon of 391 bp and provided that the NIC and NAC are negative.
- A sample will be considered negative, if it produces no band or a band of a different size and provided PIC and PAC are positive,
- 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 2003)

- 10^4 - 10^6 CFU/mL plant extract after DNA extraction using RED-Extract-N-AmpT kit

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) was modified for optimized specificity and maximum sensitivity in plant samples by Gottsberger. The protocol was validated in the 2010 test performance study.

1.2. 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.

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 about 458 bp.

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

2. Methods

Nucleic acid extraction and purification: three DNA extraction methods were evaluated in a test performance study for plant material.

2.1. Polymerase Chain Reaction – PCR

	Final concentration	Working concentration	Volume per reaction (µL)
Total reaction volume of a single PCR reaction in µL			25
1X PCR buffer	1X	10X	2.5
MgCl ₂ (or alternatives, specify)	1.5 mM	50 mM	0.75
dNTPs	0.1 mM of each of the dNTP	10 mM	0.25
Taq polymerase	1U	5U/ µL	0.2
FER1-F	0.4 µM	10 µM	1
rgER2R	0.4 µM	10 µM	1
DNA			5
PCR grade water			14.3

2.2 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.

2.3. 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 cross-reactions with the host tissue (or other matrix) and/or 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 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 PCR grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

As alternative (or in addition to) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or bacterial 18S rDNA) amplification of samples spiked with exogenous nucleic 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: in order to assigning results from PCR-based test the following criteria should be followed:

- A sample will be considered positive if it produces the amplicon of 458 bp and provided that the NIC and NAC are negative.
- A sample will be considered negative, if it produces no band or a band of a different size and provided that PIC and PAC are positive,
- Tests should be repeated if any contradictory or unclear results are obtained.

4 Performance criteria available

Performance criteria without enrichment.

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

- 10^3 - 10^4 CFU/mL plant extract after DNA extraction following Llop *et al.* (1999).

- 10^4 - 10^5 CFU/mL plant extract after DNA extraction following Taylor *et al.* (2001) modified and REDExtract-N-AmpT kit

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. The Nested-PCR in a single tube (Llop *et al.*, 2000) uses two sets of primers placed at the same time, and due to the different annealing temperatures the two PCR reactions are performed consecutively. The external primers are the same designed by McManus and Jones (1995), whilst the internal are the ones described by Llop *et al.* (2000) and both are based in sequences from pEA29.

1.2. 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.

1.3. The targeted sequences are in pEA29.

1.4. Oligonucleotides:

External primers AJ75: 5' CGT ATT CAC GGC TTC GCA GAT and AJ76: 5' ACC CGC CAG GAT AGT CGC ATA.

Internal primers PEANT1: 5' TAT CCC TAA AAA CCT CAG TGC and PEANT2: 5' GCA ACC TTG TGC CCT TTA

1.5. The amplicon size is 391bp.

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

2. Methods

Nucleic acid extraction and purification: three DNA extraction methods were evaluated in a test performance study for plant material.

2.1. Polymerase Chain Reaction – PCR

	Final concentration	Working concentration	Volume per reaction (μL)
Total reaction volume of a single PCR reaction in μL			50
PCR buffer	1X	10X	5
MgCl ₂	3 mM	50 mM	3
dNTPs	0.1 mM of each dNTP	10 mM	0.5
Taq polymerase	3U	5U/ μL	0.6
PEANT1	0.2 μM	10 μM	1
PEANT2	0.2 μM	10 μM	1
AJ75	0.00064 μM	0.1 μM	0.32
AJ76	0.00064 μM	0.1 μM	0.32
DNA			2
PCR grade water			36.25

2.2. 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.

2.3. 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 cross-reactions with the host tissue and/or 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 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 PCR grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

As alternative (or in addition to) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or bacterial 18S rDNA) amplification of samples spiked with exogenous nucleic 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: in order to assigning results from PCR-based test the following criteria should be followed:

- A sample will be considered positive if it produces the amplicon of 391 bp and provided that the NIC and NAC are negative.
- A sample will be considered negative, if it produces no band or a band of a different size and provided that PIC and PAC are positive,
- 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 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%

10.4.4. Data on Reproducibility

In IVIA: 96%

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

1. General information

1.1. The first protocol for the detection of *E. amylovora* by real-time PCR was described by Salm and Geider (2004) and used primers based on sequences of the pEA29 plasmid but the sensitivity and specificity were similar to that of the conventional PCRs. For these reasons and based on a preliminary evaluation, PCR protocols designed by Pirc *et al.* (2009) were selected for test performance study evaluation in 2009 and 2010, because the primers were based in chromosomal sequences. The one indicated based on primers from sequences from *ams* gene was evaluated in 2009 and 2010 in performance studies.

1.2. 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.

1.3. The targeted genes are *amsC* (Ams assay) and 16S-23S rRNA intergenic spacer region (ITS assay). Only primers from *amsC* gene were evaluated in the tests performance study.

1.4 Oligonucleotides:

Ams116F (5'- TCCCACATACTGTGAATCATCCA -3')

Ams189R (5'- GGGTATTTGCGCTAATTTTATTTCG -3')

Ams141T (FAM-CCAGAATCTGGCCCGCGTATACCG-TAMRA)

ITS15F (5'- TGAGTAATGAGCGAGCTAAGTGAAG - 3')

ITS93R (5'- CGCAATGCTCATGGACTCAA - 3')

ITS43T (5' - FAM-AGGCGTCAGCGCGCAGCAAC-TAMRA - 3')

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

1.6 Real-time PCR system i.e. ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) using the universal cycling conditions for all amplicons (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. 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 x 50 µL of AE buffer. The protocol for extraction using QuickPick™ SML Plant DNA Kit was as follows: 100 µL of sample was mixed with 400 µL lysis buffer and 25 µL of proteinase K, incubated for 30 minutes at 65 °C and centrifuged at 6000 g for 1 minute. 300 µL of lysate was transferred to tube 1 of a KingFisher mL tube strip. Strips contained 20 µL of MagaZorb™ Magnetic Particles and 500 µL of binding buffer (tube 1), 800 µL of wash buffer (tubes 2 and 3), 100 µL of elution buffer (tube 4) and 100 µL of water (tube 5). Instrument program Total_RNA_mL_1 in KingFisherR mL was used with minor modification: binding time in well A, 3 x 1 min release plus 2 min binding; wash in well B 15s; wash in well C, 15s; 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 3, except that only 100 µL aliquots of crude sample extract were used (Pirc *et al.*, 2009).

2.2 Polymerase Chain Reaction – PCR (for protocol using Ams primers)

	Final concentration	Working concentration	Volume per reaction (µL)
Total reaction volume of a single PCR reaction in µL			10
TaqMan Universal Master Mix (Applied Biosystems)	1x	2x	5
Ams116F	0.9 µM	10 µM	0,9
Ams189R	0,9 µM	10 µM	0,9
Ams141T	0,2 µM	10 µM	0,2
DNA			2
PCR grade water			1

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, respectively.

- Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or 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 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 PCR grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid

extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

As alternative (or in addition to) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or bacterial 18S rDNA) amplification of samples spiked with exogenous nucleic 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 indicated below was obtained using the equipment/materials and chemistry used as described in this appendix.

- A sample will be considered positive if it produces a Ct value of <38 and provided that the contamination controls are negative.
- A sample will be considered negative, if it produces a Ct of 40 or more and provided that the extraction inhibition controls are positive.
- Tests should be repeated if any contradictory or unclear results are obtained, or if the Ct value is between 38 and 40.

The cycle cut off value needs to be verified in each laboratory when implementing the test for the first time.

4. Performance criteria available

Performance criteria are provided for the PCR test without enrichment

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

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

4.2. Analytical specificity data

According to Pirc *et al.* (2009)

- Target organisms tested: 235 strains all positive.
- Non-target organisms tested: 37 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 in parallel with the real-time PCR described in Pirc *et al.* (2009) by one lab 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 after a DNA extraction as indicated in Appendix 6 and to bacterial colonies, without DNA extraction.

1.3 The target sequences are located in the chromosome.

1.4 Oligonucleotides:

hpEaF (5'- CCGTGGAGACCGATCTTTTA -3')

hpEaR (5'- AAGTTTCTCCGCCCTACGAT -3')

hpEaP (FAM- TCGTCGAATGCTGCCTCTCT-MGB)

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

1.6 Real-time PCR system i.e. Eppendorf Realplex⁴ Mastercycler Epgradient S (Eppendorf, Hamburg, Germany) using the universal 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 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, Turku, Finland) 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 x 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) and Persen *et al.* (2011).

2.2 Polymerase Chain Reaction – PCR

	Final concentration	Working concentration	Volume per reaction (µL)
Total reaction volume of a single PCR reaction in µL			20
TaqMan Universal Master Mix (Applied Biosystems)	1x	2x	10
MgCl ₂	Included in Master Mix	Included in Master Mix	
dNTPs	Included in Master Mix	Included in Master Mix	

Taq polymerase	Included in Master Mix	Included in Master Mix	
forward primer	0,5µM	10µM	1
reverse primer	0,5µM	10µM	1
probe	0,05µM	1µM	1
DNA			1
PCR grade water			6

2.3. PCR cycling conditions: 3 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, respectively.

- Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or 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 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 PCR grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: *amplification* of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

As alternative (or in addition to) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or bacterial 18S rDNA) amplification of samples spiked with exogenous nucleic 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: in order to assigning results from Real-time PCR test the following criteria should be followed:

The cycle cut off value indicated below was obtained using the equipment/materials and chemistry used as described in this appendix.

- A sample will be considered positive if it produces a Ct value of <48 and provided that the contamination controls are negative.
- A sample will be considered negative, if it produces a Ct of 50 or more and provided that the extraction inhibition controls are positive.
- Tests should be repeated if any contradictory or unclear results are obtained, or if the Ct value is 48-50.
- The cycle cut off value needs to be verified in each laboratory when implementing the test for the first time.

4. Performance criteria available (from AGES, 2010)

This test was not evaluated in the test performance studies.

4.1. Analytical sensitivity data (according to Gottsberger, 2010)
2 CFU/μL

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)

The test was developed by Temple *et al.* (2007) and Temple & Johnson (2011), and was evaluated in the 2010 test performance study, because it was considered very appropriate for laboratories which do not have PCR equipment and it 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 10⁵ CFU/mL plant extract.

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'.

Primers Fip and Bip were used at 2.4 μ M, primers F and B at 0.2 μ M final concentrations. Melting temperatures for primers were between 58 to 60°C.

LAMP Reaction Mix:

	Final concentration	Working concentration	Volume per reaction (μ L)
Total reaction volume of a single PCR reaction in μ L			50
10X ThermoPol buffer*	1x	10x	5
dNTPs	1.0 mM	10 mM	5 μ L
MgSO ₄	4 mM	100mM	2 μ L
BSA	0.4 mg/ml	10 mg/mL	2 μ L
ALB FIP	2.4 μ M	100 μ M	1.2 μ L
ALB BIP	2.4 μ M	100 μ M	1.2 μ L
ALB F	0.2 μ M	10 μ M	1 μ L
ALB B	0.2 μ M	10 μ M	1 μ L
<i>Bst</i> DNA polymerase	16 U/reaction	8U/ μ L	2 μ L
DNA			5 μ L
PCR grade water			24.6 μ L

Comentario [U1]:

Prior to starting the LAMP reaction, put a water bath at 65°C or a thermal cycler at 65°C for 55 minutes. Prepare the mix and pipette 24.6 μ L of PCR grade water into each individual 0.2 ml PCR reaction tube, next pipette 18.4 μ L of the master mix into each individual PCR reaction tube, and next pipette 2 μ L of *Bst* DNA polymerase into each individual PCR reaction tube. Finally pipette 5 μ L of template DNA. Spin tubes down in a plate spinner (1000 rpm for 30 s). Place tubes in water bath (65°C) in a holder so the reaction end is submerged, or place in a thermocycler (65°C) for 55 min. Remove tubes and allow them to cool for 10 s. 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.

4 Performance criteria available

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

- 10^5 - 10^6 CFU/mL 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 as indicated below. 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 that 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 3 washings with sterile distilled water, and kept in tubes with sterile 1% agar. Maintain the plants or the 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.

Fig. 1 : Flow chart for the diagnosis of fire blight in plants with symptoms.

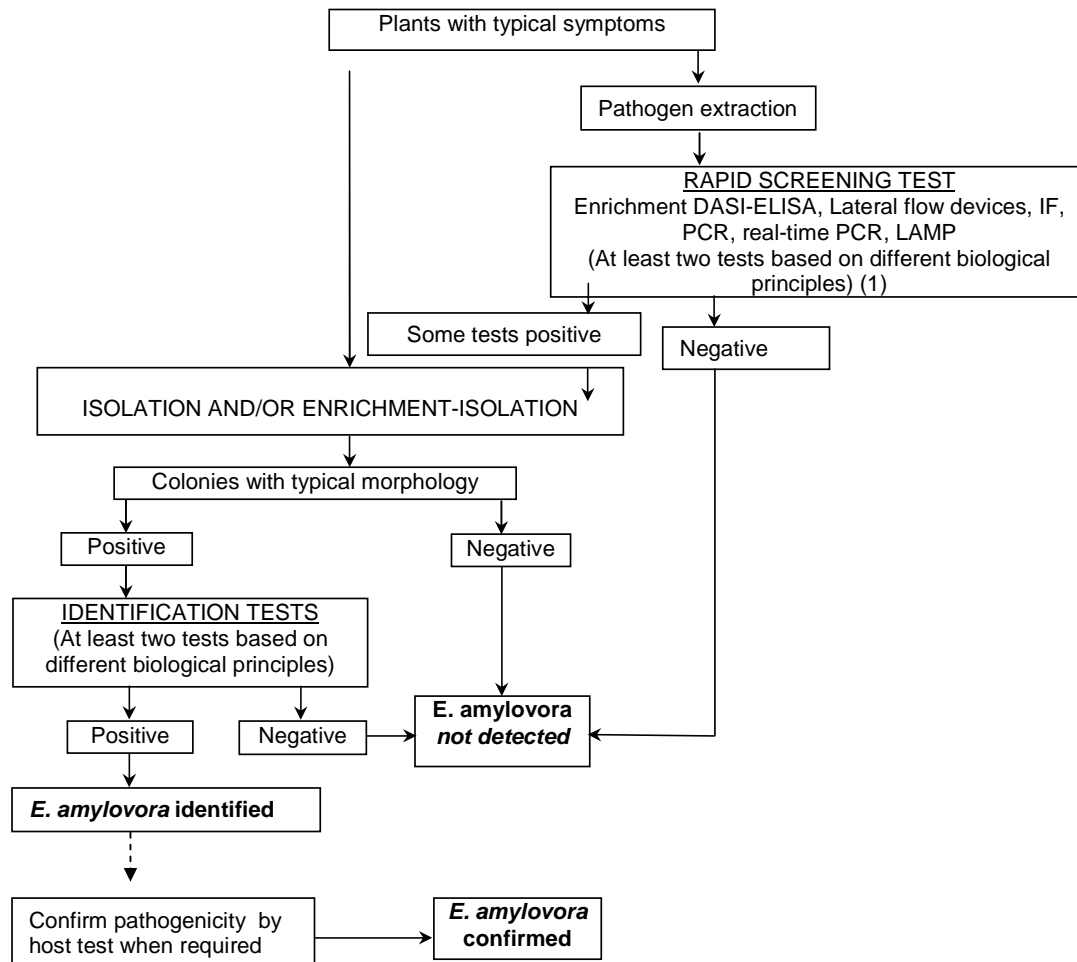


Fig. 2: Flow chart for the analysis of *Erwinia amylovora* in asymptomatic samples.

