EUROPEAN AND MEDITERRANEAN PLANT PROTECTION ORGANIZATION
ORGANISATION EUROPEENNE ET MEDITERRANEENNE POUR LA PROTECTION DES PLANTES
Summary sheet of validation data for a diagnostic test

The EPPO Standard PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* describes how validation should be conducted. It also includes definitions of performance criteria.

Laboratory contact details	National Instituite of Biology, Department of Biotechnology and Systems Biology Vecna pot 121, 1000 Ljubljana, Slovenia
Short description of the test	Real-time PCR detection of Erwinia amylovora using Ams amplicon
Date, reference of the validation report	2011-10-20 - none
Validation process according to EPPO Standard PM7/98?	yes
Is the lab accredited for this test?	no
Was the validated data generated in the framework of a project?	Euphresco
If yes, please specify	ERWINDECT
Description of the test	
Organism(s)	Erwinia amylovora(ERWIAM)
Detection / identification	detection
Method(s)	Extraction Molecular Extraction DNA RNA Molecular Extraction DNA RNA (2) Molecular Extraction DNA RNA (3) Molecular real time PCR
Method: Extraction	
Reference of the test description	
As or adapted from an EPPO diagnostic protocol	yes
EPPO Diagnostic Protocol name	PM 7/020 Erwinia amylovora (version 2)
Name of the test	PBS buffer
Other information	
Other details on the test	Extracts were prepared from symptomatic tissue; cut pieces were put into 1-2mL PBS buffer and incubated at room temperature for 10-20 minutes. Extracts were transferred to new eppendorf tubes.
Method: Molecular Extraction DNA RNA	
Reference of the test description	

As or adapted from an EPPO diagnostic protocol	yes
EPPO Diagnostic Protocol name	PM 7/020 Erwinia amylovora (version 2)
Kit	
Is a kit used	yes
Manufacturer name	QIAGEN
Specify the kit used	DNeasy Plant Mini Kit
Kit used following the manufacturer's instructions?	
Other information	
Method: Molecular Extraction DNA RNA (2)	
Reference of the test description	
Kit	
Is a kit used	yes
Manufacturer name	BIONOBILE
Specify the kit used	QuickPick Plant DNA kit
Kit used following the manufacturer's instructions?	
Other information	
Other details on the test	QuickPick SML Plant DNA kit (Bionobile) The extraction method using QuickPick SML Plant DNA kit (Bionobile) was further validated with real-time PCR with two additional experiments and calculated common LOD value
Method: Molecular Extraction DNA RNA (3)	
Reference of the test description	
As or adapted from an EPPO diagnostic protocol	yes
EPPO Diagnostic Protocol name	PM 7/020 Erwinia amylovora (version 2)
As or adapted from an IPPC diagnostic protocol	yes
IPPC diagnostic Protocol name	ISPM 27 Annex 13 DP 13: Erwinia amylovora (version 2016)
Name of the test	DNA extraction according to Llop et al. (1999)
Is the test modified compared to the reference test	no
Kit	
Is a kit used	no
Other information	
Other details on the test	modified Llop et al. 1999 (Simple DNA)
Method: Molecular real time PCR	
Reference of the test description	

As or adapted from an EPPO diagnostic protocol	yes
EPPO Diagnostic Protocol name	PM 7/020 Erwinia amylovora (version 2)
Name of the test	Real-time PCR (Pirc et al. 2009)
As or adapted from an IPPC diagnostic protocol	yes
IPPC diagnostic Protocol name	ISPM 27 Annex 13 DP 13: Erwinia amylovora (version 2016)
Kit	
Is a kit used	no
Other information	
Other details on the test	Pirc et al. using ams New test which will be included in PM 7/020(2) (approval 2012) Pirc, M., Ravnikar, M., Tomlinson, J., Dreo, T. (2009). Improved fireblight diagnostics using quantitative real-time PCR detection of Erwinia amylovora chromosomal DNA. Plant Pathology, 58(5), 872-881. EUPHRESCO final report of the project: Development and validation of innovative diagnostic tools for the detection of fire blight (Erwinia amylovora) (ERWINDECT) http://www.euphresco.org/downloadFile.cfm?id=662
Are the performance characteristics included in the EPPO diagnostic protocol?	**yes**
Performance Criteria :	
Organism 1.:	**Erwinia amylovora(ERWIAM)**
Analytical sensitivity	
What is smallest amount of target that can be detected reliably?	see full report
Diagnostic sensitivity	
Proportion of infected/infested samples tested positive compared to results from the standard test, see appendix 2 of PM 7/98	see full report
Standard test(s)	Nested PCR (Llop et al., 2000)
Analytical specificity - inclusivity	
Number of strains/populations of target organisms tested	423 strains of Erwinia amylovora were tested (list of tested strains are in Annex 1 and Annex 2)
Specificity value	
Analytical specificity - exclusivity	
Number of non-target organisms tested	97 non-target organisms or tissues (list of tested organisms and tissues are in Annex 3)
Specificity value	No cross reactivity was observed (for details see full report).
Diagnostic Specificity	

Proportion of uninfected/uninfested samples (true negatives) testing negative compared to results from a standard test	Not done
Specify the test(s)	Not done
Reproducibility	
Provide the calculated % of agreement for a given level of the pest (see PM 7/98)	Reproducibility at: - LOD concentration: Three different symptomatic samples (previously tested as negative using standard methods) were spiked at LOD level (□104 cells/ml) and aliquoted. Evaluation of reproducibility was tested in combination with QuickPick™ SML Plant DNA extraction method. Number of replicates were 7 (sample 1), 6 (sample 2) and 9 (sample 3). Sample 1: Average Cq value=33,81, SD=0,74, CV 2,21% Sample 2: Average Cq value=33,87, SD=0,82, CV 2,43% Sample 3: Average Cq value=33,68, SD=0,66, CV 1,97% 100% agreement for positive result. - Medium concentration: DNA was extracted from Erwinia amylovora suspension with medium level of concentration with QuickPick™ SML Plant DNA extraction method and aliquoted. Real- time PCR was performed 7 times. The average Cq values in all runs were 29,82 (SD 0,43 and CV 1,45%)- 100% agreement for positive results - High concentration: DNA was extracted from Erwinia amylovora suspension with high level of concentration with QuickPick™ SML Plant DNA extraction method and aliquoted. Real- time PCR was performed 7 times. The average Cq values in all runs were 23,05 (SD 0,44 and CV 1,92%)- 100% agreement for positive results
Repeatability	
Provide the calculated % of agreement for a given level of the pest (see PM 7/98)	As we got very reproducible results in all tested concentrations (low/medium/high) in combination of different days and operators we didn't test this separately.
Test performance study	
Test performance study?	yes
Brief details of the test performance study and its output.It available, link to published article/report	1. Ring test 2009 (4 partners - Final report available on http://www.euphresco.org/downloadFile.cfm?id=473.) 2. Ring test 2010 (14 laboratories - Final report is not available yet) In year 2009 real time PCR with Ams amplicon was first included in ring test among four project partners in Euphresco Erwindect project (final report of the project is available on webpage: http://www.euphresco.org/downloadFile.cfm?id=662. To standardize the procedure of real time PCR as much as possible all chemicals were provided to all partners. For real time PCR different instruments were used among partners and different reaction volumes. Instruments and PCR condition used by different partners in real time PCR analysis are shown in table 4.3.2.6 in final report. The ring test was composed of two parts: Part 1: Testing of the DNA

	isolated by the partner 4 (QuickPick™ SML Plant DNA extraction method) Results of the four laboratories were in high accordance (Table 4.2.3.7 in final report). No template control and DNA extracted from E. amylovora negative plant material gave negative results in all laboratories. The lowest concentration of target bacteria tested, corresponding to 1.8 x 10E3 cfu/mL plant extract was positive in all cases with minimum two out of three parallel reactions positive. Higher concentrations of E. amylovora were detected in all cases. This corresponds to specificity and sensitivity values of 1 with 0 rates of false positive and false negative results. Part 2: Each partner (4) prepared their own non-spiked and spiked samples (final concentrations: 107, 106, 104, 103, 102 cfu/ml) using plant material sent out by Partner 2 and bacterial suspension of E. amylovora strain CFBP 1430. DNA from this material was isolated using modified Taylor DNA extraction. Sensitivity in spiked pear sample was 10E3 cfu/ml, as expected from preliminary studies and theoretical limits of real time PCR method. On spiked plant samples, prepared by ring-test partners, real time PCR had analytical sensitivity of 0.67 and specificity of 0.83. False positive rate was 0.17, most likely due to contaminations during preparation of spiked samples especially as in one case also classical PCR was positive. It is generally accepted that the rooms for preparation of samples, extraction of DNA and real time PCR reaction preparation should be separated this could not be guaranteed by all ringtest partners. Based on experience of using real time PCR in routine analyses it is expected that by using proper procedure to minimize the risk of cross-contaminations the level of false positive results can be greatly reduced. False negative rate - do we report it above LOD, this would be more informative was 0.33, due to samples with concentrations below theoretical and practical sensitivity of the method. While symptomatic fire-blight samples with such low concentrations are not expected this the sensitivity can be further increased by analyzing a bigger volume of sample DNA. In year 2010 real time PCR with Ams amplicon was included in ring test among 14 laboratories (AGES, Austria; CDR, Spain; IVIA, Spain; LRBV, Spain; LNPV, France; FS, Marocco; INRA, Marooco; NAK, The Netherlands; PD, The Netherland; MAF, New Zealand; RCPQ, Russia, NIB, Slovenia; UBFA, Serbia; OSU, USA). - Final report is not available yet .
Other information	
Any other information considered useful	Real – time PCR were in ring test 2009 performed on different PCR cycling instruments and reaction valume. Also different software fore signal acquisition and analysis were used. Despite differences Ct values, efficiency of mplification and theoretical cycle threshold value for one copy

	detection were comparable in most cases (see in ERWINDECT final report Fig 4.3.2.1). Real – time PCR (Ams assay) were transfered also to SmartCycler_ instrument. Comparison of analytical sensitivity was performed. All results are available in publication Tanja Dreo, Manca Pirc and Maja Ravnikar ; Real-time PCR, a method fit for detection and quantification of Erwinia amylovora ; http://www.springerlink.com/content/x27m424n1r9m73v8/
The following complementary files are available online:	• Erwinia amylovora Real-time PCR AMs amplicon full report

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