EUROPEAN AND MEDITERRANEAN PLANT PROTECTION ORGANIZATION
ORGANISATION EUROPEENNE ET MEDITERRANEENNE POUR LA PROTECTION DES PLANTES
(11-17239)
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.

Target Organism	Erwinia amylovora	
Short description	Real-time PCR detection of Erwinia amylovora using Ams amplicon	
Laboratory contact details	National Institiute of Biology, Department of Biotechnology and Systems Biology Vecna pot 121, 1000 Ljubljana, Slovenia	
Date and reference of the validation report	2011-10-20 - none	
Validation process according to EPPO Standard PM 7/98:	Yes	
Reference of the test description	PM 7/020(1) 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	
Is the test the same as described in the EPPO DP?	Yes	
Is the lab accredited for this test?	No	
Plant species tested (if relevant)	Different plant species that are host for Erwinia amylovora (Malus domestica, Pyrus communis, Cydonia oblonga, Cotoneaster sp., Chaenomeles sp., Crategus sp., Pyracantha sp., Sorbus sp.,)	
Matrices tested (if relevant)	Symptomatic extracts were prepared from different plant parts with symptoms (subcortical tissues from twigs, leaves and fruits)	
List of methods used		
Method for extraction / isolation / baiting of target organism from matrix	X	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. Three different extraction methods were performed and evaluated with real – time PCR 1- modified Llop et al. 1999 (Simple DNA) 2 – Dneasy Plant Mini Kit (Qiagen) 3 – 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
Molecular methods, e.g. hybridization, PCR and real time PCR	X	Real time PCR using Ams amplicon
Serological methods: IF, ELISA, Direct Tissue Blot Immuno Assay		
Plating methods: selective isolation		
Bioassay methods: selective enrichment in host plants, baiting, plant test and grafting.		
Pathogenicity test		
Fingerprint methods: protein profiling, fatty acid profiling & DNA profiling		
Morphological and morphometrical methods intended for identification		
Biochemical methods: e.g. enzyme electrophoresis, protein profiling		
Other		
<u>Analytical sensitivity (= limit of detection)</u>		
What is smallest amount of target that can be detected reliably?	see full report	
<u>Diagnostic sensitivity</u>		
Proportion of infected/infested samples tested positive compared to results from the standard test , see appendix 2 of PM 7/98	see full report	
Specify the standard test	Nested PCR (Llop et al., 2000)	
<u>Analytical specificity</u>		
Specificity value		
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)	
Number of non-target organisms tested	97 non-target organisms or tissues (list of tested organisms and tissues are in Annex 3)	
Cross reacts with (specify the species)	No cross reactivity was observed (for details see full report).	
<u>Diagnostic Specificity</u>		
Proportion of uninfected/uninfested samples (true negatives) testing negative compared to results from a standard test	Not done	
Specify the standard test	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
Include brief details of the test performance study and its output.It available, provide a 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 ring-test 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 .
<u>Other information</u>	
Any other information considered useful **e.g. robustness, ease of performing the test, etc.**	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. Despide differences Ct values, efficiency of amplification 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:	• Detailed information on the validation of the Real-time PCR detection of Erwinia amylovora using Ams amplicon