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	Detection of FD and BN by real time PCR
Date, reference of the validation report	2011-12-30 - NIB; Report on suitability testing: Validation report on testing of FD and BN phytoplasma in grapevine samples using real time PCR (Record number: D0022/11)
Validation process according to EPPO Standard PM7/98?	yes
Is the lab accredited for this test?	yes
Was the validated data generated in the framework of a project?	
Description of the test	
Organism(s)	Grapevine flavescence dorée phytoplasma (PHYP64) 'Candidatus Phytoplasma solani' (PHYPSO)
Detection / identification	detection
Method(s)	Molecular Extraction DNA RNA Molecular real time PCR
Method: Molecular Extraction DNA RNA	
Reference of the test description	
As or adapted from an EPPO diagnostic protocol	yes
EPPO Diagnostic Protocol name	PM 7/079 Grapevine flavescence dorée phytoplasma (version 2)
As or adapted from an IPPC diagnostic protocol	no
Other information	
Method: Molecular real time PCR	
Reference of the test description	
As or adapted from an EPPO diagnostic protocol	yes
EPPO Diagnostic Protocol name	PM 7/079 Grapevine flavescence dorée

	phytoplasma (version 2)
Name of the test	Real-time PCR according to Hren et al. (2007)
As or adapted from an IPPC diagnostic protocol	no
Other information	
Other details on the test	Presented are validation data for diagnosis of FD and BN using Real Time PCR with two sets of oligonucleotide primers and probes specific for FD and BN, respectively, and one set of oligonucleotide primers and probes universal for phytoplasma. The methodology is published by Hren et al. (2007). Validation was performed on ABI PRISM 7900 HT apparatus using 7900 HT Fast Sequence Detection System (Applied Biosystems), and chemical reagents from 1x TaqMan Universal PCR Master Mix (Applied Biosystems).
Are the performance characteristics included in the EPPO diagnostic protocol?	**yes**
Performance Criteria :	
Organism 1.:	**Grapevine flavescence dorée phytoplasma(PHYP64)**
Analytical sensitivity	
What is smallest amount of target that can be detected reliably?	Determined using nonlinear model (programming environment R) based on the Cq value of the BN dilutions (BN amplicon) and FD dilutions (FD and Uni amplicons) in a positive grapevine sample. The following dilutions of the sample DNA were tested per amplicons (in parenthesis: the average Cq value at 10^0): Uni (20.68): non-diluted up to 24.3x10^5 x diluted; FD (24.02): non-diluted up to 24.3x10^5 x diluted; BN (26.30): non-diluted up to 24.3x10^4 x diluted. Conclusion: Real Time PCR analitical sensitivity for amplicons that are included for FD and BN phytoplasma diagnostic in grapevine samples is represented as Cq values with 95% probability of detection for the amplicons as follows (in parenthesis: the dilutions of the sample DNA at calculated Cq values): Uni: 35.55 (10^4 < x < 3x10^4); FD: 35.71 (10^3 < x < 10^4); BN: 37.84 (3x10^3 < x < 9x10^3).
Diagnostic sensitivity	
Proportion of infected/infested samples tested positive compared to results from the standard test, see appendix 2 of PM 7/98	Determined in 153 grapevine samples and compared with a standard diagnostic method (nested PCR): FD amplicon: 100%; BN amplicon: 99%; FD+BN+Uni amplicon: 98%. Additionally, comparison was done using nested PCR method on an EY1 (FD) - and SE (BN) - positive sample in series of 10x dilutions (DNA isolated from a healthy grapevine sample spiked with target phytoplasma DNA). The experiment was repeated twice (two sets of independently prepared series of dilutions). Results for BN: Nested PCR: last positive signal was obtained at the 10^6 dilution (a weak band

	occurred in both sets of dilutions); Real Time PCR: last positive signal was obtained at the 10^6 dilution (Ct 34.6 in first, and Ct 35.8 in a second set of dilutions). Results for FD: Nested PCR: last positive signal was obtained at the 10^3 dilution (weak band in a first set of dilutions); Real Time PCR: last positive signal was obtained at 10^6 dilution (Ct 37.3 in a first set of dilutions). Results of the comparison of the two methods for a second set of dilutions: last positive signal with Real Time PCR was obtained at the 10^6 dilution, while with nested PCR it was obtained at the 10^7 dilution. Conclusion: diagnostic sensitivity of the Real Time PCR method is comparable to a sensitivity of the nested PCR method.
Standard test(s)	Nested PCR for BN: PCR with STOL11f2/r1 primer pair (Daire et al., 1997) followed by a nested PCR reaction with STOL11f3/r2 (Clair et al., 2003). Nested PCR for FD: PCR with FD9f/r primer pair (Daire et al., 1997) followed by a nested PCR reaction with FD9f3b/r2 (Clair et al., 2003).
Analytical specificity - inclusivity	
Number of strains/populations of target organisms tested	BN amplicon: 100; FD amplicon: 10; Uni amplicon: 17.
Specificity value	Percentage of accurate results: Uni: 98,8%; FD: 100%; BN: 100%. Percentage of false positives: Uni: 1,6%; FD: 0%; BN: 0%. Percentage of false negatives: Uni: 0%; FD: 0%; BN: 0%. Explanation in regard of specificity of the amplicons (Hren et al., 2007): a) BN gene (referred as BN): Stolbur group (16SrXII) (includes BN phytoplasma); primers and probe binding occurs to a Stoll11 genome fragment. b) FD gene (further referred as FD): Elm yellows group (16SrV) (includes FD phytoplasma); primers and probe binding occurs to a FD9 genome fragment (sec Y gene). c) UniRNA (further referred as Uni): Phytoplasmas; primer and probe binding to 16S rRNA. In FD and BN phytoplasma diagnosis, Uni amplicon is used to confirm a positive result of the FD amplicon. Uni amplicon amplifies FD and BN phytoplasma, and also other phytoplasma from various groups (e.g. phytoplasma groups 16SrI, 16SrIII in 16SrX). Uni amplicon shows a clear preferrence for FD phytoplasma (the difference in Cq value between amplicons Uni and FD typically falls between -1 and -4). When BN is present in grapevine samples, the Cq value of Uni amplicon is for at least 4 cycles higher than the Cq value of BN amplicon (Hren et al., 2007). Consequently, the samples that test positive only for BN do not generate a positive signal when using only Uni amplicon. On the other hand, when a grapevine sample does test positive with Uni amplicon only, it can be concluded that it is infected with a type of phytoplasma other than FD or BN, or the FD phytoplasma is present in the sample in a very low concentration, or there are inhibitors present in the

	sample that have a stronger effect on the specific amplicon than on the Uni amplicon.
Analytical specificity - exclusivity	
Number of non-target organisms tested	BN amplicon: 79; FD amplicon: 169; Uni amplicon: 63.
Specificity value	Cross reactivity was tested using: other phytoplasmas that are kept in C.roseus (AY, WX, AP, PD, ESFY, MA); various grapevine cultivars showing no disease symptoms; 5 + 34 bacterial isolates that could be present in grapevine as epiphytes or sprophytes; phytoplasmas that are kept in C.roseus: EY1 (Elm yellows group) /SE (Stolbur group); BN/FD – infected grapevine (different cultivars). Conclusion: There is no cross reactivity with either other phytoplasmas nor with healthy grapevine. FD gene and BN gene do not cross react with any of the 39 bacterial isolates. Uni RNA cross reacted with only 1 out of the 39 bacterial isolates (Cq of 34.2 for more than 10^5 copies of genome per reaction). In addition, 'in silico' analysis of BN and FD amplicons indicated no significant sequence homology with non targets.
Diagnostic Specificity	
Proportion of uninfected/uninfested samples (true negatives) testing negative compared to results from a standard test	Determined in 153 grapevine samples and compared with a standard diagnostic method (nested PCR): FD amplicon: 100%; BN amplicon: 78%*; FD+BN+Uni amplicon: 63%*. *The results indicate that the Real Time PCR method is more sensitive than the nested PCR method since the presence of phytoplasma was detected in 14 more grapevine samples using Real Time PCR than using nested PCR method. Notably, 13 out of 14 grapevine samples were showing weak phytoplasma disease symptoms (1 sample tested positive using Real Time PCR only, and did not show any phytoplasma disease symptoms).
Specify the test(s)	Nested PCR for BN: PCR with STOL11f2/r1 primer pair (Daire et al., 1997) followed by a nested PCR reaction with STOL11f3/r2 (Clair et al., 2003). Nested PCR for FD: PCR with FD9f/r primer pair (Daire et al., 1997) followed by a nested PCR reaction with FD9f3b/r2 (Clair et al., 2003).
Reproducibility	
Provide the calculated % of agreement for a given level of the pest (see PM 7/98)	Percentage of identical results: 100% (test results were always positive for positive samples). Nr. of samples per 1 amplicon: 2; nr. of operators: 2; nr. of devices: 2; nr. of parallels per 1 sample: 14-29; for details see complementary file ""Validation report..."".
Repeatability	
Provide the calculated % of agreement for a given level of the pest (see PM 7/98)	There is a high repeatability for all three amplicons (100% in all samples with Cq value that is for 3 lower than LOD). Nr. of samples per 1 amplicon: 3 (high, medium, low); nr. of parallels per 1 sample:

	1-3 x 5; for details see complementary file ""Validation report..."".
Organism 2.:	**'Candidatus Phytoplasma solani'(PHYPSO)**
Analytical sensitivity	
What is smallest amount of target that can be detected reliably?	Determined using nonlinear model (programming environment R) based on the Cq value of the BN dilutions (BN amplicon) and FD dilutions (FD and Uni amplicons) in a positive grapevine sample. The following dilutions of the sample DNA were tested per amplicons (in parenthesis: the average Cq value at 10^0): Uni (20.68): non-diluted up to 24.3x10^5 x diluted; FD (24.02): non-diluted up to 24.3x10^5 x diluted; BN (26.30): non-diluted up to 24.3x10^4 x diluted. Conclusion: Real Time PCR analitical sensitivity for amplicons that are included for FD and BN phytoplasma diagnostic in grapevine samples is represented as Cq values with 95% probability of detection for the amplicons as follows (in parenthesis: the dilutions of the sample DNA at calculated Cq values): Uni: 35.55 (10^4 < x < 3x10^4); FD: 35.71 (10^3 < x < 10^4); BN: 37.84 (3x10^3 < x < 9x10^3).
Diagnostic sensitivity	
Proportion of infected/infested samples tested positive compared to results from the standard test, see appendix 2 of PM 7/98	Determined in 153 grapevine samples and compared with a standard diagnostic method (nested PCR): FD amplicon: 100%; BN amplicon: 99%; FD+BN+Uni amplicon: 98%. Additionally, comparison was done using nested PCR method on an EY1 (FD) - and SE (BN) - positive sample in series of 10x dilutions (DNA isolated from a healthy grapevine sample spiked with target phytoplasma DNA). The experiment was repeated twice (two sets of independently prepared series of dilutions). Results for BN: Nested PCR: last positive signal was obtained at the 10^6 dilution (a weak band occurred in both sets of dilutions); Real Time PCR: last positive signal was obtained at the 10^6 dilution (Ct 34.6 in first, and Ct 35.8 in a second set of dilutions). Results for FD: Nested PCR: last positive signal was obtained at the 10^3 dilution (weak band in a first set of dilutions); Real Time PCR: last positive signal was obtained at 10^6 dilution (Ct 37.3 in a first set of dilutions). Results of the comparison of the two methods for a second set of dilutions: last positive signal with Real Time PCR was obtained at the 10^6 dilution, while with nested PCR it was obtained at the 10^7 dilution. Conclusion: diagnostic sensitivity of the Real Time PCR method is comparable to a sensitivity of the nested PCR method.
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Analytical specificity - inclusivity	
Number of strains/populations of target organisms tested	BN amplicon: 100; FD amplicon: 10; Uni amplicon: 17.
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Analytical specificity - exclusivity	
Number of non-target organisms tested	BN amplicon: 79; FD amplicon: 169; Uni amplicon: 63.
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Reproducibility	
Provide the calculated % of agreement for a given level of the pest (see PM 7/98)	Percentage of identical results: 100% (test results were always positive for positive samples). Nr. of samples per 1 amplicon: 2; nr. of operators: 2; nr. of devices: 2; nr. of parallels per 1 sample: 14-29; for details see complementary file ""Validation report..."".
Repeatability	
Provide the calculated % of agreement for a given level of the pest (see PM 7/98)	There is a high repeatability for all three amplicons (100% in all samples with Cq value that is for 3 lower than LOD). Nr. of samples per 1 amplicon: 3 (high, medium, low); nr. of parallels per 1 sample: 1-3 x 5; for details see complementary file ""Validation report..."".
Test performance study	
Test performance study?	yes
Brief details of the test performance study and its output.It available, link to published article/report	Participation in inter-laboratory method evaluation (»Ring test detection of grapevine phytoplasmas – Flavescence doree and Bois noir«, organized by the French national plant protection laboratory, LNPV). Herein discussed diagnostic protocol is comparable with the current French diagnostic protocol (Real Time PCR, published by Pelletier et al., 2009).
Other information	
Any other information considered useful	In time period between 2006-2011 1581 grapevine samples were tested using this method. There was no observed impact of grapevine cultivars on the test results. BN was confirmed using Real Time PCR in 36 different grapevine cultivars, while FD was confirmed in 15 different grapevine cultivars. Specificity of the Real Time PCR method for

	diagnosis of FD phytoplasma in respect of results from its RFLP and/or sequencing of PCR products: 118 (92,2%) out of 128 grapevine samples that tested FD-positive using Real Time PCR method, also tested FD-positive using PCR-RFLP and/or sequencing of the FD9 gene sequence. We could not confirm a nested PCR result with RFLP analysis in 9 samples tested with nested PCR method because there was no PCR reaction product obtained (most probable explanation: nested PCR is less sensitive than Real Time PCR (see Figure 4 in Hren et al., 2007). In one sample we did not confirm FD phytoplasma but did confirm ALY phytoplasma, which is not arguable from the molecular point of view where ALY isolates could show greater similarity with FD isolates than the similarity among various FD phytoplasma genetic groups; moreover, the role of ALY in FD phytoplasma epidemiology is not excluded (Angelini et al., 2001, Amaud et al., 2007). Data about suitability of DNA extraction are available in complementary file ""Validation report..."". For identification of critical points and estimation of the uncertainty of measurement see complementary file ""Identification of critical points..."".
The following complementary files are available online:	• Identification of critical points and estimation of the uncertaintly of measurement • Validation report on testing of FD and BN phytoplasma in grapevine samples using Real Time PCR

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