**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.

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| **Laboratory contact details** | National Instituite of Biology, Department of Biotechnology and Systems Biology<br>Vecna pot 121, 1000 Ljubljana, Slovenia |
| **Short description of the test** | Detection of ‘Candidatus Phytoplasma mali’, ‘Ca. P. pyri’ and ‘Ca. P. prunorum’ by real-time PCR |
| **Date, reference of the validation report** | 2014-05-28 - Validation report on the testing of phytoplasma of the apple proliferation group using real time PCR |
| **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?** | |
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| **Description of the test** | |
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| **Organism(s)** | 'Candidatus Phytoplasma prunorum' (PHYPPR)<br>'Candidatus Phytoplasma mali' (PHYPMA)<br>'Candidatus Phytoplasma pyri' (PHYPPY) |
| **Detection / identification** | detection |
| **Method(s)** | Molecular Extraction DNA RNA<br>Molecular real time PCR |
| **Method: Molecular Extraction DNA RNA** | |
| ***Reference of the test description*** | |
| **As or adapted from an EPPO diagnostic protocol** | no |
| **As or adapted from an IPPC diagnostic protocol** | no |
| **Reference of the test** | MEHLE, Nataša, NIKOLIC, Petra, RUPAR, Matevž, BOBEN, Jana, RAVNIKAR, Maja, DERMASTIA, Marina. 2013. Automated DNA extraction for large numbers of plant samples. In: DICKINSON, Matthew (ed.), HODGETTS, Jennifer (ed.). Phytoplasma: methods and protocols, (Methods in Molecular Biology, vol. 938), (Springer Protocols). New York: Humana Press: 139-145. |
| ***Kit*** | |
| **Is a kit used** | no |

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| ***Other information*** | |
| **Method: Molecular real time PCR** | |
| ***Reference of the test description*** | |
| **As or adapted from an EPPO diagnostic protocol** | no |
| **As or adapted from an IPPC diagnostic protocol** | no |
| **Reference of the test** | NIKOLIC, Petra, MEHLE, Nataša, GRUDEN, Kristina, RAVNIKAR, Maja, DERMASTIA, Marina. 2010. A panel of real-time PCR assays for specific detection of three phytoplasmas from the apple proliferation group. Mol. cell. probes, 24, 5: 303-309. MEHLE, Nataša, NIKOLIC, Petra, GRUDEN, Kristina, RAVNIKAR, Maja, DERMASTIA, Marina. 2013. Real-time PCR for specific detection of three phytoplasmas from the apple proliferation group. In: DICKINSON, Matthew (ed.), HODGETTS, Jennifer (ed.). Phytoplasma : methods and protocols, (Methods in Molecular Biology, vol. 938), (Springer Protocols). New York: Humana Press: 269-281. |
| **Is the test modified compared to the reference test** | no |
| ***Kit*** | |
| **Is a kit used** | no |
| ***Other information*** | |
| **Are the performance characteristics included in the EPPO diagnostic protocol?** | **yes** |
| **Performance Criteria :** | |
| **Organism 1.:** | **'Candidatus Phytoplasma prunorum'(PHYPPR)** |
| **Analytical sensitivity** | |
| **What is smallest amount of target that can be detected reliably?** | Not applicable (calculated values (maximum dilution of DNA detected) are a guide and are not used for diagnostic purposes). Dilutions of sample DNA for amplicons (in parentheses is given the average Cq value at 10^1): ESFY (25.7): 10^1 x diluted up to 10^7 x diluted; Cq value of 0.95 for detection probability for amplicons (in parentheses is given dilution of sample DNA): ESFY: 33.2 (10^3<x<10^4); Dilutions of sample DNA for amplicons (in parentheses is given the average Cq value at 10^1): AP (22.5): 10^1 x diluted up to 10^7 x diluted; Cq value of 0.95 for detection probability for amplicons (in parentheses is given dilution of sample DNA): AP: 34.1 (10^4<x<10^5); |
| **Diagnostic sensitivity** | |
| **Proportion of infected/infested samples tested positive compared to results from the standard test, see appendix 2 of PM 7/98** | The sensitivity of the nested PCR (f01/r01) and qPCR for each phytoplasma type were compared. Positive DNA sample was 10-fold serially diluted in water. Two independent studies revealed that the |

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| | detection sensitivity of qPCR for all of the three phytoplasma species was higher than of nested PCR. Additionally, field fruit tree samples were analysed using qPCR and with nested PCR. Correlation between a nested PCR (f01/r01) and qPCR: ESFY (no. of samples: 92): 100%. |
| **Standard test(s)** | nested PCR (f01/r01) + RFLP |
| **Analytical specificity - inclusivity** | |
| **Number of strains/populations of target organisms tested** | ESFY: 50 |
| **Specificity value** | Percentage of accurate results: AP: 100%; Percentage of false positives: AP: 0%; Percentage of false negatives: AP: 0%; The specificities of all three of the qPCR sets of primers/ probes were determined by testing cross-reactivity with the following: - 17 DNA samples of phytoplasma strains cultivated in C. roseus and Pennisetum purpureum; - DNA from two field samples of Vitis vinifera, diagnostically predetermined to be infected with the Flavescence dorée and Bois noir phytoplasma; - DNA extracted from leaf veins or root phloem tissue of symptomatic and asymptomatic fruit trees (total of 241 samples of fruit trees); - several pathogenic bacteria isolates and bacterial isolates that can be present as epiphytes or saprophytes on the fruit trees (total of 28 isolates). The testing of the range of targets and non-targets provided the following data. The AP assay consistently detected only DNA from ‘Ca. P. mali’; the ESFY assay only detected DNA of ‘Ca. P. prunorum’; and the PD assay only detected DNA from ‘Ca. P. pyri’; regardless of the source of DNA (periwinkle or fruit tree). There was no cross-reactivity with DNA from any unrelated phytoplasma reference strains or isolated from field-grown fruit-tree material. Similarly, the possibility of cross-reactivity with non-phytoplasma DNA was excluded after the assays applied did not give any positive amplification signal using DNA from four common bacterial pathogens or from 24 non-identified bacterial isolates from fruit trees. 'In silico' analysis: The NCBI BLAST analyses confirmed the specificity of the AP probe for ‘Ca. P. mali’, the ESFY assay for ‘Ca. P. prunorum’, and the PD assay for ‘Ca. P. pyri’. The alignment of all of the publically available sequences for ‘Ca. P. mali’, ‘Ca. P. prunorum’ and ‘Ca. P. pyri’ revealed some mismatches within the probe sequences. However, those isolates have not been tested yet with the designed protocol, and thus its ability to recognize these phytoplasma cannot be ruled out completely. |
| **Analytical specificity - exclusivity** | |
| **Number of non-target organisms tested** | ESFY: 178. |
| **Specificity value** | |
| **Diagnostic Specificity** | |

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| **Proportion of uninfected/uninfested samples (true negatives) testing negative compared to results from a standard test** | ESFY (no. of samples: 92): 97.8%. A lower percentage of diagnostic specificity of the qPCR is due to a higher sensitivity of qPCR method than nested PCR method. |
| **Specify the test(s)** | nested PCR (f01/r01) + RFLP |
| **Reproducibility** | |
| **Provide the calculated % of agreement for a given level of the pest (see PM 7/98)** | Percentage of identical results is 100% at least in samples with estimated high or medium phytoplasma amount. No. of samples per 1 amplicon: 2; No. of operators: 5 ; No. of devices: 2. ESFY (mean Cq value: 27.30; No. of qPCR runs: 37; STDEV (Cq): 0.28): 100% (positive in all qPCR runs); ESFY (mean Cq value: 31.82; No. of qPCR runs: 37; STDEV (Cq): 0.64): 100% (positive in all qPCR runs); |
| **Repeatability** | |
| **Provide the calculated % of agreement for a given level of the pest (see PM 7/98)** | Repeatability for all three assays is 100% in all samples with Cq values that are 3 Cq lower than LOD. ESFY (average Cq: 23-27.2; No. of parallels: 5x5; STD (Cq): 0.05-0.11): 100% positive parallels; ESFY (average Cq: 27.2-30.2; No. of parallels: 2x5; STD (Cq): 0.11-0.20): 100% positive parallels; ESFY (average Cq: 30.2-33.2*; No. of parallels: 4x5; STD (Cq): 0.20-0.37): 100% positive parallels; *Cq LOD(95%) |
| **Organism 2.:** | **'Candidatus Phytoplasma mali'(PHYPMA)** |
| **Analytical sensitivity** | |
| **What is smallest amount of target that can be detected reliably?** | Not applicable (calculated values (maximum dilution of DNA detected) are a guide and are not used for diagnostic purposes). Dilutions of sample DNA for amplicons (in parentheses is given the average Cq value at 10^1): AP (22.5): 10^1 x diluted up to 10^7 x diluted; Cq value of 0.95 for detection probability for amplicons (in parentheses is given dilution of sample DNA): AP: 34.1 (10^4<x<10^5); |
| **Diagnostic sensitivity** | |
| **Proportion of infected/infested samples tested positive compared to results from the standard test, see appendix 2 of PM 7/98** | The sensitivity of the nested PCR (f01/r01) and qPCR for each phytoplasma type were compared. Positive DNA sample was 10-fold serially diluted in water. Two independent studies revealed that the detection sensitivity of qPCR for all of the three phytoplasma species was higher than of nested PCR. Additionally, field fruit tree samples were analysed using qPCR and with nested PCR. Correlation between a nested PCR (f01/r01) and qPCR: AP (no. of samples: 62): 100%; |
| **Standard test(s)** | nested PCR (f01/r01) + RFLP |
| **Analytical specificity - inclusivity** | |
| **Number of strains/populations of target organisms tested** | AP: 69 |

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| **Specificity value** | Percentage of accurate results: AP: 100%; Percentage of false positives: AP: 0%; Percentage of false negatives: AP: 0%; The specificities of all three of the qPCR sets of primers/ probes were determined by testing cross-reactivity with the following: - 17 DNA samples of phytoplasma strains cultivated in C. roseus and Pennisetum purpureum; - DNA from two field samples of Vitis vinifera, diagnostically predetermined to be infected with the Flavescence dorée and Bois noir phytoplasma; - DNA extracted from leaf veins or root phloem tissue of symptomatic and asymptomatic fruit trees (total of 241 samples of fruit trees); - several pathogenic bacteria isolates and bacterial isolates that can be present as epiphytes or saprophytes on the fruit trees (total of 28 isolates). The testing of the range of targets and non-targets provided the following data. The AP assay consistently detected only DNA from ‘Ca. P. mali’; the ESFY assay only detected DNA of ‘Ca. P. prunorum’; and the PD assay only detected DNA from ‘Ca. P. pyri’; regardless of the source of DNA (periwinkle or fruit tree). There was no cross-reactivity with DNA from any unrelated phytoplasma reference strains or isolated from field-grown fruit-tree material. Similarly, the possibility of cross-reactivity with non-phytoplasma DNA was excluded after the assays applied did not give any positive amplification signal using DNA from four common bacterial pathogens or from 24 non-identified bacterial isolates from fruit trees. 'In silico' analysis: The NCBI BLAST analyses confirmed the specificity of the AP probe for ‘Ca. P. mali’, the ESFY assay for ‘Ca. P. prunorum’, and the PD assay for ‘Ca. P. pyri’. The alignment of all of the publically available sequences for ‘Ca. P. mali’, ‘Ca. P. prunorum’ and ‘Ca. P. pyri’ revealed some mismatches within the probe sequences. However, those isolates have not been tested yet with the designed protocol, and thus its ability to recognize these phytoplasma cannot be ruled out completely. |
| **Analytical specificity - exclusivity** | |
| **Number of non-target organisms tested** | AP: 154 |
| **Specificity value** | |
| **Diagnostic Specificity** | |
| **Proportion of uninfected/uninfested samples (true negatives) testing negative compared to results from a standard test** | AP (no. of samples: 62): 100%; A lower percentage of diagnostic specificity of the qPCR is due to a higher sensitivity of qPCR method than nested PCR method. |
| **Specify the test(s)** | nested PCR (f01/r01) + RFLP |
| **Reproducibility** | |
| **Provide the calculated % of agreement for a given level of the pest (see PM 7/98)** | Percentage of identical results is 100% at least in samples with estimated high or medium phytoplasma amount. No. of samples per 1 amplicon: 2; No. of operators: 4 No. of devices: 1 |

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| | AP (mean Cq value: 28.37; No. of qPCR runs: 45; STDEV (Cq): 0.27): 100% (positive in all qPCR runs); AP (mean Cq value: 33.65; No. of qPCR runs: 45; STDEV (Cq): 0.81): 100% (positive in all qPCR runs); |
| **Repeatability** | |
| **Provide the calculated % of agreement for a given level of the pest (see PM 7/98)** | Repeatability for all three assays is 100% in all samples with Cq values that are 3 Cq lower than LOD. AP (average Cq: 22-28.1; No. of parallels: 5x5; STD (Cq): 0.05-0.08): 100% positive parallels; AP (average Cq: 28.1-31.1; No. of parallels: 3x5; STD (Cq): 0.07-0.38): 100% positive parallels; AP (average Cq: 31.1-34.1*; No. of parallels: 3x5; STD (Cq): 0.30-0.77): 100% positive parallels; *Cq LOD(95%) |
| **Organism 3.:** | **'Candidatus Phytoplasma pyri'(PHYPPY)** |
| **Analytical sensitivity** | |
| **What is smallest amount of target that can be detected reliably?** | Not applicable (calculated values (maximum dilution of DNA detected) are a guide and are not used for diagnostic purposes). Dilutions of sample DNA for amplicons (in parentheses is given the average Cq value at 10^1): PD (25.7): 10^1 x diluted up to 10^6 x diluted. Cq value of 0.95 for detection probability for amplicons (in parentheses is given dilution of sample DNA): PD: 37.8 (10^4<x<10^5). |
| **Diagnostic sensitivity** | |
| **Proportion of infected/infested samples tested positive compared to results from the standard test, see appendix 2 of PM 7/98** | The sensitivity of the nested PCR (f01/r01) and qPCR for each phytoplasma type were compared. Positive DNA sample was 10-fold serially diluted in water. Two independent studies revealed that the detection sensitivity of qPCR for all of the three phytoplasma species was higher than of nested PCR. Additionally, field fruit tree samples were analysed using qPCR and with nested PCR. Correlation between a nested PCR (f01/r01) and qPCR: PD (no. of samples: 44): 100%; |
| **Standard test(s)** | nested PCR (f01/r01) + RFLP |
| **Analytical specificity - inclusivity** | |
| **Number of strains/populations of target organisms tested** | PD: 31. |
| **Specificity value** | Percentage of accurate results: PD: 100%. Percentage of false positives: PD: 0%. Percentage of false negatives: PD: 0%. The specificities of all three of the qPCR sets of primers/ probes were determined by testing cross-reactivity with the following: - 17 DNA samples of phytoplasma strains cultivated in C. roseus and Pennisetum purpureum; - DNA from two field samples of Vitis vinifera, diagnostically predetermined to be infected with the Flavescence dorée and Bois noir phytoplasma; - DNA extracted from leaf veins or root phloem tissue of symptomatic and asymptomatic fruit trees (total of 241 samples of fruit trees); - several |

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| | pathogenic bacteria isolates and bacterial isolates that can be present as epiphytes or saprophytes on the fruit trees (total of 28 isolates). The testing of the range of targets and non-targets provided the following data. The AP assay consistently detected only DNA from ‘Ca. P. mali’; the ESFY assay only detected DNA of ‘Ca. P. prunorum’; and the PD assay only detected DNA from ‘Ca. P. pyri’; regardless of the source of DNA (periwinkle or fruit tree). There was no cross-reactivity with DNA from any unrelated phytoplasma reference strains or isolated from field-grown fruit-tree material. Similarly, the possibility of cross-reactivity with non-phytoplasma DNA was excluded after the assays applied did not give any positive amplification signal using DNA from four common bacterial pathogens or from 24 non-identified bacterial isolates from fruit trees. 'In silico' analysis: The NCBI BLAST analyses confirmed the specificity of the AP probe for ‘Ca. P. mali’, the ESFY assay for ‘Ca. P. prunorum’, and the PD assay for ‘Ca. P. pyri’. The alignment of all of the publically available sequences for ‘Ca. P. mali’, ‘Ca. P. prunorum’ and ‘Ca. P. pyri’ revealed some mismatches within the probe sequences. However, those isolates have not been tested yet with the designed protocol, and thus its ability to recognize these phytoplasma cannot be ruled out completely. |
| **Analytical specificity - exclusivity** | |
| **Number of non-target organisms tested** | PD: 164. |
| **Specificity value** | |
| **Diagnostic Specificity** | |
| **Proportion of uninfected/uninfested samples (true negatives) testing negative compared to results from a standard test** | PD (no. of samples: 44): 87.5%; A lower percentage of diagnostic specificity of the qPCR is due to a higher sensitivity of qPCR method than nested PCR method. |
| **Specify the test(s)** | nested PCR (f01/r01) + RFLP |
| **Reproducibility** | |
| **Provide the calculated % of agreement for a given level of the pest (see PM 7/98)** | Percentage of identical results is 100% at least in samples with estimated high or medium phytoplasma amount. No. of samples per 1 amplicon: 2; No. of operators: 4 No. of devices: 2 PD (mean Cq value: 31.79; No. of qPCR runs: 18; STDEV (Cq): 0.30): 100% (positive in all qPCR runs); PD (mean Cq value: 36.03; No. of qPCR runs: 18; STDEV (Cq): 0.58): 89% (positive in 89% of qPCR runs). |
| **Repeatability** | |
| **Provide the calculated % of agreement for a given level of the pest (see PM 7/98)** | Repeatability for all three assays is 100% in all samples with Cq values that are 3 Cq lower than LOD. PD (average Cq: 25-31.8; No. of parallels: 6x5; STD (Cq): 0.08-0.28): 100% positive parallels; PD (average Cq: 31.8-34.8; No. of parallels: 3x5; STD (Cq): 0.05-0.37): 100% positive parallels; PD |

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| | (average Cq: 34.8-37.8*; No. of parallels: 4x5; STD (Cq): 0.20-1.55): 95% positive parallels. *Cq LOD(95%) |
| **Test performance study** | |
| **Test performance study?** | yes |
| **Brief details of the test performance study and its output.It available, link to published article/report** | Inter-laboratory comparisons for detection methods for ‘Ca. P. mali’, ‘Ca. P. pyri’ and ‘Ca. P. prunorum’ (Euphresco project: Interlaboratory comparison and validation of detection methods for phytoplasmas of phytosanitary concern in European orchards). Conclusions: those qPCR assays were found to be appropriate for the diagnosing AP, PD and ESFY. |
| **Other information** | |
| **Any other information considered useful** | Selectivity: There was no observed impact of fruit tree cultivar on the test results. Up to the end of 2013, AP was confirmed using qPCR in at least 22 different apple cultivars, ESFY in at least 18 different stone fruit cultivars (7 peach, 7 apricot, 4 plum cultivars) and PD in at least 13 different pear cultivars. Additionally, we detected ESFY in Prunus spinosa, in vector Cacopsylla pruni (in eggs, larvae and adults), and in the potential vector Asymmetrasca decedens. Full validation report is added - see Validation report qPCR AP group. Possible sources and components of uncertainty in real-time PCR testing for AP, PD and ESFY, their impact and the measures applied to reduce uncertainty were identified - see Identification of critical points_Appendix 2. |

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