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	Council for Agricultural Research and Economics–Research Centre for Plant Protection and Certification Via Carlo Giuseppe Bertero, 22, 00156 Rome, Italy
Short description of the test	Diagnostic protocol for the detection and identification of 'Candidatus Liberibacter solanacearum' in carrot seeds (DNA extraction method, real-time PCR and conventional PCR)
Date, reference of the validation report	2017-05-25 - Ilardi V. , V. Lumia, E. Di Nicola, M. Tavazza, 2018. Identification, intra and inter-laboratory validation of a diagnostic protocol for ‘Candidatus Liberibacter solanacearum’ in carrot seeds. European Journal of Plant Pathology https://doi.org/10.1007/s10658-018-01606-w
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?	Other_project
If yes, please specify	ASPROPI
Description of the test	
Organism(s)	'Candidatus Liberibacter solanacearum'(LIBEPS)
Detection / identification	detection and identification
Method(s)	Molecular Extraction DNA RNA Molecular Conventional PCR 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	Ilardi et al. (2018) European Journal of Plant Pathology https://doi.org/10.1007/s10658-018-01606-w
Kit	

Is a kit used	yes
Manufacturer name	QIAGEN
Specify the kit used	DNeasy Plant Mini Kit
Kit used following the manufacturer's instructions?	yes
Other information	
Other details on the test	The International Seed Federation (ISF, 2016) recommends testing samples of 20 g of Apiaceae seeds divided into two sub-samples of 10 g each. The ISF DNA extraction protocol (2016) was used with some modification. Seeds were washed by shaking them for 30 min in 0.5% Triton X-100 and, after several rinses, they were left to soften in 1 / 6 water overnight. The seeds were crushed with a mechanical homogenizer in heavy plastic bags (Bioreba) in 1:10 (w/v) of a modified Trimethylammonium bromide (CTAB) buffer (2,5% CTAB, NaCl 1.4 M, Tris-HCl 1 M pH 8.0, EDTA 0.5 M, pH 8.0, PVP-40 1%, 30 mM ascorbic acid). 400 µg of RNase A was added to 500 µl of homogenate (corresponding to 50 seeds), and after incubation at 65 °C for 30 min, total genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's instructions. DNA was eluted in 100 µl of AE buffer provided by the kit.
Method: Molecular Conventional PCR	
Reference of the test description	
As or adapted from an EPPO diagnostic protocol	yes
EPPO Diagnostic Protocol name	PM 7/143 'Candidatus Liberibacter solanacearum' (version 1)
Name of the test	Conventional end-point PCR (Ravindran et al., 2011)
Other information	
Other details on the test	conventional PCR as reported by Ravindran et al., (2011) Plant Disease 95.12: 1542-1546
Method: Molecular real time PCR	
Reference of the test description	
As or adapted from an EPPO diagnostic protocol	yes
EPPO Diagnostic Protocol name	PM 7/143 'Candidatus Liberibacter solanacearum' (version 1)
Name of the test	Real-time PCR adapted by ANSES from Li et al. 2009
Is the test modified compared to the reference test	yes modified from Li et al. (2009) Journal of Microbiological Methods 78:59–65. The primers and probes were 'Candidatus Liberibacter spp. specific HLBr primer and HLBp probe, Ca. L. solanacearum

	specific LsoF primer. Deviations from the reference: PCR reagents (Universal master mix II no UNG -applied biosystem), each primer and probe concentrations (400nM and 150nM, respectively), DNA (1 µl) reaction volume (15µl). Amplification condition: 1 cycle 95°C/10 min, 45 cycles 95°C/15sec and 60°C/60 sec.
Other information	
Reaction type	Probe
Are the performance characteristics included in the EPPO diagnostic protocol?	**yes**
Performance Criteria :	
Organism 1.:	**'Candidatus Liberibacter solanacearum'(LIBEPS)**
Analytical sensitivity	
What is smallest amount of target that can be detected reliably?	The limit of detection (LOD), calculated with the total DNA extract of CaLsol infected seeds, was of 10^-2 and 10^-3 dilution for the conventional and real-time PCR, respectively. For the real-time PCR, the LOD was also evaluated with purified pTXZC18 diluted with water. Five copies of the target were detected with Ct values of 34.57 ± 0.428 in 100% of the experiments (24/24)
Analytical specificity - inclusivity	
Number of strains/populations of target organisms tested	32 target organisms were tested: 1. ISPAVE_VIb_1 ‘Berlicum’ carrot seed infected by CaLsol haplotype E (Ilardi et al., 2016) 2. 4Flakkée ‘Flakkée’ CaLsol infected carrot seed 3. 5Maestro ‘Maestro’ CaLsol infected carrot seed 4. ISPAVE_VIb_6 ‘Nantese 3’ carrot seed infected by CaLsol haplotype D (Ilardi et al., 2016) 5. 8Berlicum ‘Berlicum2’ CaLsol infected carrot seed 6. ISPAVE_VIb_9 ‘Berlicum 2’ carrot seed infected by CaLsol haplotype E (Ilardi et al., 2016) 7. 10Berlicum ‘Berlicum 2’ CaLsol infected carrot seed 8. ISPAVE_VIb_11 ‘Falkkée’ carrot seed infected by CaLsol haplotype D (Ilardi et al., 2016) 9. ISPAVE_VIb_15 ‘Mezza lunga nantese’ carrot seed infected by CaLsol haplotype E (Ilardi et al., 2016) 10. ISPAVE_VIb_17 ‘Berlicum’ carrot seed infected by CaLsol haplotype D (Ilardi et al., 2016) 11. C-AV ‘Nantese migliorata 2’ CaLsol infected carrot seed 12. C1 carrot seed infected by CaLsol haplotype D 13. C2 carrot seed infected by CaLsol haplotype D 14. C3 carrot seed infected by CaLsol haplotype D 15. C4 carrot seed infected by CaLsol haplotype D 16. C5 carrot seed infected by CaLsol haplotype E/D 17. C6 carrot seed infected by CaLsol haplotype D 18. P4 parsley seed infected by CaLsol haplotype D 19. P1 parsley seed infected by CaLsol haplotype E 20. P2 parsley seed infected by CaLsol haplotype E 21. P3 parsley seed infected by CaLsol haplotype E 22. 1P ‘Riccio Verde’ CaLsol infected parsley seed 23. 2P ‘Riccio Verde’ CaLsol infected parsley seed 24. 3P ‘Comune’ CaLsol

	infected parsley seed 25. 4P ‘Comune 2 multifoglia’ CaLsol infected parsley seed 26. 5P ‘Gigante’ CaLsol infected parsley seed 27. 6P ‘Gigante’ CaLsol infected parsley seed 28. 7P ‘Gigante d’Italia’ CaLsol infected parsley seed 29. P 1 SCS ‘Gigante di Napoli’ CaLsol infected parsley seed 30. P 4 SCS ‘Gigante d’Italia’ CaLsol infected parsley seed 31. P 5 SCS ‘prezzemolo Comune 2’ CaLsol infected parsley seed 32. P 7 SCS ‘Riccio Muschiato’ CaLsol infected parsley seed 33. S1 ‘Sedano D’Elne’ CaLsol infected celery seed 34. S-AV ‘Sedano D’Elne’ CaLsol infected celery seed 35. CaLsol control pTXZC18 plasmid with the CaLsol 16S rDNA target (Li et al., 2009) kindly provided by Li 2009. In the test performance study with 11 laboratories the following samples were tested: C4 carrot seed infected by CaLsol haplotype D, ISPAVE_VIb_1 ‘Berlicum’ carrot seed infected by CaLsol haplotype E, C-AV ‘Nantese migliorata 2’ CaLsol infected carrot seed, and for real-time PCR also CaLsol control pTXZC18 plasmid.
Specificity value	
<u>Analytical specificity - exclusivity</u>	
Number of non-target organisms tested	37 non-target organisms were tested: 1. 12Nantese2 ‘Nantese2’ CaLsol free carrot seed 2. 14Berlicum2 ‘Berlicum2’ CaLsol free carrot seed 3. 16LungaB. ‘Lunga di Berlicum’ CaLsol free carrot seed 4. P 2 SCS ‘Gigante di Napoli’ CaLsol free parsley seed 5. P 3 SCS ‘Gigante di Napoli’ CaLsol free parsley seed 6. P 6 SCS ‘Comune 2’ CaLsol free parsley seed 7. P-AV ‘Gigante di Napoli’ CaLsol free parsley seed 8. 2Berlicum ‘Berlicum’ CaLsol free carrot seed 9. 3Bolero ‘Bolero F1’ CaLsol free carrot seed 10. 7Nantesa3 ‘Nantesa3’ CaLsol free carrot seed 11. 13Nantese2 ‘Nantese2’ CaLsol free carrot seed 12. S2 ‘Peros Rendy’ CaLsol free celery seed 13. S3 ‘Sedano D’Elne’ CaLsol free celery seed 14. F1 ‘Montebianco’ CaLsol free fennel seed 15. F3 ‘Wadenromen ’ CaLsol free fennel seed 16. F4 ‘Romanesco’ sel. Circeo CaLsol free fennel seed 17. F-AV ‘Wadenromen’ CaLsol free fennel seed 18. 1519 Pseudomonas fluorescens 19. 1174 P. putida 20. 1182 P. marginalis from chicory 21. 1146 P. syringae pv syringae from lemon 22. 1001 Agrobacterium tumefaciens 23. 1235 Erwinia herbicola ISF438 24. 1030 Xantomonas campestris pv campestris from cabbage 25. 1049 Xantomonas arboricola pv corylina from turnip 26. 1240 Pectobacterium carotovora from artichoke 27. 1433 Pectobacterium carotovora from zucchini 28. 04-500 X. campestris pv begoniae from carrot 29. 11-267N2 Pseudomonas sp from fennel 30. 1432 P. viridiflava from tomato 31. Ferr1 Phytoplasma stolbur (solani 16SrXII-A) 32. PAV 1 Unknown bacterium from carrot seed 33. PAV 2 Unknown bacterium from carrot seed 34. PAV 3 Unknown bacterium from carrot seed 35. PAV 4 Unknown bacterium from carrot seed 36. PAV 5 Unknown

	bacterium from carrot seed 37. PAV 6 Unknown bacterium from carrot seed In the test performance study with 11 laboratories the following samples were tested: F-AV ‘Wadenromen’ CaLsol free fennel seed, F1 ‘Montebianco’ CaLsol free fennel seed, 3Bolero ‘Bolero F1’ CaLsol free carrot seed, 04-500 X. campestris pv begoniae from carrot,11-267N2 Pseudomonas sp from fennel)
Specificity value	None of them
Diagnostic Specificity	
Proportion of uninfected/uninfested samples (true negatives) testing negative compared to results from a standard test	real-time PCR: 100% conventional PCR: 81.5%
Reproducibility	
Provide the calculated % of agreement for a given level of the pest (see PM 7/98)	real-time PCR: 100% conventional PCR: 100% calculated with the total DNA extract of CaLsol infected seeds at 10^-2 and 10^-3 dilution for the conventional and real-time PCR, respectively. For the real-time PCR, was also evaluated with Five copies of purified pTXZC18 diluted with water.
Repeatability	
Provide the calculated % of agreement for a given level of the pest (see PM 7/98)	real-time PCR: 100% conventional PCR: 100% 2 different operators for real-time PCR and 3 for conventional PCR. 2 different equipments for real-time PCR and 2 for conventional PCR. Calculated with the total DNA extract of CaLsol infected seeds at 10^-2 and 10^-3 dilution for the conventional and real-time PCR, respectively. For the real-time PCR, was also evaluated with Five copies of purified pTXZC18 diluted with water.
Test performance study	
Test performance study?	yes
Brief details of the test performance study and its output.It available, link to published article/report	Ilardi V. , V. Lumia, E. Di Nicola, M. Tavazza, 2018. Identification, intra and inter-laboratory validation of a diagnostic protocol for ‘Candidatus Liberibacter solanacearum’ in carrot seeds. European Journal of Plant Pathology https://doi.org/10.1007/s10658-018-01606-w TPS was performed by ten laboratories of the Italian Regional Plant Protection Service (IRPPS), widespread throughout the country, and CREA-DC-Rome laboratory. For real-time and conventional PCR, the oligonucleotides and reagents, including water, were sent to the participants. For the end-point PCR and real-time PCR, Go Taq G2Flexi DNA polymerase (Promega) and TaqMan Universal Master Mix II (applied biosystem) were provided, respectively. Each sample was tested by the participants in triplicate (technical replicates). To test the DNA extraction protocol, CaLsol infected and CaLsol free seeds, were provided together with the buffers and the DNeasy Plant Mini Kit (Qiagen, Germany). real-time PCR Li et al., 2009 Diagnostic sensitivity 98.6% Diagnostic specificity 100.0%

	Relative accuracy 99.0% Accordance 98.2% Concordance 98.0% COR* 1.11 end-point PCR Ravindran et al., 2011 Diagnostic sensitivity 100% Diagnostic specificity 81.5% Relative accuracy 88.9% Accordance 82.2% Concordance 80.0% COR* 1.15 seed DNA extract evaluated by real-time PCR Li et al., 2009 Diagnostic sensitivity 100.0% Diagnostic specificity 95.0% Relative accuracy 98.75% Accordance 97.81% Concordance 97.5% COR* 1.14 seed DNA extract evaluated by end-point PCR Ravindran et al., 2011 Diagnostic sensitivity 90.74% Diagnostic specificity 100.0% Relative accuracy 93.82% Accordance 90.12% Concordance 88.20% COR* 1.22 *Concordance odds ratio= accordance x (100 – concordance)/concordance x (100 – accordance), to address the variability of the method within and between laboratories, calculated as indicated by ISO 16140:2003.

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