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 Institiute of Biology, Department of Biotechnology and Systems Biology Vecna pot 121, 1000 Ljubljana, Slovenia
Short description of the test	Detection of Plant Viruses Using Nanopore High-Throughput Sequencing: Validation Report
Date, reference of the validation report	2023-05-29 - Report on suitability testing D0005/23
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?	no
Description of the test	
Organism(s)	Ipomovirus lycopersici(TOMMOV)
Detection / identification	detection and identification
Method(s)	Molecular Extraction DNA RNA Molecular HTS
Method: Molecular Extraction DNA RNA	
Reference of the test description	
Kit	
Is a kit used	yes
Manufacturer name	QIAGEN
Specify the kit used	RNeasy Plant Mini Kit
Kit used following the manufacturer's instructions?	yes Minor modification: 2-mercaptoethanol was not added. Total RNA is eluted twice with 50 µL (total of 100 µL) of RNase-free water pre-warmed to 65°C.
Other information	
Method: Molecular HTS	
Reference of the test description	
As or adapted from an EPPO diagnostic protocol	no
New test being considered for inclusion in the next version of the EPPO diagnostic protocol?	yes

As or adapted from an IPPC diagnostic protocol	no
Reference of the test	Pecman et al. 2022.Systematic comparison of nanopore and illumina sequencing for the detection of plant viruses and viroids using total RNA sequencing approach. Frontiers in microbiology. 2022, vol. 13, p. 1-14. Pecman et al. Detection of plant viruses using nanopore sequencing based metagenomic approach (Chapter 17) In: Methods Molecular Biology, Vol. 2732, Vitantonio Pantaleo and Laura Miozzi (Eds): Viral Metagenomics. Springer Nature. IN PRESS.
Is the test modified compared to the reference test	no
Other information	
Other details on the test	The protocol is based on sequencing on MinION sequencer (Oxford Nanopore Technologies) using the cDNA-PCR protocol for sequencing ribosomal RNA-depleted total RNA.
Performance Criteria :	
Organism 1.:	**Ipomovirus lycopersici(TOMMOV)**
Analytical sensitivity	
What is smallest amount of target that can be detected reliably?	Tomato mild mottle virus (ToMMoV, PV-0993) dilutions (6x, 10x, 30x, 60x, 100x, and 1000x) prepared by mixing RNA from this isolate with RNA extracted from healthy tomato leaves were tested. Up to three replicates were analysed for selected dilutions. The highest dilution that gave a positive result, when using 600,000 filtered reads (the lowest expected number of reads in case of using PCR-cDNA barcoding kit SQK-PCB111.24): 100x. In addition, bioinformatic subsampling of data from tomato mild mottle virus (ToMMoV, PV-0993 and PV-1015) , and also from cowpea mild mottle virus (CPMMV) PV-0907 and squash vein yellowing virus (SqVYV) PV-1348 (all from the DSMZ collection) was performed. This was done for 90,000,000 (approximately 500,000 reads), 50,000,000 (approximately 270,000 reads), 10,000,000 (approximately 50,000 reads), 5,000,000 (approximately 30,000 reads), and 1,000,000 (approximately 5,000 reads) nucleotides. Each subsampling was replicated 5 times. The subsample with the lowest nucleotide number (1,000,000), corresponding to a nearly 100-fold dilution, still yielded positive detection results.
Analytical specificity - inclusivity	
Number of strains/populations of target organisms tested	In silico testing for the presence of tomato mild mottle virus (ToMMoV) and several other selected viruses (beet curly top virus (BCTV), from cowpea mild mottle virus (CPMMV), lettuce infectious yellows virus (LIYV), melon yellowing-associated virus (MYaV), SqVYV, tomato chocolate virus

	(ToChV) and tomato marchitez virus (ToMarV)) in the NCBI viral reference database (updated 5.6.2022) resulted in the detection of seven of the eight selected viruses. The genome reference of ToChV, which was not found in the NCBI viral reference database, was added, and this completed database was further used. Testing of the range of targets showed that all results were consistent with expected results. The list of targets tested: Beet curly top virus (Geminiviridae, Curtovirus): total RNA obtained from OMID EINI (Institut für Zuckerrübenforschung Abteilung Phytomedizin, Holtenser Landstraße 77, 37079 Göttingen) Cowpea mild mottle virus (Betaflexiviridae, Carlavirus): isolates from DSMZ collection PV-0907 Squash vein yellowing virus (Potyviridae, Ipomovirus): isolates from DSMZ collection PV-1348 Tomato mild mottle virus (Potyviridae, Ipomovirus): two isolates from DSMZ collection PV-0993 and PV-1015.
Specificity value	100%
Analytical specificity - exclusivity	
Number of non-target organisms tested	Testing of the range of non-targets showed that all results were consistent with expected results. The list of non-targets tested: Four healthy Solanum lycopersicum samples of different cultivars, six bulk samples (three samples of tomatoes consisted of six plants, one sample of tomatoes consisted of seven plants, one sample of peppers consisted of seven plants, one sample of cucurbits consisted of seven plants).
Specificity value	100%
Reproducibility	
Provide the calculated % of agreement for a given level of the pest (see PM 7/98)	100% (Evaluated on following samples controls: 1. three repetitions of tomato mild mottle virus (ToMMoV , DSMZ collection PV-0993), diluted 10-times 2. sequencing of ERCC control 3. sequencing of Phaseolus vulgaris endorna virus (PvEV) control 4. sequencing of RCS control).
Repeatability	
Provide the calculated % of agreement for a given level of the pest (see PM 7/98)	100% (Evaluated on The tenfold dilution of tomato mild mottle virus (ToMMoV , DSMZ collection PV-0993) was tested on the flowcell G using MinION sequencer (ONT) in two replicates (once diluted in Solanum lycoprersicum cv. Money Maker and once in S. lycopersicum Roma).
Test performance study	
Test performance study?	no
Other information	
Any other information considered useful	Selectivity RNA extracted from ToMMoV (DSMZ PV-0993) was spiked into RNA of different tomato cultivars (Moneymaker, Roma, bulk sample of

	different tomato cultivars) so that a 6- or 10-fold dilution was produced. No significant influence of plant cultivar on test results was detected.
The following complementary files are available online:	• Validation_report_nanopore • Validation_report_nanopore_measurement_uncertainty

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