Naslov poročila / Title

DETECTION OF PLANT VIRUSES USING NANOPORE HIGH-THROUGHPUT SEQUENCING: VALIDATION REPORT

Anja Pecman, Veronika Bukvič, Ana Vučurović, Irena Bajde, Jakob Brodarič, Nataša Mehle, Denis Kutnjak

Številka poročila Report Number	D0005/23
Tip poročila Report Type	Validation report
Naslov serije Series Title	Poročila o preizkušanju ustreznosti Reports on suitability testing
Kraj Place	Ljubljana, Slovenija Ljubljana, Slovenia
Inštitut Institution	Nacionalni inštitut za biologijo, Oddelek za biotehnologijo in sistemsko biologijo National Institute of Biology, Department of Biotechnology and Systems Biology
Datum Date	29.5.2023
Število strani Page number	18
Jezik Language	English
Prispevek avtorjev Author contribution	Anja Pecman: participation in the design of the study, performance of the analyses (wet and dry lab), supervising, data collection, writing of the report Veronika Bukvič: wet and dry lab analysis performed Ana Vučurović: dry lab analysis performed Irena Bajde: wet lab analysis performed Jakob Brodarič: wet lab analysis performed Nataša Mehle: conception of the study, coordination of the study, review of the report, preparation of the annexes to the report 29.5.2023 Denis Kutnjak: participated in the design of the study, supervised and approved the report and annexes to the report 29.5.2023
Citat / Referenca	Pecman, A., Bukvič, V., Vučurović, A., Bajde, I., Brodarič, J., Mehle, N., Kutnjak, D. 2023. Detection of plant viruses using nanopore high-throughput sequencing: validation report. National Institute of Biology, Ljubljana, Slovenia.

Zaključki/ Conclusions

Nanopore high-throughput sequencing with protocols 02D-Pos100, 02D-Pos101 and 02D-Pos102 is suitable for accurate diagnosis of tomato mild mottle virus (ToMMoV) in tomato leaves.

Posledice za dosedanje delo / Consequences for the current work

Test can be used as fully validated (see Appendix 1).

Materiali in metode / Materials and Methods

Procedure that was the subject of validation: 02D-Pos100 and 02D-Pos101 (cover procedure: 02D-Pos102).

To generate validation data, runs were performed with eight flowcells using MinION sequencer from Oxford Nanopore Technologies (ONT) (Table 1). Programs used for bioinformatic analysis are listed in Table 2. The samples included in the analysis are listed in Table 3, and the controls used are listed in Table 4 and 5.

Table 1: Details about ONT MinION flowcells and used references for mapping

MinIon flowcell	Date	lab book ID (02-LD) or test ID (VAL)	PCR-cDNA barcoding kit	MinION Mk1B sequencer ID	Name of Refseq database used
A	16.11.2021	02-LD243	SQK-PCB109	MN21448	20211105
B	30.6.2022	02-LD408	SQK-PCB109	MN21448	20220506 and 20220715_ToChV1_2
C	9.9.2022	02-LD412	SQK-PCB109	MN33718	20220715_ToChV1_2
D	22.9.2022	02-LD412	SQK-PCB109	MN21448	20220715_ToChV1_2
E	24.2.2023	VAL1	SQK-PCB111.24	MN21448	20230113_ YHR174W_ToChV1_2
F	9.3.2023	VAL2	SQK-PCB111.24	MN21448	20230113_ YHR174W_ToChV1_2
G	23.3.2023	VAL3	SQK-PCB111.24	MN21448	20230113_ YHR174W_ToChV1_2
H	7.4.2023	VAL4	SQK-PCB111.24	MN21448	20230113_ YHR174W_ToChV1_2

Table 2: Programs used for bioinformatic analysis

Program	Version used
nanoQC	0.9.2
NanoStat	1.1.2
NanoFilt	2.6.0
minimap2	2.17-r941
miniasm	0.3-r179
awk	4.1.4
racon	1.4.13
CLC Genomic Workbench	20
Putty	Not relevant
WinSCP	Not relevant

Table 3: Details about samples included in the study

Sample name	Sample description
Pecman 2022*	Sample (I), cDNA-PCR sequencing of rRNA-depleted totRNA, contains: pepino mosaic virus (PepMV-EU), pepino mosaic virus (PepMV-CH), tomato mosaic virus (ToMV), tomato chlorosis virus (ToCV), southern tomato virus (STV), tomato yellow leaf curl virus (TYLCV), columnea latent viroid (CLVD)
BCTV sample 1	Beet curly top virus: total RNA obtained from OMID EINI (Institut für Zuckerrübenforschung Abteilung Phytomedizin, Holtenser Landstraße 77, 37079 Göttingen)
CPMMV PV0907	cowpea mild mottle virus: DSMZ, PV0907
SqVYV PV1348	squash vein yellowing virus: DSMZ, PV1348
ToMMoV PV0993	tomato mild mottle virus: DSMZ, PV0993
ToMMoV PV1015	tomato mild mottle virus: DSMZ, PV1015
Bulk sample 1 (A)	bulk sample of seven peppers: D598/21, D650/21, D693/21, D712/21, D724/21, D742/21, D767/21. Nucleic acid of alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV), cucumber mosaic virus satellite RNA (CMV-satRNA), bell pepper alphaendornavirus (BPEV), broad bean wilt virus 1 (BBWV1), potato virus Y (PVY) were confirmed using Illumina sequencing approach whit 934548 reads analysed.**
Bulk sample 2 (A)	bulk sample of seven zucchini: D524/21, D603/21, D647/21, D689/21, D721/21, D770/21, D786/21. Nucleic acid of cucumber mosaic virus (CMV), yucchini yellow mosaic virus (ZYMV), cucurbit aphid-borne yellows virus (CABYV) were confirmed using Illumina sequencing approach whit 1290156 reads analysed. **
Bulk sample 3 (A)	bulk sample of seven tomatoes: D1136/21, D535/21, D562/21, D589/21, D599/21, D601/21, D718/21. Nucleic acid of potato virus S (PVS), potato virus Y (PVY), tomato spotted wilt virus (TSWV), tomato fruit blotch virus (ToFBV) were confirmed using Illumina sequencing approach whit 669390 reads analysed. **

Bulk sample 4 (A)	bulk sample of six tomatoes: D521/21, D561/21, D655/21, D657/21, D699/21, D725/21. Nucleic acid of tomato spotted wilt virus (TSWV), cucumber mosaic virus (CMV), cucumber mosaic virus satellite RNA (CMV-satRNA), southern tomato virus (STV) were confirmed using Illumina sequencing approach whit 821688 reads analysed. **
Bulk sample 5 (A)	bulk sample of six tomatoes: D738/21, D743/21, D749/21, D750/21, D752/21, D753/21. Nucleic acid of tomato mosaic virus (ToMV), tomato spotted wilt virus (TSWV) were confirmed using Illumina sequencing approach whit 259742 reads analysed. **
Bulk sample 6 (A)	bulk sample of six tomatoes: D444/21, D517/21, D550/21, D564/21, D651/21, D653/21. Nucleic acid of cucumber mosaic virus (CMV) were confirmed using Illumina sequencing approach whit 766518 reads analysed. **
Bulk sample 1 (C)	bulk sample of six tomatoes: D541/22, D542/22, D594/22, D681/22, D682/22, D707/22. Nucleic acid of cucumber mosaic virus (CMV), cucumber mosaic virus satellite RNA (CMV-satRNA) were confirmed using nanopore sequencing (02-LD412).
Bulk sample 2 (C)	bulk sample of six tomatoes: D711/22, D713/22, D715/22, D747/22, D749/22, D788/22. Nucleic acid of potato virus Y (PVY), tomato mosaic virus (ToMV) were confirmed using nanopore sequencing (02-LD412).
Bulk sample 3 (C)	bulk sample of six tomatoes: D790/22, D791/22, D814/22, D834/22, D849/22, D858/22. Nucleic acid of potato virus Y (PVY), tomato mosaic virus (ToMV), physostegia chlorotic mottle virus (PhCMoV) were confirmed using nanopore sequencing (02-LD412).
Bulk sample 4 (C)	bulk sample of six tomatoes: D487/22, D511/22, D554/22, D555/22, D619/22, D620/22. Nucleic acid of potato virus Y (PVY), potato virus S (PVS), potato virus M (PVM), tomato spotted wilt virus (TSWV) were confirmed using nanopore sequencing (02-LD412).
Bulk sample 5 (C)	bulk sample of six tomatoes with RNA extraction ID D622/22, D680/22, D714/22, D735/22, D748/22, D750/22 with detected nucleic acid of potato virus Y (PVY) confirmed using nanopore sequencing (02-LD412).
Bulk sample 1 (D)	bulk sample of six peppers: D277/22, D556/22, D597/22, D625/22, D627/22, D683/22. Nucleic acid of tomato spotted wilt virus (TSWV), pepper cryptic virus 2 (PCV-2), bell pepper alphaendornavirus (BPEV) were confirmed using nanopore sequencing (02-LD412).
Bulk sample 2 (D)	bulk sample of six peppers: D709/22, D712/22, D716/22, D835/22, D850/22, D851/22. Nucleic acid of tomato spotted wilt virus (TSWV), cucumber mosaic virus (CMV), pepper cryptic virus 2 (PCV-2), bell pepper alphaendornavirus (BPEV) were confirmed using nanopore sequencing (02-LD412).
Bulk sample 3 (D)	bulk sample of six peppers: D686/22, D708/22, D746/22, D852/22, D855/22, D857/22. Nucleic acid of potato virus Y (PVY), cucumber mosaic virus (CMV), pepper cryptic virus 2 (PCV-2), bell pepper alphaendornavirus (BPEV), broad bean wilt virus 1 (BBWV-1), broad bean wilt virus 2 (BBWV-2) were confirmed using nanopore sequencing (02-LD412).
Bulk sample 4 (D)	bulk sample of six peppers: D710/22, D1270/22, D1272/22, D1273/22, D1275/22, D1276/22. Nucleic acid of tomato spotted wilt virus (TSWV),

	cucumber mosaic virus (CMV), bell pepper alphaendornavirus (BPEV), broad bean wilt virus 2 (BBWV-2) were confirmed using nanopore sequencing (02-LD412).
Bulk sample 5 (D)	bulk sample of six peppers: D711/22, D713/22, D715/22, D747/22, D749/22, D788/22. Nucleic acid of potato virus Y (PVY), tomato mosaic virus (ToMV), tomato spotted wilt virus (TSWV), physostegia chlorotic mottle virus (PhCMoV), tomato fruit blotch virus (ToFBV) were confirmed using nanopore sequencing (02-LD412).
S.lyc ID195	Leaves of *Solanum lycopersicum* cv. RioGrande (internal ID 195)
S.lyc ID332	Leaves of *Solanum lycopersicum* cv. Roma (internal ID 332)
S.lyc. ID460	Leaves of *Solanum lycopersicum* cv. Moneymaker (internal ID 460)
S. lyc. DSMZ	Leaves of noninfected *Solanum lycopersicum* from DSMZ collection

*PECMAN, Anja, ADAMS, Ian, GUTIÉRREZ-AGUIRRE, Ion, FOX, Adrian, BOONHAM, Neil, RAVNIKAR, Maja, KUTNJAK, Denis. 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, Anja, KOGEJ ZWITTER, Zala, MEHLE, Nataša, VUČUROVIĆ, Ana, RAVNIKAR, Maja, KUTNJAK, Denis. Uporaba visokozmogljivega sekvenciranja za iskanje karantenskih rastlinskih virusov = The use of high-throughput sequencing for detection of quarantine plant viruses. In: TRDAN, Stanislav (ed.). 15. slovensko posvetovanje o varstvu rastlin z mednarodno udeležbo = 15th Slovenian Conference on Plant Protection with International Participation : zbornik predavanj in referatov = leactures and papers : 1.-2. marec 2022, Portorož, Slovenija. Ljubljana: Društvo za varstvo rastlin Slovenije: = Plant Protection Society of Slovenia, 2022. p. 268-274.

Table 4: Controls included in the procedure

Control name	Description	Aim
NAD5	For each sample the RNA extraction performance was verified by real-time RT-PCR using a set of oligonucleotide primers and probe for NAD5	- Internal positive control for RNA extraction from each individual sample
PvEV	Alien control (a well-characterised sample that contains a virus not expected to be present in the analysed samples): RNA extracted from *Phaseolus vulgaris* cv. Black Turtle infected with endornavirus (the RNA extraction of PvEV and all further steps were performed together/in parallel with the samples)	- Monitoring contamination throughout the process (role of negative control) - Ensuring detection of specific target (not expected in the samples analysed) when used at low concentration (role of positive control throughout the process)
ERCC	ERCC RNA Spike-In Control Mixes (Invitrogen™, #4456740): control added to every sample and PvEV	- Additional positive control for steps after DNase digestion for each sample
RCS	RNA Control Expansion (EXP-RCS00, Oxford Nanopore Technologies, UK) (positive control present in high concentration)	- To monitor the success of the library preparation and sequencing step (to monitor possible barcode cross-talk during sequencing)

Table 5: Graphical representation of the controls included in the procedure (green colour means that the control is included in the step)

Step in the procedure	NAD5	PvEV	ERCC	RCS
RNA extraction				
DNase digestion				
Plant ribosomal RNA depletion, polyadenilation				
Library preparation, sequencing, bioinformatic analysis				

Validation procedure was following the steps described in 02D-Pos64. This include:

- Initiative to introduce new method: see Appendix 2
- Validation plan and instructions: see Appendix 3
- Validation procedure: according to EPPO standard PM 7/98 (see also 02D-Nav23), considering the specific guidelines of EPPO PM 7/151. Details are described below.

Analytical specificity

'*In silico*' check for the presence of selected viruses (Table 6) in the NCBI viral reference database: manually by searching for their names in the NCBI viral reference database updated on 5.6.2022. If the viral sequence of any of those listed viruses was not found in database, it was subsequently added.

Table 6: List of selected viruses

Virus name (Family, Genus)	Virus abbreviation
Beet curly top virus (*Geminiviridae, Curtovirus*)	BCTV
Cowpea mild mottle virus (*Betaflexiviridae, Carlavirus*)	CPMMV
Lettuce infectious yellows virus (*Closteroviridae, Crinivirus*)	LIYV
Melon yellowing-associated virus (*Betaflexiviridae, Carlavirus*)	MYaV
Squash vein yellowing virus (*Potyviridae, Ipomovirus*)	SqVYV
Tomato chocolate virus (*Secoviridae, Torradovirus*)	ToChV
Tomato marchitez virus (*Secoviridae, Torradovirus*)	ToMarV
Tomato mild mottle virus (*Potyviridae, Ipomovirus*)	ToMMoV

Tested targets (selected viruses were analysed using procedure 02D-Pos100 and 02D-Pos101):

- BCTV sample 1
- CPMMV PV0907
- SqVYV PV1348
- ToMMoV PV0993
- ToMMoV PV1015

Tested non targets (samples without viruses and samples with other viruses were analysed using procedure 02D-Pos100 and 02D-Pos101):

- S. lyc. ID460
- S. lyc ID332
- S. lyc ID195
- S. lyc. DSMZ
- Bulk sample 1 (A)
- Bulk sample 2 (A)
- Bulk sample 3 (A)
- Bulk sample 4 (A)
- Bulk sample 5 (A)
- Bulk sample 6 (A)

Analytical sensitivity

Bioinformatic subsampling of data from CPMMV PV0907, SqVYV PV1348, ToMMoV PV0993, and ToMMoV PV1015 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.

Further validation was performed only for ToMMoV and for a selected matrix: tomato leaves. For this part of the further validation, we selected the sample of ToMMoV isolate PV0993, which had a lower virus titer compared to ToMMoV isolate PV1015 (Figure 1). Equimass dilutions (mixing equal mass of nucleic acids) in two or more dillutions of the RNA of this isolate was prepared in RNA extracted from healthy tomato leaves (S. lyc. ID460). Up to three replicates were analysed for selected dilutions.

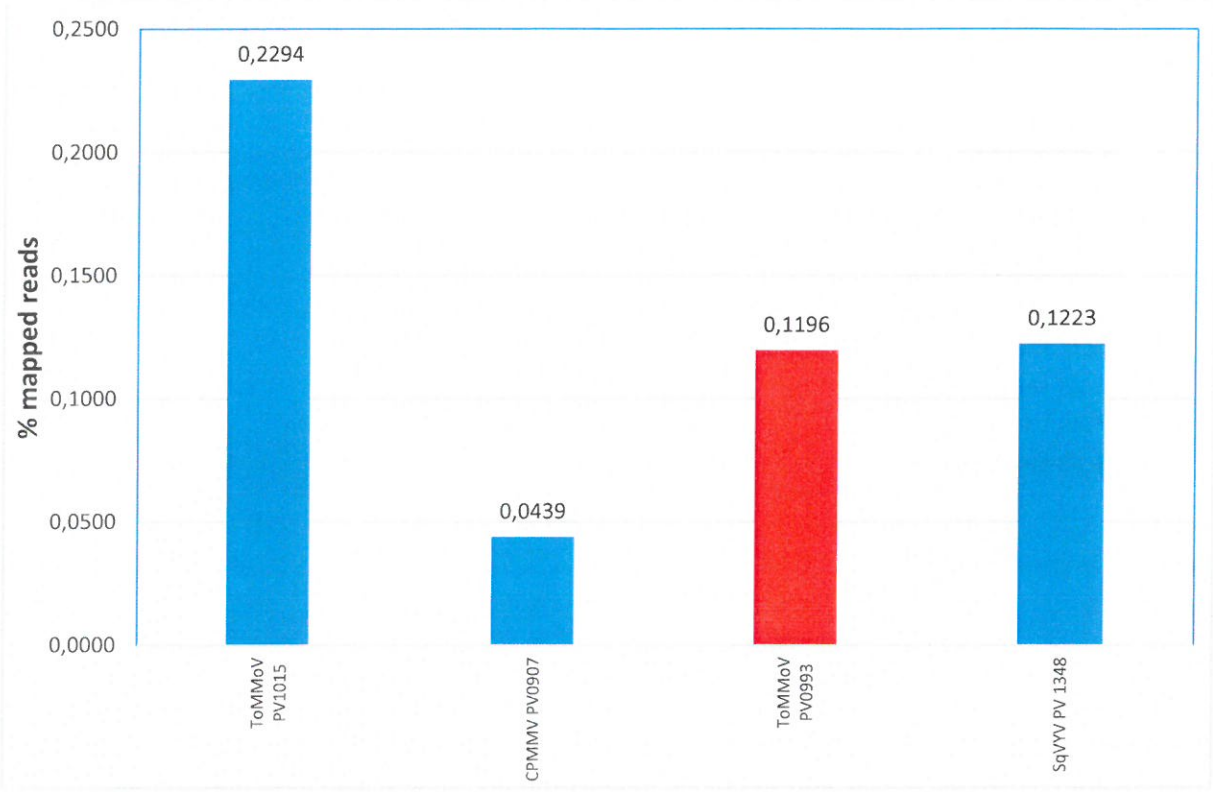

Figure 1: % of mapped viral reads to selected reference (flowcell B) for individual sequenced samples.

The number of filtered reads of all tested samples sequenced on flowcell A-G using MInION sequencer (ONT) was also checked to determine the lowest expected data outputs per sample. Then, subsampling to lowest expected outputs was performed for all the datasets derived from analysed dilutions of ToMMoV in five repetitions. Bioinformatic analyses were then repeated on the subsampled datasets.

Selectivity

RNA extracted from ToMMoV PV0993 was spiked into different RNA of tomato cultivars (samples S. lyc., ID460, S. lyc ID332 and Bulk sample 2 (C)) so that a 6- or 10-fold dilution was produced. These samples were tested, and the results compared among each other.

Repeatability

The tenfold dilution of ToMMoV PV0993 was tested on the flowcell G using MinION sequencer (ONT) in two replicates (once diluted in S. lyc. ID460 and once in S. lyc ID332).

In addition, repeatability was evaluated based on the results of the ERCC control. This control was added to each sample before the ribosomal RNA depletion step.

Reproducibility

Following samples/controls were included for the reproducibility evaluation:

- three repetitions of ToMMoV PV0993, diluted 10-times
- sequencing of ERCC control
- sequencing of PvEV control
- sequencing of RCS control

Rezultati / Results

Analytical specificity

In silico testing for the presence of listed quarantine viruses in the NCBI viral reference database (updated on 5.6.2022) resulted in the detection of seven out of the eight selected viruses (Table 7). 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 and non-targets provided the data presented in Table 8. All results agreed with the expected results (inclusivity and exclusivity: 100%).

Table 8: NCBI accession numbers of selected viruses included in the viral reference database

Virus abbreviation	NCBI acc. no.	Inclusion in the reference database from 5.6.2022	Inclusion in the updated reference database
BCTV	NC_001412	yes	yes
CPMMV	NC_014730	yes	yes
LIYV	NC_003617 NC_003618	yes	yes
MYaV	NC_038324	yes	yes
SqVYV	NC_010521	yes	yes
ToChV	FJ560490.1, FJ560489.1	no	yes
ToMarV	NC_010987 NC_010988	yes	yes
ToMMoV	NC_038920	yes	yes

Table 8: Results of analytical specificity testing

Sample	Virus/es present in a sample (expected results)	Results of analytical specificity testing			Is there a discrepancy between the expected and tested results?	% of accurate results
		Selected quarantine viruses detected (Table 5)	Other quarantine viruses detected (EU 2021/2285)	Other viruses detected		
BCTV sample 1	BCTV	BCTV	no	no	no	100%
CPMMV PV0907	CPMMV	CPMMV	no	no	no	100%
SqVYV PV1348	SqVYV	SqVYV	no	no	no	100%
ToMMoV PV0993	ToMMoV	ToMMoV	no	no	no	100%
ToMMoV PV1015	ToMMoV	ToMMoV	no	no	no	100%
Bulk sample 1 (A)*	AMV, CMV, CMV-satRNA, BPEV, BBWV1, PVY	no	no	AMV, CMV, CMV-satRNA, BPEV, BBWV1, PVY	no	100%
Bulk sample 2 (A)*	CMV, ZYMV, CABYV	no	no	CMV, ZYMV, CABYV	no	100%
Bulk sample 3 (A)*	PVS, PVY, TSWV, ToFBV	no	no	PVS, PVY, TSWV, ToFBV	no	100%
Bulk sample 4 (A)*	TSWV, CMV, CMV-satRNA, STV	no	no	TSWV, CMV, CMV-satRNA, STV	no	100%
Bulk sample 5 (A)*	ToMV, TSWV	no	no	ToMV, TSWV	no	100%
Bulk sample 6 (A)*	CMV	no	no	CMV	no	100%
S. lyc. ID460	none	no	no	no	no	100%
S. lyc. ID332	none	no	no	no	no	100%
S. lyc. ID195	none	no	no	no	no	100%
S. lyc. DSMZ	none	no	no	no	no	100%

*for detailed analysis see: PECMAN, Anja, KOGEJ ZWITTER, Zala, MEHLE, Nataša, VUČUROVIĆ, Ana, RAVNIKAR, Maja, KUTNJAK, Denis. Uporaba visokozmogljivega sekvenciranja za iskanje karantenskih rastlinskih virusov = The use of high-throughput sequencing for detection of quarantine plant viruses. In: TRDAN, Stanislav (ed.). 15. slovensko posvetovanje o varstvu rastlin z mednarodno udeležbo = 15th Slovenian Conference on Plant Protection with International Participation : zbornik predavanj in referatov = leactures and papers : 1.-2. marec 2022, Portorož, Slovenija. Ljubljana: Društvo za varstvo rastlin Slovenije: = Plant Protection Society of Slovenia, 2022. p. 268-274.

Analytical sensitivity

Results of bioinformatical subsampling are shown in Figure 2 as fraction of reference covered by reads (%). The subsample with the lowest nucleotide number (1,000,000), corresponding to a nearly 100-fold dilution, still yielded positive detection results.

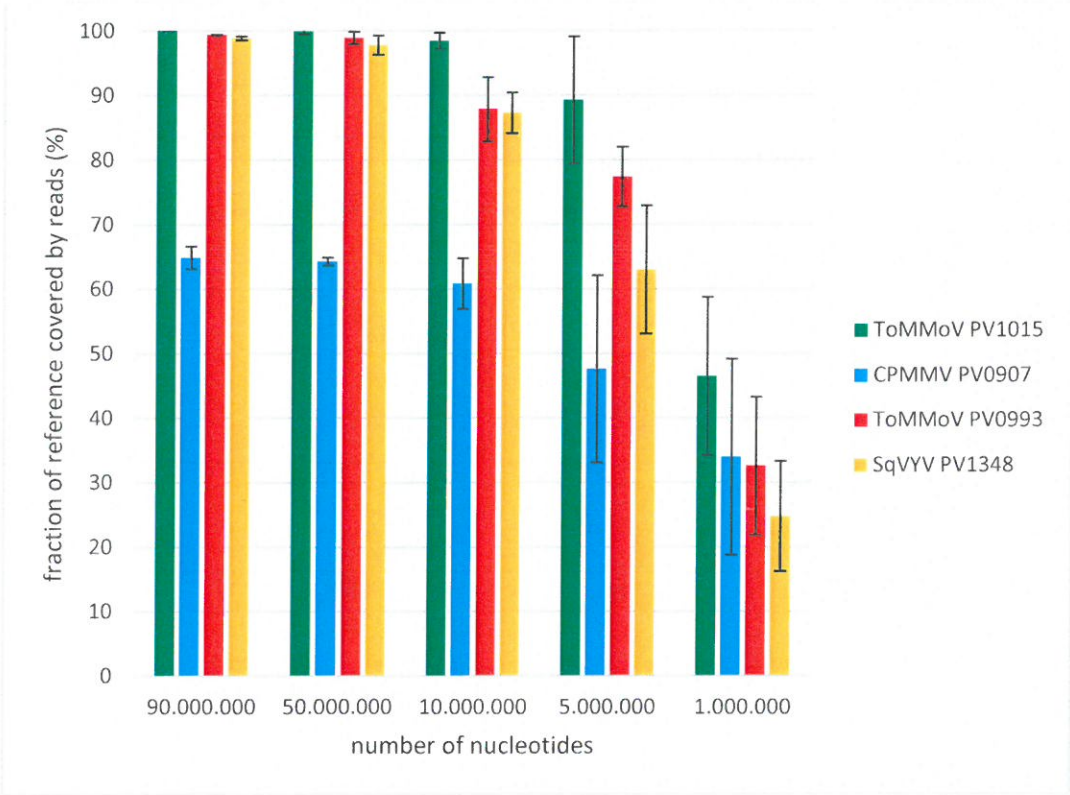

Figure 2: Fraction of reference covered by reads (%) for subsamples of CPMMV PV0907, SQVYV PV1348, ToMMoV PV0993 and ToMMoV PV1015. Error bars represent standard deviation of 5 repetitions.

The analytical sensitivity results obtained by testing the ToMMoV dilutions are shown in Table 9. The highest dilution that gave a positive result, when using complete datasets, was 100x. In addition, bioinformatical subsampling and analysis was performed on 100,000 filtered reads (the lowest expected output using SQK-PCB109 kit = older kit version) and 600,000 filtered reads (the lowest expected output using SQK-PCB111.24 kit = currently used kit version). Both outputs were determined as the lowest expected, when number of all filtered reads was compared across the samples (Figure 3). In the case of 100,000 filtered reads, the highest dilution of ToMMoV that would yield a positive result would be 60x, but not all replicates tested would yield a positive result (e.g., at a 10x dilution, 14 of 15 replicates (93%) would yield a positive result) (Table 9). In the case of 600,000 filtered reads (the lowest expected number of reads in case of currently used kit version), all tested

dilutions, except 1000x, would give positive results. According to our procedure, the maximum number of samples in a bulk is 6. And from this experiment, we can conclude that we can confidently detect ToMMoV even if only one of six samples in a bulk is infected with a virus.

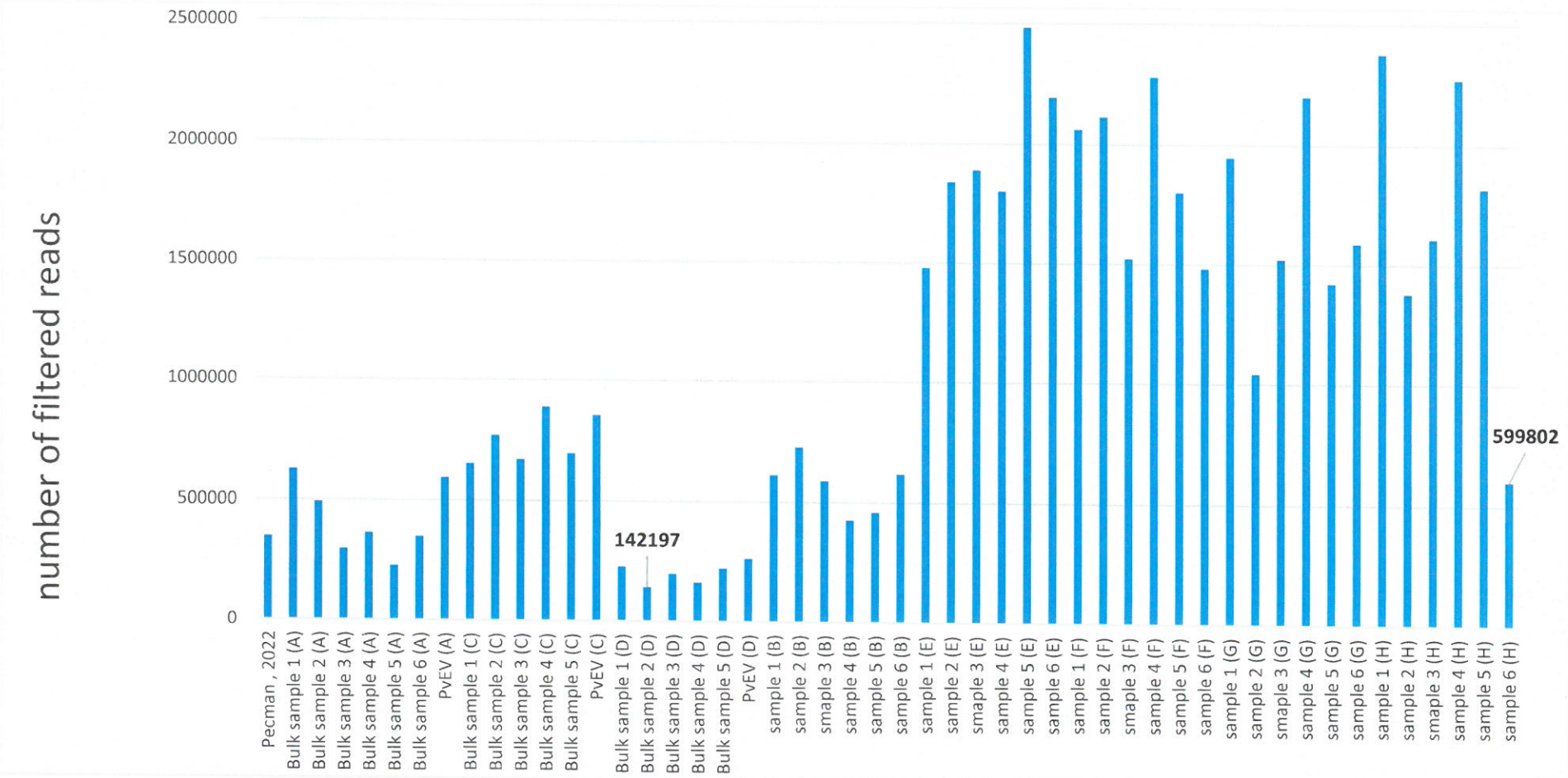

Figure 3: Number of filtered reads across all investigated samples for MInION flowcell A-H (samples analysed on flowcells A, C and D are specified in Table 3; for samples analysed on flowcell B see lab book 02-LD408; for samples analysed on flowcells E, F, G and H see filled forms for the tests with the following IDs: VAL1, VAL2, VAL3 and VAL4). Kit used on MinION flowcells A-D was SQK-PCB109 and on MinION flowcells E-H SQK-PCB111.24. Two datasets with the lowest read number for each of the used flow cell types are designated.

Table 9: Results of analytical sensitivity analysis based on dilutions of ToMMoV PV0993 RNA in RNA extracted from healthy tomato leaves (S. lyc. ID460)

Dilution	MinION flowcell	Number of filtered reads	% of mapped target reads	Number of detected target reads	Virus detected according to 02D-Pos101	Number of target reads in subsamples of 100,000 filtered reads (average of five repetitions) and number of positive replicates of 5 tested	Number of target reads in subsamples of 600,000 filtered reads (average of five repetitions) and number of positive replicates of 5 tested
6x	E	1801844	0.0167	301	yes	15.4 (5/5)	102 (5/5)
10x	F	2278379	0.0052	118	yes	6.8 (5/5)	30.8 (5/5)
	G	1521981	0.0034	51	yes	3.8 (5/5)	17.6 (5/5)
	H	1608942	0.0030	48	yes	1.4 (4/5)	17.2 (5/5)
30x	G	2198169	0.0007	15	yes	0.4 (1/5)	3.8 (5/5)
	H	2271095	0.0013	29	yes	0.8 (3/5)	8.4 (5/5)
60x	G	1422434	0.0005	7	yes	0.2 (1/5)	2.8 (5/5)
	H	1821516	0.0011	20	yes	1.4 (4/5)	7 (5/5)
100x	F	1799832	0.0005	9	yes	0 (0/5)	3.2 (5/5)
1,000x	F	1482617	0	0	no	0 (0/5)	0 (0/5)

Selectivity

No significant influence of plant cultivar on test results was detected (Table 10).

Table 10: Selectivity test on different cultivars. Data on the percentage of mapped target reads are given in the table.

Sample and dilution tested	MinION flowcell	Tomato cultivars in which ToMMV was spiked (sample name)			Virus detected
		S.lyc ID460 (% of mapped reads)	S.lyc ID332 (% of mapped reads)	Different cultivars: Bulk sample 2 (C) (% of mapped reads)	
ToMMoV PV0993 10x	G, G	0.0034	0.0033	nt	yes
ToMMoV PV0993 6x	E, H	0.0167	nt	0.0120	yes

nt – not tested

Repeatability

Two samples of 10-fold diluted ToMMoV PV0993 analysed on the same MinION flowcell yielded a highly similar percentage of mapped reads (see Table 10). Furthermore, in all cases, we were able to confirm the presence of the ERCC control in all six samples tested within the same flowcell using MinION sequencer (ONT) with percentage of mapped reads within set ranges. The percentage of mapped reads from the ERCC control for each sample and for all analyzed MinION flowcells is shown in Table 11. There were some differences in the percentage of mapped reads, but these are not critical to the final results.

Table 11: % of mapped ERCC reads

MInION flowcell	Barcode of sample	WET/DRY lab operator	Number of filtered reads	Number of detected target reads	% of mapped reads	average % of mapped reads	standard deviation	Detected within set ranges
B	1	AP/AP	610270	5277	0.8647	1.3507	1.1101	yes
	2		725837	6437	0.8868			
	3		586881	4994	0.8509			
	4		427789	5035	1.1770			
	5		460803	16572	3.5963			
	6		615993	4486	0.7283			
C	1	VERB/VERB	650713	30611	4.7042	4.0220	0.4932	
	2		769019	30547	3.9722			
	3		669092	25144	3.7579			

	4		888281	40270	4.5335			yes
	5		694531	24030	3.4599			
	6		854828	31663	3.7040			
D	1	VERB/ VERB	228458	8478	3.7110			
	2		142197	6158	4.3306			
	3		197752	8103	4.0976			
	4		162347	6313	3.8886			
	5		221680	7050	3.1803			yes
	6		263724	10812	4.0997	3.8846	0.4042	
E	1	IB/VERB	1480407	58341	0.8647			
	2		1839324	54792	0.8868			
	3		1890382	38163	0.8509			
	4		1801844	37852	1.1770			
	5		2482460	18200	3.5963			
	6		2192478	29493	0.7283	2.1863	1.1455	yes
F	1	IB/VERB	2062114	43326	3.9409			
	2		2112681	23041	2.9789			
	3		1523399	18806	2.0188			
	4		2278379	25983	2.1007			yes
	5		1799832	23924	0.7331			
	6		1482617	22075	1.3452	1.3975	0.3728	
G	1	JB/ANV	1946978	9524	2.1010			
	2		1042972	2904	1.0906			
	3		1521981	9813	1.2345			
	4		2198169	16066	1.1404			
	5		1422434	9592	1.3292			yes
	6		1588303	20315	1.4889	0.6828	0.3348	
H	1	JB/ANV	2376297	47844	0.4892			
	2		1380927	21276	0.2784			
	3		1608942	14940	0.6448			
	4		2271095	27396	0.7309			
	5		1821516	29133	0.6743			yes
	6		599802	7624	1.2790	0.6513	0.3210	
Average percentage of mapped reads and standard deviation for all MinION flowcells						2.0250	1.4699	

Reproducibility

Reproducibility was evaluated on up to 8 flowcells using MinION sequencer (ONT) (for differences, see Table 1). Wet lab analysis were performed by four different members of personnel, and dry lab analysis by three different members of personnel. In all cases, we were able to detect the target (100%

reproducibility). There were some differences in the percentage of mapped reads (for ERCC see Table 11; for ToMMoV, PvEV and RCS see Table 12), but these are not critical to the final results (detection of targets).

Table 12: Results of reproducibility testing

Sample (dilution) / control name	MinION flowcell	WET/DRY lab operator	Number of filtered reads	Number of detected target reads	% of mapped reads	average % of mapped reads	standard deviation	Target detected
ToMMoV PV0993 (10x)	F	IB/VERB	2278379	118	0.0052			yes
	G	JB/ANV	1521981	51	0.0034			yes
	H	JB/ANV	1608942	48	0.0030	0.0038	0.0012	yes
RCS	E	IB/VERB	439051	426234	97.0807			yes
	F	IB/VERB	229119	219983	96.0126			yes
	G	JB/ANV	315207	303582	96.3119			yes
	H	JB/ANV	165917	156541	94.3490	95.9386	1.1513	yes
PvEV1	A	AP/AP	592192	107	0.0181			yes
	B	AP/AP	460803	126	0.0273			yes
	C	VERB/VERB	854828	55	0.0064			yes
	D	VERB/VERB	263724	13	0.0049			yes
	E	IB/VERB	2192478	107	0.0049			yes
	F	IB/VERB	1523399	66	0.0043			yes
	G	JB/ANV	1042972	13	0.0012			yes
	H	JB/ANV	599802	31	0.0052	0.0091	0.0089	yes
PvEV2	A	AP/AP	592192	203	0.0343			yes
	B	AP/AP	460803	168	0.0365			yes
	C	VERB/VERB	854828	155	0.0181			yes
	D	VERB/VERB	263724	78	0.0296			yes
	E	IB/VERB	2192478	148	0.0068			yes
	F	IB/VERB	1523399	84	0.0055			yes
	G	JB/ANV	1042972	51	0.0049			yes
	H	JB/ANV	599802	75	0.0125	0.0185	0.0132	yes
PvEV3	A	AP/AP	592192	392	0.0662			yes
	B	AP/AP	460803	222	0.0482			yes
	C	VERB/VERB	854828	441	0.0516			yes
	D	VERB/VERB	263724	126	0.0497			yes
	E	IB/VERB	2192478	225	0.0103			yes
	F	IB/VERB	1523399	121	0.0079			yes
	G	JB/ANV	1042972	21	0.0020			yes
	H	JB/ANV	599802	67	0.0112	0.0309	0.0254	yes