FITO	SAMPLE **IDENTIFICATION OF CRITICAL POINTS AND ESTIMATION OF THE UNCERTAINTY OF MEASUREMENT**	Label: 02D-Vzo07-01 Page: 1/7 Level of confidentiality: PT-I, Z Valid since: 06-07-2012

Scope	Detection of tomato mild mottle virus (ToMMoV) in tomato leaves using nanopore high-throughput sequencing
Method (name, Pos/Nav)	Nanopore high-throughput sequencing (02D-Pos100 and 02D-Pos101)
Harmful organism	Tomato mild mottle virus (ToMMoV)
Type of sample (description, Pos/Nav)	Tomato leaves; RNA extracted with RNeasy procedure (02D-Pos54, 02D-Pos37)
Remarks	/
Authorized analyst (signature, date)	Denis Kutnjak 29.5.2023
Other providers (signature, date)	Anja Pecman Ana Vučurović 29.5.2023 Irena Bajde 29.5.2023 Jakob Brodarič 29.5.2023 Veronika Bukvič 29.5.2023
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A step in the process	Possible impact on the result	Measures applied to reduce uncertainty	Document that defines the measures
Sampling: type of sample	Unequal distribution of target virus in plant samples.	The sampling procedure is clearly specified and personnel well trained. Different parts are taken from the same plant and bulked together for testing. Upon receipt the samples are verified as suitable for analysis.	Yearly Program of monitoring of plant pathogens (Letni Program preiskav za ugotavljanje navzočnosti škodljivih organizmov rastlin) 02D-Pos11, 02D-Pos102
Sampling: time	Seasonal variation of target virus concentration in samples.	The time of sampling is specified. Upon receipt the sample is verified as suitable for analysis i.e whether it has been sampled at a suitable period of time/ season.	Yearly Program of monitoring of plant pathogens (Letni Program preiskav za ugotavljanje navzočnosti škodljivih organizmov rastlin)
Sampling: labelling of samples	Incorrect labelling of samples – the result of an analysis is recorded under the wrong sample.	Although the labelling of the samples is not entirely under our direct control, the labelling of the samples is clearly specified. Upon receipt of sample, the client's label on the sample is checked to ensure that it matches	Yearly Program of monitoring of plant pathogens (Letni Program preiskav za ugotavljanje navzočnosti škodljivih organizmov

		the label on the sampling form.	rastlin) 02R-Nav05, 02D-Nav01
Transport of the sample to the laboratory	Transport of samples at high temperatures, and freezing and thawing of samples during transport, can cause damage to the samples, which may result in a reduced possibility of detecting target virus in these samples.	The means of delivery to the laboratory are specified and upon receipt, the status of the samples is checked visually.	Yearly Program of monitoring of plant pathogens (Letni Program preiskav za ugotavljanje navzočnosti škodljivih organizmov rastlin) 02D-Pos11, 02D-Pos102
Receipt of the sample	The sample has not been delivered to the responsible person – the analyses are not carried out on time, or not at all.	All employees are familiar with the instructions for receipt of samples.	02R-Nav05, 02R-Sez07
Documenting the sample	Information about the delivered sample does not reach the responsible person - the analyses are not carried out on time, or not at all, or the wrong analyses are performed.	Instructions for the recording of the newly delivered samples are specified.	02R-Sez07, 02D-Nav01, 02D-Nav16
Storage of the sample	Long-term storage of samples at high temperatures, and freezing and thawing of samples, can cause damage to samples, which may result in a reduced possibility of detecting target virus in these samples.	The place for storage of samples is specified as is the time in which samples need to be processed.	02R-Nav05, 02D-Pos102
Selection of tests	An unsuitable test is performed	The battery of tests is specified together with a well-defined sequence of procedures for performing the tests.	02D-Pos102
Analysts (wet and dry lab)	Not familiar with the method – erroneous execution.	The method is carried out only by qualified and competent trained analysts.	02R-Sez04, Competence of the staff will be continuously monitored: by

		Monitoring of continuing competence of the staff.	monitoring PvEV, ERCC and RCS controls and/or by participating in proficiency tests (these will be summarized in a Report of suitability testing)
Preparation of the sample for analysis	Contamination among samples. Choosing an unsuitable part of the sample; insufficient homogenisation of the sample.	Sample preparation and sample homogenization procedures are specified, including the manner of carrying out the work, to prevent contamination among samples. Only responsible authorised persons carry out these procedures. The choice of the sample part is well specified. Homogenisation procedure (with subsequently performed RNA extraction) is controlled using: - internal positive control for RNA extraction (PIC): a PIC (e.g., NAD5) is performed for each sample separately - alien control – PvEV: monitoring contamination throughout the process (role of negative control) and ensuring detection of specific target (not expected in the samples analysed) when used at low concentration (role of positive control throughout the process).	02D-Pos102, 02D-Pos100, 02D-Pos101, 02D-Pos37, 02D-Pos54, 02R-Sez04
Extraction	Choosing an unsuitable method for extraction of RNA; an error when carrying	The choice and performance of the extraction procedure are well specified. They are	02D-Pos37, 02D-Pos54, 02D-Pos102, 02D-Pos100,

	out the extraction procedure; contamination during the extraction procedure.	carried out only by responsible authorised persons. The alien control (PvEV) is included in the extraction procedure. Adequacy of RNA extraction procedure is verified for every individual sample by amplification of a PIC (e.g., NAD5).	02D-Pos101, 02R-Sez04
Preparation of bulk samples	Bulk sample does not contain the same amount of RNA from each sample	The amount of added RNA in a bulk is calculated based on the measurement of RNA concentration for all samples.	02D-Pos100, 02D-Obr162
DNase digestion	False negative results due to an error in performing the DNase digestion step	Alien control PvEV has also in this step the function of a positive control	02D-Pos100, 02D-Pos101
Plant ribosomal RNA depletion, polyadenilation	False negative results due to an error in performing the plant ribosomal RNA depletion and/or polyadenylation steps	In addition to the PvEV, these steps are also controlled by the ERCC control.	02D-Pos100, 02D-Pos101
Library preparation, sequencing, bioinformatic analysis	False negative results due to an error in performing the library preparation, sequencing and/or bioinformatic analysis	In addition to the PvEV and ERCC, these steps are also controlled by the RCS control.	02D-Pos100, 02D-Pos101
Impact of inhibitors	False negative results due to the presence of the inhibitors in the RNA extract.	An internal positive control for RNA extraction from each individual sample (e.g., NAD5) is also used to monitor the presence of the inhibitors in the RNA extract. If the presence of inhibitors is suspected, RNA extraction could be repeated using an alternative (different) extraction procedure. In addition, potential inhibition after the DNase digestion step is controlled by ERCC added to each sample.	02D-Pos102, 02D-Pos54 02D-Pos100, 02D-Pos101

Impact of the matrix on specificity	New and unknown matrix - the presence of nucleotide sequences, some of which are similar to the target virus.	Confirmatory tests are performed for all cases showing the presence of the nucleotide sequence of ToMMoV or other quarantine viruses.	02D-Pos102
Impact of the matrix on sensitivity	New and unknown matrix – decreased sensitivity of the method.	The effect of matrix on sensitivity is controlled by ERCC addition to each sample. However, if low levels of the target virus are expected (e.g., asymptomatic samples), a single RNA sample may be analysed instead of a bulk sample.	02D-Pos100, 02D-Pos101 02D-Pos102
Possibility of contamination	False-positive results.	Alien control (PvEV) is used to monitor contamination throughout the process (role of negative control). Measures to prevent contamination are well defined including carrying out individual steps of the analytic procedure in separate rooms or chambers.	02D-Pos100, 02D-Pos101, 02D-Pos18
Impact of laboratory materials and equipment	Impact on the results: wrong laboratory material and/or equipment used. Contamination of the laboratory materials and/or equipment Use of uncalibrated or unvalidated equipment.	Negative and positive controls (PvEV, ERCC and RCS) are always included. All equipment is regularly maintained and calibrated. The suitability of material and equipment is verified by comparison of the results of positive controls (PvEV, ERCC and RCS).	02D-Pos100, 02D-Pos101 This will be continuously monitored (summarized in a Report of suitability testing).
Impact of the pipetting	Non- accurate pipetting of small volumes. Increased possibility of contamination: pipetting tips without filters.	Only calibrated pipettes are used. Measures to reduce impact include are clearly described (use of pipette tips with filters in all critical steps, UV chambers, etc). Alien control PvEV is used	02D-Pos100, 02D-Pos101, 02D-Pos18

		throughout the process (monitoring contamination throughout the process, ensuring detection of specific target when used at low concentration).	
Impact of the chemicals	False negative or false positive results due old chemicals, improper storage of chemicals, improper choice of chemicals, poor quality of a lot of chemicals, contaminated chemicals	Alien control PvEV is used throughout the process (monitoring contamination throughout the process, ensuring detection of specific target when used at low concentration). Expiration dates of chemicals and lot numbers are recorded.	02D-Pos100 and associated forms (from 02D-Obr162 to 02D-Obr167)
Impact of the environment	Impact of temperature when pipetting small volumes.	Before pipetting, the temperature of the liquid is allowed to equilibrate to room temperature. The temperature of the rooms is specified and checked to ensure that it is within specification.	02D-Obr162, 02D-Obr163, 02D-Obr164, 02D-Obr165, 02D-Obr166, 02D-Obr167
Impact of the equipment and/ or reagent changes	Changes to the equipment or reagent can lead to incorrect results	Negative and positive controls (PvEV, ERCC, and RCS) are always included. These controls are also used to evaluate new equipment and changes in critical reagents (see 02D-Nav24). The suitability of new reagents and equipment is verified by comparing the results of the PvEV, ERCC, and RCS controls with the results of these controls from previous runs.	02D-Pos101 02D-Nav24 02A-Nav01 This comparison (verification) will be summarized in a Report of suitability testing.
Impact of the software or database changes	Changes to the software or database can lead to incorrect results	In case of a change of software or database version, a bioinformatic analysis of the PvEV of the previous run should be performed prior to the bioinformatic analysis of the samples.	02D-Pos101
Analysis of the results, determination and entry of the test results	False-negative results or false-positive results (cross-talk).	Data analysis, and interpretation of control and sample results are clearly specified.	02D-Pos101, 02D-Vzo17

		Confirmatory tests are performed for all cases showing the presence of the nucleotide sequence of ToMMoV or other quarantine viruses.	02D-Pos102
End result of testing – a result that provides the final confirmation of presence/absence	The final result depends on the partial results of different tests - no clearly defined rules for communicating the final result could lead to wrong conclusions. Incorrect presentation of the final results can confuse the client.	The criteria for finalising the samples are clearly stated. Only the responsible authorised person (s) may approve the final test result. Presentation of the final test results is specified.	02D-Pos102, 02R-Sez07, 02D-Pos11, 02D-Pos18
Informing clients and issuing test reports	Final results not sent to all parties that are eligible according to the contract for receiving these results. Delay in informing a customer about the results of the analyses.	There are clearly stated guidelines for issuing the final results and reports. All correspondence with the client is archived to ensure traceability.	02D-Nav01 Yearly Program of monitoring of plant pathogens (Letni Program preiskav za ugotavljanje navzočnosti škodljivih organizmov rastlin)
Archiving	A complaint from a customer about the execution of the analyses, court request in case of legal proceedings.	Test reports from previous years archived together with traceability from receiving sample to issuing test reports. All positive RNA extracts are kept in the collection of RNA.	02D-Vzo01, 02D-Pos18, 02D-Nav01, 02R-Nav02, 02R-Nav08
Other/remarks	New strains of ToMMoV capable of infecting tomatoes may arise.	The procedure itself would detect all strains of ToMMoV based on generic property of HTS method.	/