NACIONALNI INŠTITUT ZA **BIOLOGIJO**
NATIONAL INSTITUTE OF **BIOLOGY**

European Union Reference Laboratory for Pests of Plants on Viruses, Viroids and Phytoplasmas

Co-funded by the European Union

EURL-Virology

(European Union Reference Laboratory for Pests of Plants on Viruses, Viroids and Phytoplasmas)

TESTING OF CHRYSANTHEMUM STEM NECROSIS VIRUS BY REAL-TIME RT-PCR

VALIDATION REPORT

VERSION 30 APRIL 2024

The validation was performed by the National Institute of Biology, Department of Biotechnology and Systems Biology, Microbiology Unit, Laboratory for detection of viruses, viroids and phytoplasmas, Večna pot 121, Ljubljana, Slovenia, a partner of the European Union Reference Laboratory for pests of plants on viruses, viroids and phytoplasmas.

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Suggested Citation:

Mehle, N., Bajde, I., Brodarič, J., & Vučurović A., 2024. Validation report on the testing of Chrysanthemum stem necrosis virus by real-time RT-PCR (version April, 30 2024). EURL-Virology; National Institute of Biology, Ljubljana, Slovenia.

Contents

1 Scope of validation

Detection and identification of Chrysanthemum stem necrosis virus (CSNV).

Method	Real time RT-PCR as described in Appendix 6 of EPPO PM7/139(1) Tospoviruses
Harmful organism	Chrysanthemum stem necrosis virus
Sample type	Leaves of host plants (e.g., Chrysanthemum, tomato)

2 Description of the method

2.1 RNA extraction

Freeze-dried leaf tissue (representing 100 mg fresh leaf weight) was ground in 450 µL of buffer RLT from the RNeasy Plant Mini Kit (Qiagen). RNA was then extracted from the entire homogenate according to the manufacturer's instructions. Total RNA was eluted twice with 50 µL (total of 100 µL) of RNase-free water pre-warmed to 65 °C. To monitor the RNA extraction procedure, a real-time RT-PCR amplifying plant nad5 transcript (*nad5;* Botermans et al., 2013) was included in the analysis (data not shown).

2.2 Real-time RT-PCR

CSNV specific real-time RT-PCR was used as described in Appendix 6 of EPPO PM7/139(1) Tospoviruses.

Reagents used: AgPath-IDTM One-step RT-PCR kit (Ambion)

Equipment used: Applied Biosystems 7900HT Fast, QuantStudio7 Pro, QuantStudio7 Flex and PRISM ViiA7.

Cq cut-off value determined with these reagents and equipment: 36.5.

3 Validation procedure

Validation was performed according EPPO PM7/98 5) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity.

3.1 Analytical specificity

'In-silico' comparison of the amplicon sequence (sequence comprising primers and probe: TGAATTTGAGGAAGAACAGAACCANNTTGCATTCAACTTCCNNNNNNNNTGCAATGACA ACCTGGATCAG) to sequences in the genomic libraries (sequences available at the NCBI nucleotide database on 18 March 2024) has been done. In addition, primer blast with forward and reverse primers was done using Primer-Blast tool at NCBI against "nr" database (on 18 March 2024).

Tested targets (Table 1):

- 3 different CSNV isolates
- gBlocks representing fragments of CSNV sequences of the nucleocapsid (N) gene (listed are accession numbers of NCBI sequences used for designing the gBlocks): LC496394.1, NC 027719.1, LC719278.1, AB600871.1, AB600873.1. These gBlocks have been tested in concentrations about 1 million copies per reaction (10^{-5} dilution).

Tested non-targets (Table 1):

- 24 isolates of six different tospovirus species from American Clade 1, i.e., Alstroemeria necrotic streak virus (ANSV), Groundnut ringspot virus (GRSV), Impatiens necrotic spot virus (INSV), Melon severe mosaic virus (MSMV), Tomato chlorotic spot virus (TCSV), Tomato spotted wilt virus (TSWV)
- Two different tospovirus species from Asian Clade 1, i.e., Capsicum chlorosis virus (CaCV) and Watermelon silver mottle virus (WSMoV)
- Three isolates of two different tospovirus species from Eurasian Clade, i.e., Iris yellow spot virus (IYSV) and Tomato yellow ring virus (TYRV)
- Chrysanthemum plant infected with pospiviroid Chrysanthemum stunt viroid (CSVd)

3.2 Analytical sensitivity

To determine the analytical sensitivity of the real-time RT-PCR test, the following experiments were carried out:

- Analysis of serial dilutions of RNA of CSNV PV-0529* in RNA of chrysanthemum leaves, which was infected with CSVd (PV-0735)
- Analysis of serial dilutions of RNA of CSNV PV-0529* in water

- Analysis of serial dilutions of RNA of CSNV NIB V 038 in water

* The RNA of CSNV PV-0529 was extracted from the same freeze-dried plant material, but on different days.

3.3 Selectivity

Leaves of different tospovirus host plants were tested as part of the analytical specificity evaluation (see Table 1), and these results were also used for the selectivity evaluation. In addition, data from the analysis of official samples were evaluated regarding these performance characteristics.

3.4 Repeatability

RNA dilutions with high, medium and low concentrations of CSNV NIB V 038 were tested in five replicates in the same real-time RT-PCR run.

3.5 Reproducibility

Reproducibility evaluated on RNA extracts:

- Two dilutions of the CSNV PV-0529 RNA sample (dilutions 10^{-1} and 10^{-2}) were tested in 13 runs (on 13 different days). These were performed by three different operators (IB, JB, TJ) and with one of the following devices: ABI 7900HT Fast, ABI QuantStudio7 Pro, ABI QuantStudio7 Flex, ABI PRISM ViiA7
- Two dilutions of the CSNV PV-1219 RNA sample (dilutions 10^{-1} and 10^{-2}) were tested in nine runs (on 9 different days). These were performed by two different operators (IB, JB) and with one of the following devices: ABI 7900HT Fast, ABI QuantStudio7 Pro (two different machines)

Reproducibility evaluated on freeze-dried homogenates of leaf tissue:

- DSMZ (Germany) prepared several aliquots of freeze-dried homogenates of leaf tissue. These were aliquots of CSNV isolates from the DSMZ collection: PV-0529, PV-0529 mixed with leaves of CSNV negative chrysanthemums (ratio: 1:1), PV-1219 and PV-1219 mixed with leaves of CSNV negative chrysanthemums (ratio: 1:3). Four aliquots of each preparation were included in the reproducibility assessment. RNA extractions and real-time RT-PCRs have been performed by two different operators (IB, JB) and with one of the following devices: ABI 7900HT Fast, ABI QuantStudio7 Pro. In real-time RT-PCR, each RNA extract was tested in three wells.

Table 1. Isolates used for validation. Targeted CSNV isolates are in red shaded rows.

Virus*	Isolate	Collection	Origin	Collection year	Original host plant	Type of sample received	Lab host plant
Genus *Orthotospovirus, American Clade 1*							
ANSV	PV-1027	DSMZ, Germany	Colombia	before 2011	*Alstroemeria* sp.	fresh	*Nicotiana rustica*
CSNV	PV-0529	DSMZ, Germany	Unknown	before 1998	*Chrysanthemum* sp.	fresh	*N. benthamiana*
CSNV	PV-1219	DSMZ, Germany	Slovenia	October 2001	*Chrysanthemum* sp.	lyophilized	*N. benthamiana*
CSNV	NIB V 038	NIB, Slovenia	Slovenia	2001	*Chrysanthemum* sp.	frozen	*N. benthamiana*
GRSV	PV-0205	DSMZ, Germany	South Africa	before 1988	*Arachis hypogaea*	fresh	*N. benthamiana*
INSV	PV-0281	DSMZ, Germany	Germany	before 1999	*Anemone coronaria*	fresh	*N. benthamiana*
INSV	PV-0280	DSMZ, Germany	USA	1990	*Hippeastrum* sp	lyophilized	*N. benthamiana*
INSV	PV-0485	DSMZ, Germany	unknown	1998	*Gloxinia* sp.	lyophilized	*N. benthamiana*
INSV	PV-1123	DSMZ, Germany	Germany	2014	*Lobelia speciosa*	lyophilized	*N. clevelandii*
INSV	PV-1189	DSMZ, Germany	Germany	2016	*Ocimum basilicum*	lyophilized	*N. benthamiana*
MSMV	VE440	IPSP CNR, Italy	Mexico	2007	melon	lyophilized	*N. benthamiana*
TCSV	PV-0390	DSMZ, Germany	Brazil	before 1994	pepper	fresh	*N. rustica*
TCSV	PV-0391	DSMZ, Germany	Brazil	1994	pepper	lyophilized	*N. rustica*
TSWV	PV-0182	DSMZ, Germany	Bulgaria	before 1988	*N. tabacum*	fresh	*N. rustica, N. benthamiana*
TSWV	PV-1175	DSMZ, Germany	Hungary	before 2015	pepper	fresh	*N. tabacum* cv. Xanthi, *N. rustica*
TSWV	PV-0393	DSMZ, Germany	Bulgaria	before 1993	*N. tabacum*	fresh	*N. tabacum cv.* Samsun
TSWV	PV-0204	DSMZ, Germany	Germany	before 1988	*Impatiens hawkeri* New-Guinea	fresh	*N. rustica*
TSWV	/	WSU, USA	USA		tomato	frozen	*N. benthamiana*
TSWV	108-19	UB-FA, Serbia	Serbia	2019	tomato cv Wrestler	frozen	*N. tabacum, Solanum lycopersicum*
TSWV	109-19	UB-FA, Serbia	Serbia	2019	tomato cv Wrestler	frozen	*N. tabacum, S. lycopersicum*
TSWV	France 77	INRA, France	France	2008	chilli pepper (RB strain)	fresh	*C. annuum* cv. Yolo wonder
TSWV	/	CREA, Italy	Italy, Lazio	2011	pepper	lyophilized	*C. annuum*
TSWV	/	CREA, Italy	Bulgaria	2012	tomato	lyophilized	*S. lycopersicum*
TSWV	/	CREA, Italy	Italy, Calabria	2014	hot pepper	lyophilized	*C. annuum*
TSWV	/	CREA, Italy	Italy, Lazio	2015	pepper	lyophilized	*C. annuum*
TSWV	/	CREA, Italy	Italy, Lazio	2016	lisianthus	lyophilized	*Lysianthus*

TSWV	21007721	NPPO-NL, NVWA	Netherlands	2001	*Ligularia*	fresh	*N. benthamiana*
Genus *Orthotospovirus, Asian Clade 1*							
CaCV	PV-0864	DSMZ, Germany	Thailand	2005	tomato	lyophilized	*N. benthamiana*
WSMoV	PV-0283	DSMZ, Germany	Taiwan	1991	tomato	lyophilized	*N. benthamiana*
Genus *Orthotospovirus, Eurasian Clade*							
IYSV	PV-0528	DSMZ, Germany	unknown	1998	leek	lyophilized	*N. benthamiana*
TYRV	PV-0526	DSMZ, Germany	Iran	1998	tomato	lyophilized	*N. benthamiana*
TYRV	PV-0532	DSMZ, Germany	Iran	1998	tomato	lyophilized	*N. benthamiana*
Genus *Pospiviroid*							
CSVd	PV-0735	DSMZ, Germany	Germany	Dec 2001 or before	chrysanthemum cv Dream Time	lyophilized	*Chrysanthemum*
Healthy tomato leaves							
/	/	DSMZ, Germany	unknown	unknown	tomato	lyophilized	*S. lycopersicum*

*for virus species name see EPPO PM7/139(1)

4 Results of validation

4.1 Evaluation of RNA extraction

The performance of RNA extraction was verified for each sample by real-time RT-PCR using a set of oligonucleotide primers and a probe for *nad5*. RNA extraction was considered successful if *nad5* was detected with a Cq value below 33.6. If this was not achieved, the RNA extraction was repeated.

4.2 Analytical specificity

In silico NCBI BLAST and Primer blast gave the same results. In total there are 24 publicly available CSNV sequences containing the target sequence of the CSNV amplicon available at NCBI GenBank database. *In silico* NCBI BLAST analyses confirmed the specificity of the CSNV amplicon. However, multiple alignment of all the publicly available sequences of CSNV, plus the sequence of one isolate available at NIB (isolate from Gerbera D680/02 described in Boben et al. (2007)), revealed some mismatches between available CSNV sequences and the primers and probe sequences (Figure 1). The oligonucleotide primers and probe sequences are only identical with the isolate AF067068 from the NCBI (which was used to design primers and probe). All other isolates have one or two mismatches with primer/probe sequences. These differences are as follows:

- One nucleotide difference in the reverse primer sequence: of 15 available CSNV sequences with one difference in the nucleotide sequence at the reverse primer annealing site, three isolates were tested by CSNV-specific real-time RT-PCR: MF093685.1 (NIB V 038), MW051793.1 (DSMZ PV-0529) and OL584373 (DSMZ PV-1219), and in all these three cases, CSNV-specific real-time RT-PCR yielded a Cq value between 11 and 17 (Table 3). It can be concluded that this one difference in nucleotide sequence at the reverse primer annealing site has no influence on the detection of CSNV.
- Two nucleotide differences in the reverse primer sequence: Sequence of LC496394.1. The possibility of detecting this CSNV sequence was verified by laboratory testing of gBlock LC496394.1.
- A nucleotide difference in the forward primer sequence and in the reverse primer sequence: Sequences of AB438998.1 and AB600873.1. The possibility of detecting these CSNV sequences was verified by laboratory testing of gBlock AB600873.1.
- A nucleotide difference in the probe and in the reverse primer sequence:

- Sequence of AB600871.1. The possibility of detecting this CSNV sequence was verified by laboratory testing of gBlock AB600871.1
- Sequence of NC_027719.1. The possibility of detecting this CSNV sequence was verified by laboratory testing of gBlock NC_027719.1.
- Sequences of LC719278.1, LC719279.1, LC719280.1, LC719281.1. The possibility of detecting these CSNV sequences was verified by laboratory testing of gBlock LC719278.1.

All sequences containing the difference in the probe sequence concern isolates originating from Japan (Table 2).

In summary, the inclusivity assessed with three CSNV isolates and with five CSNV gBlocks is 100%. The range of Cq values obtained for the CSNV isolates tested, was between 11 and 17, and for gBlocks between 16 and 24 (Table 3).

Testing of 29 non-target tospoviruses and CSVd resulted in 100% exclusivity when a Cq cut-off value 36.5 was considered (Table 3).

Figure 1. Alignment of the CSNV primers and probe and the partial sequences of the nucleocapsid protein (N) gene of CSNV isolates. 24 sequences were obtained from the NCBI GenBank database, and one sequence (Gerbera Slo ID of isolate D680/02) was obtained during studies conducted at NIB.

Table 2. Metadata for CSNV sequences available in NCBI

Accession number	Isolate	Host plant	Collection year	Year of submission to the GenBank	Country	Region/Location
AF067068.1	Chry1	Chrysanthemum	/	1999	Brazil	/
MF093685.1	NIB-V38	Chrysanthemum	2001	2017	Slovenia	/
LC126118.1	Kr	Chrysanthemum x morifolium 'Jinba'	2014	2016	South Korea	Gyeongsangnam-do
OP289529.1	CSNV-Ca	Capsicum annuum	2019	2023	Brazil	/
KF493771.1	TcCh07A	Chrysanthemum	2007	2015	Japan	Tochigi Prefecture
LC061220.1	Gm2	Chrysanthemum	/	2015	South Korea	/
LC061219.1	Gm1	Chrysanthemum	/	2015	South Korea	/
OL584373.1	DSMZ PV-1219	Chrysanthemum	/	2022	Slovenia	/
KC894721.1	Lis 519	Lisianthus	2012	2014	Brazil	/
MW051793.1	DSMZ PV-0529	Chrysanthemum	/	2021	/	/
KC525102.1	CSNV_ChrysLO_FVR_ILVO2012	Chrysanthemum x morifolium cultivar Ludo Orange	2012	2013	Belgium	/
JQ764839.1	/	Chrysanthemum	2011	2012	/	/
AB600872.1	TcCh07A	Chrysanthemum	2007	2015	Japan	Tochigi
AB600870.1	CbCh07A	Chrysanthemum	2007	2015	Japan	Chiba
AB597291.1	/	/	2009	2011	Japan	Toyama
LC496394.1	Oita_2015	Capsicum annuum	2015	2023	Japan	Oita
NC_027719.1	PD4412741	Chrysanthemum x morifolium Ramat	2009	2018	Japan	/
LC719281.1	cine	Senecio cruenius	2019	2023	Japan	/
LC719280.1	cyc	Cyclamen persicum	2018	2023	Japan	/
LC719279.1	glo	Gomphrena globosa	2019	2023	Japan	/
LC719278.1	bego	Begonia tuberhybrida	2019	2023	Japan	/
AB600873.1	HiCh06A L1	Chrysanthemum	2006	2015	Japan	Hiroshima
AB600871.1	GnCh07S	Chrysanthemum	/	2015	Japan	Gunma
AB438998.1	HiCh06A L1	Chrysanthemum	2006	2009	Japan	Hiroshima

Table 3. Results of testing target and non-target viruses with a CSNV-specific real-time RT-PCR ()

Virus*	Isolate	RNA dilution tested	Real-time RT-PCR replicate 1 (Cq)	Real-time RT-PCR replicate 2 (Cq)
Genus *Orthotospovirus, American Clade 1*				
ANSV	PV-1027	undiluted	neg	neg
CSNV	PV-0529	10-fold dilution	16.6	16.7
CSNV	PV-1219	undiluted	11.2	11.1
CSNV	NIB V 038	undiluted	16.6	16.5
CSNV	gBlock LC496394.1	10^{-5} dilution	23.5	23.1
CSNV	gBlock AB600873.1	10^{-5} dilution	18.5	18.5
CSNV	gBlock of AB600871.1	10^{-5} dilution	18.3	18.4
CSNV	gBlock of NC027719.1	10^{-5} dilution	17.8	18.1
CSNV	gBlock of LC719278.1	10^{-5} dilution	15.7	15.7
GRSV	PV-0205	undiluted	neg	neg
INSV	PV-0281	10-fold dilution	neg	neg
INSV	PV-0280	undiluted	neg	neg
INSV	PV-0485	undiluted	neg	neg
INSV	PV-1123	undiluted	36.5	neg
INSV	PV-1189	undiluted	neg	neg
MSMV	VE440	10-fold dilution	neg	neg
TCSV	PV-0390	undiluted	neg	neg
TCSV	PV-0391	undiluted	neg	neg
TSWV	PV-0182	10-fold dilution	neg	neg
TSWV	PV-1175	100-fold dilution	neg	neg
TSWV	PV-0393	10-fold dilution	neg	neg
TSWV	PV-0204	undiluted	neg	neg
TSWV	WSU, USA	undiluted	neg	neg
TSWV	108-19	undiluted	neg	neg
TSWV	109-19	undiluted	neg	neg
TSWV	France 77	undiluted	neg	neg
TSWV	CREA, Italy 2011	undiluted	37.3	neg
TSWV	CREA, Italy 2012	undiluted	neg	neg
TSWV	CREA, Italy 2014	undiluted	neg	neg
TSWV	CREA, Italy 2015	undiluted	neg	neg
TSWV	CREA, Italy 2016	undiluted	37.2	neg
TSWV	21007721	undiluted	neg	neg
Genus *Orthotospovirus, Asian Clade 1*				
CaCV	PV-0864	undiluted	neg	neg
WSMoV	PV-0283	undiluted	neg	neg
Genus *Orthotospovirus, Eurasian Clade*				
IYSV	PV-0528	undiluted	neg	neg
TYRV	PV-0526	undiluted	neg	neg
TYRV	PV-0532	undiluted	neg	neg
Genus *Pospiviroid*				
CSVd	PV-0735	undiluted	neg	neg
Healthy tomato leaves				
tomato	DSMZ, Germany	undiluted	neg	neg

Results of two parallel tests are shown: Cq value is given when an exponential amplification curve was observed, while »neg« is given for cases without an exponential amplification curve.

4.3 Analytical sensitivity

Based on a dilution series prepared from RNA of CSNV isolates diluted in RNA of CSVd-infected chrysanthemum leaves or in water, the analytical sensitivity was 10^{-4}. However, CSNV in a dilution of 10^{-5} was also detected in some replicates (Table 4). The differences in Cq values obtained with CSNV PV-0529 dilutions prepared in water and in chrysanthemum RNA are most likely due to differences in RNA extraction (see Reproducibility section).

Table 4. Results of testing the CSNV dilutions with a CSNV-specific real-time RT-PCR

Sample		Real-time RT-PCR replicate 1 (Cq)	Real-time RT-PCR replicate 2 (Cq)
CSNV PV-0529 dilutions in RNA of chrysanthemum	10^{-1}	22.6	22.2
	10^{-2}	26.2	25.7
	10^{-3}	29.9	29.4
	10^{-4}	32.5	33.3
	10^{-5}	36.4	36.9
	10^{-6}	neg	neg
CSNV PV-0529 dilutions in water	10^{-1}	17.1	17.2
	10^{-2}	21.6	21.6
	10^{-3}	26.9	26.9
	10^{-4}	30.5	30.7
	10^{-5}	35.5	36.1
	10^{-6}	neg	neg
	10^{-7}	neg	neg
CSNV NIB V 038 dilutions in water	undiluted	16.6	16.5
	10^{-1}	19.8	19.7
	10^{-2}	25.7	25.5
	10^{-3}	30.0	30.2
	10^{-4}	33.6	34.9
	10^{-5}	36.3	neg
	10^{-6}	neg	neg
	10^{-7}	neg	neg

Results of two parallel tests are shown: Cq value is given when an exponential amplification curve was observed, while »neg« is given for cases without an exponential amplification curve.

4.4 Selectivity

No reactions were observed in samples of *Chrysanthemum spp*. (1), *Solanum lycopersicum* (2), *Capsicum annuum* (4) and other host plants of tospoviruses that were not infected with CSNV (see Tables 1 and 2). Also, when applying the test to official samples (e.g. several leaf samples of different varieties of *S. lycopersicum*, *Chrysanthemum* spp., *Gerbera* sp., *C. annuum*), no cross-reactions with the host tissue were observed (data not shown).

The test was successfully used for the detection of CSNV in four official samples of *Chrysanthemum* spp. and in one official sample of *Gerbera* sp. (data not shown).

4.5 Repeatability

Repeatability has been evaluated on CSNV NIB V 038 RNA dilutions. The repeatability was 100% (Table 5).

Table 5: Testing of RNA dilutions of CSNV NIB V 038 by CSNV-specific real-time RT-PCR in five replicates.

	Undiluted sample	Dilution: 10^{-3}	Dilution: 10^{-4}
Replicate 1 (Cq)	16.2	30.7	34.9
Replicate 2 (Cq)	15.7	30.4	35.5
Replicate 3 (Cq)	15.9	30.6	36.2
Replicate 4 (Cq)	16.0	30.4	37.6
Replicate 5 (Cq)	15.9	30.6	34.8
Average (Cq)	**15.9**	**30.6**	**35.8**
Standard deviation (Cq)	**0.18**	**0.12**	**0.14**
Positive replicates (%)	**100%**	**100%**	**100%**

4.6 Reproducibility

Two dilutions of RNA from two CSNV isolates analysed in 9 (PV-1219) and 13 (PV-0529) runs of real-time RT-PCR, respectively, showed that the reproducibility of the test was 100% (Figure 2). Dilutions with a higher amount of target RNA (10^{-1}) resulted in a mean Cq value of 16.6 (PV-0529)/ 19.4 (PV-1219) with a standard deviation of 0.64 (PV-0529)/ 0.45 (PV-1219). The mean Cq values for the dilutions with a lower amount of target RNA (10^{-2}) were 19.7 (PV-0529)/ 22.6 (PV-1219) with a standard deviation of 0.48 (PV-0529)/ 0.46 (PV-1219).

The test also showed to be 100% reproducible when it was evaluated using freeze-dried homogenates of leaf tissue, which means that the RNA extraction step was also included in the evaluation. However, as expected, a higher variation in Cq values was obtained in this case (Table 6).

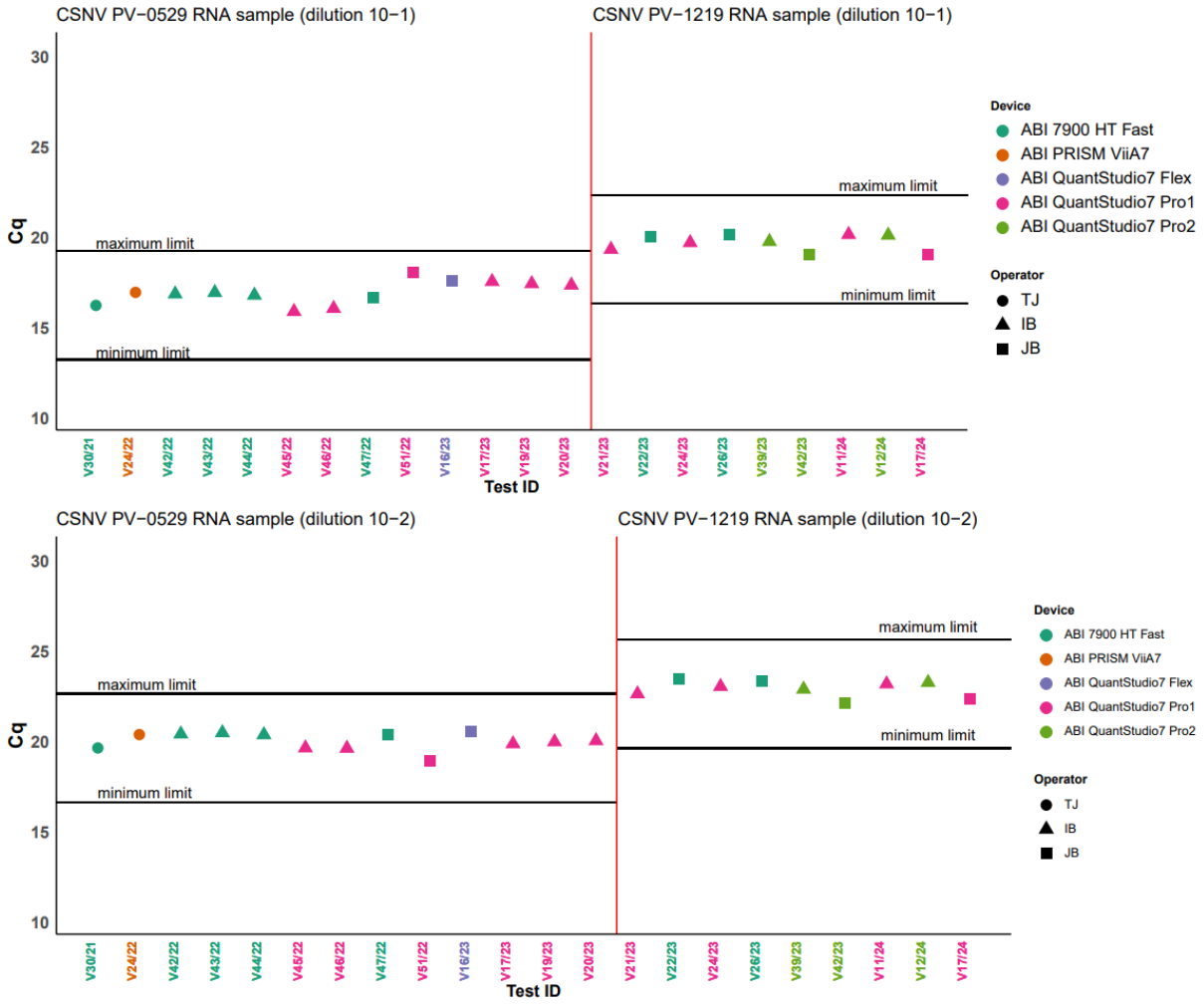

Figure 2: Run sequence plot of the repeated analysis of RNA extracts of dilutions of CSNV PV-0529 and PV-1219. The different PCR cyclers and operators are indicated. Cq values are shown, using the same threshold in each run. The upper (maximum accepted) and lower limits (minimum accepted) are set to the Cq value of the first test ± 3 Cq.

Table 6: Multiple testing of freeze-dried homogenates of leaf tissue infected with CSNV by CSNV-specific real-time RT-PCR.

Test ID	CSNV PV-0529	CSNV PV-0529 + negative chrysanthemum (ratio: 1:1)	CSNV PV-1219	CSNV PV-1219 + negative chrysanthemum (ratio: 1:3)
V20/23	Pos (17.4)	Pos (17.4)	Pos (16.8)	Pos (23.0)
V21/23	Pos (15.8)	Pos (20.3)	Pos (16.2)	Pos (22.7)
V24/23	Pos (15.8)	Pos (18.7)	Pos (16.8)	Pos (21.7)
V26/23	Pos (16.6)	Pos (15.8)	Pos (11.1)	Pos (13.6)
Positive replicates (%)	**100 %**	**100 %**	**100 %**	**100 %**

For the samples in which CSNV was detected (indicated as »Pos«), the average Cq values of three replicates are given in parentheses.

5 Conclusions

Criteria		Results
Analytical sensitivity	Tested concentrations	Dilutions of CSNV isolates in water and in RNA of chrysanthemum
	LOD	10^{-4}
Analytical specificity	Number of tested samples	No of targets tested: 8 (3 isolates + 5 gBlocks) No of non-targets tested: 30 (29 isolates of other tospoviruses and one CSVd)
	Inclusivity	100%
	Exclusivity	100%
Selectivity	Impact of the matrix	No effect of the matrix on the test result detected
Repeatability	No. of replicates, percentage of identical results	No. of samples tested: 3 (high, medium and low target concentration) No. of replicates tested: 5 Percentage of identical results (positive replicates) is 100%
Reproducibility	No. of replicates, percentage of identical results	No. of target isolates tested: 2 (for each isolate different dilutions were evaluated) No. of operators: up to 3 No. of real-time PCR instruments: up to 5 No. of different days: up to 13 Percentage of identical results (positive replicates) is 100%

6 Reference

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