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Rubio, L.; Giménez, K.; Romero, J.; Font San Ambrosio, MI.; Alfaro Fernández, AO.; Galipienso, L. (2022). Detection and absolute quantitation of watermelon mosaic virus by real-time RT-PCR with a TaqMan probe. Journal of Virological Methods. 300:1-5. https://doi.org/10.1016/j.jviromet.2021.114416

The final publication is available at

https://doi.org/10.1016/j.jviromet.2021.114416

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Additional Information

1 **Detection and absolute quantitation of watermelon mosaic virus by real-time RT-PCR with a TaqMan**
2 **probe**

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11

12 Keywords: WMV, potyvirus, cucurbits, viral accumulation, diagnostics

13
14

15 **Abstract**

16 Watermelon mosaic virus (WMV) causes serious damage to several crops worldwide, mainly cucurbits. Disease
17 control is based on preventing spread and search for natural resistances for plant breeding, which requires tools
18 for sensitive detection and precise quantitation. We developed a procedure based on reverse transcription
19 followed by real-time quantitative polymerase chain reaction (RT-qPCR) with a primer pair and a TaqMan®
20 probe specific for WMV. Primers and probes were designed from conserved sequence stretches to target a wide
21 range of WMV isolates. A standard curve performed with transcripts enabled estimation of WMV RNA copies
22 per ng of total RNA, with a wide dynamic range and sensitivity (10^4 to 10^{11}). This RT-qPCR was assayed with
23 field samples from different cucurbits and used to evaluate the temporal accumulation in pumpkin plants.

24

25 Watermelon mosaic virus (WMV), genus *Potyvirus* of the family *Potyviridae*, is one of the major
26 viruses producing severe damage in cucurbit crops in countries with temperate or Mediterranean climates, but
27 it also infects some legumes, carrots, orchids, and weeds (Lecoq and Desbiez, 2012). Symptoms vary with the
28 WMV isolate and cultivars and consist in plant stunting, leaf mosaic, vein banding and/or fruit malformation
29 and discoloration. WMV is transmitted in a non-persistent manner by at least 35 species aphids (Lecoq and
30 Desbiez, 2012). WMV, as other potyviruses, has a genome composed of a single positive sense single-stranded
31 RNA molecule of about 10 kb long containing a single long open reading frame (ORF). The ORF is translated
32 into a large polyprotein, which is cleaved into the mature proteins: P1, helper component (HC), P3, 6K1,
33 cylindrical inclusion (C1), 6K2, nuclear inclusion A (NIa), viral protein linked genome (VPg), nuclear inclusion
34 B (NIb) and coat protein (CP) (Urcuqui-Inchima, 2001; Revers and Garcia, 2015). The P3 cistron also encodes
35 a second protein, PIPO, which is generated by +2 frame shift (Chung et al., 2008).
36 Disease management based on preventing WMV spread by controlling its aphid vectors (insecticide
37 treatment, trap and barrier crops, mineral oils and breeding resistant cultivars to aphids) has low impact (Lecoq
38 and Desbiez, 2012). Resistant watermelon plants were obtained by genetic engineering (Lin et al., 2012) but the
39 use of genetically modified organisms (GMOs) is banned in many countries because of the public concern on
40 the potential ecological impact. Despite the considerable efforts of plant breeders to search genetic sources
41 enabling to evade WMV infection, only partial resistance has been achieved in melon, cucumber (*Cucumis*
42 *melo*, *Cucumis sativus*), and squash (*Curcubita maxima* and *Cucurbita moschata*) whereas no resistance has
43 been found in pumpkin or zucchini (*Cucurbita pepo*) (Martín-Hernández and Picó, 2021).
44 Fast, specific, and sensitive methods for virus detection and precise quantitation are essential for disease
45 management, such as searching new resistance sources (Rubio et al., 2020). WMV detection can be achieved
46 by serological methods such as enzyme-linked immunosorbent assay (Dietzgen and Herrington, 1991) dot-blot
47 immunoassay and tissue-blot immunoassay (Mostafa and Abou-Ela, 2011) and molecular methods such as dot-
48 blot hybridization (Juan et al., 2007), reverse-transcription polymerase chain reaction (RT-PCR) (Aguiar et al.,
49 2019, Kwon et al., 2014) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Zhao
50 et al., 2016) . However, some of these methods are not very sensitivity, which can affect virus diagnosis (Rubio
51 et al., 2020), and none is apt for a precise quantitation that is needed for evaluating partial resistance in breeding
52 programs. Quantitative real-time PCR (qPCR) is a rapid, sensitive, specific and a high reproducible technique
53 that avoids most of the problems of other techniques (Mackay et al., 2002) and has been used for the detection
54 and sensitive quantitation of several plant viruses (Debreczeni et al., 2011; Ferriol et al., 2011) to evaluate
55 genetic resistance (Soler et al., 2015), cross protection (Hanssen et al., 2010) and how virus titer affects the virus
56 transmission rate and dispersal by insect vectors (Ferriol et al., 2013; Debreczeni et al., 2014). Here, a procedure
57 based on reverse transcription (RT) followed by qPCR was developed for sensitive detection and absolute
58 quantitation of WMV and tested with field cucurbit samples and a time-course assay.
59 To design accurate primers and a TaqMan probe able to detect a wide range of WMV isolates and
60 minimize false negatives (Kralik and Ricchi, 2017; Rubio et al., 2020), the nucleotide diversity of the complete
61 coat protein gene (CP) from worldwide WMV isolates (Moreno et al., 2004; Desbiez et al., 2007; Desbiez and

62 Lecoq, 2008; Hajizadeh et al., 2007; Sharifi et al., 2008) was considered. Thus, nucleotide sequences of WMV
63 CP from different hosts and geographical origins were retrieved from GenBank (Table 1) and aligned with the
64 CLUSTALW algorithm implemented in the program MEGA-X (Kumar et al., 2018). Primers WMV-1F (5'-
65 GGGCAAGAGAAGCAATAGCA-3') and WMV-1R (5'-GTGGACCCATACCCAACAAA-3'),
66 encompassing a region of 164 nucleotides, and the TaqMan® probe A (5'Fam-
67 CACACTGCAAGGGACGTAAAA-Tamra3') were designed with the free online software Primer3
68 (Untergasser et al., 2012) and Primer Premier 5 (Lalitha, 2000) from conserved sequence stretches (Fig. 1).
69 Some WMV isolates contain a one or two polymorphic sites in the primer or probe regions, but their positions
70 were intended not to affect binding and the consequent RT-qPCR (Rubio et al., 2020). Also, these primer and
71 probe sequences showed no similarity with other potyviruses when analyzed by BLAST
72 (https://blast.ncbi.nlm.nih.gov/) and gave negative reaction when they were tested with other cucurbit
73 potyviruses such as Zucchini yellow mosaic virus and Moroccan watermelon mosaic virus (data not shown).
74 To generate a standard curve to absolutely quantify WMV, RT-qPCR was performed with serial dilutions *of in*
75 *vitro* positive-sense RNA transcripts. First, an infectious clone of WMV isolate Vera (Aragonés et al. 2019) was
76 used as a template for PCR with primers T7-WMV-1F (a modified version of primer WMV-1F containing the
77 T7-promoter at its 5' terminus) and WMV-1R. PCR was performed in a reaction mixture (20 µl) containing
78 PCR buffer, 1.5 mM MgCl2, 1 mM of each dNTP, 0.5 U of TaqDNA polymerase (Invitrogen), and 0.2 mM of
79 each primer. Thermocycling conditions included 2 min denaturation at 94 ºC, 40 cycles of 94 ºC for 20 s, 55 ºC
80 for 20 s and 72 ºC for 25 s, and an extension step at 72 ºC for 5 min. The PCR product was confirmed by
81 electrophoretic analysis in 2% agarose gels. *In vitro* RNA transcripts were generated from the PCR product by
82 using the Megascript T7 kit (Ambion) following the manufacturer's instructions and incubating at 37ºC for 4
83 hours. Transcripts were treated with RNase-free DNase (Turbo-DNA-free, Ambion), purified with the Rnaid
84 w/Spin Kit (Q-BIO gene) and its integrity and purity were evaluated by electrophoresis in RNAse free
85 conditions in 2% agarose gel. Transcript concentration (µg/µl) was evaluated with a spectrophotometer
86 NanoDrop™ 1000 (Thermo Scientific) and stored at -80 ºC until use. To calculate the number of single stranded
87 RNA (ssRNA) copies, micrograms of ssRNA were converted to picomoles by considering the average
88 molecular weight of a ribonucleotide (340 pg) and the number of bases of the transcript (Nb). The formula pmol
89 of ssRNA = µg of ssRNA × (10^6 pg/1 µg) × (1 pmol/340 pg) × (1/Nb) and Avogrado's constant (6.023 × 10^{23}
90 molecules/mol) were used to estimate the number of ssRNA copies. Ten-fold serial dilutions containing from
91 10^{11} to 10^1 RNA copies were prepared with total RNA extracts from uninfected cucumber (*Cucumis sativus*)
92 plants. RT-qPCR was performed in a LightCycler®480 (Roche Molecular Diagnostics, Basel, Switzerland) in
93 a reaction mixture (25 µl) containing 12.5 µl LightCycler® 480 Probes Master (Roche Molecular Diagnostics),
94 15 U of RT Multiscribe Reverse Transcriptase (Applied Biosystems), 2 U RNase Inhibitor (Applied
95 Biosystems), different concentrations of each forward and reverse primer and the TaqMan®MGB probe.
96 Cycling conditions consisted of reverse transcription at 48 ◦C for 30 min, incubation at 95 ◦C for 10 min, and
97 40 cycles of 95 ◦C for 15 s and 60 ◦C for 1 min. Negative control samples included water and RNA extracts
98 from non-infected cucumber, melon, watermelon (*Citrullus lanatus*), and pumpkin plants. The standard curve

99 was obtained with three replicates per dilution by plotting threshold cycle (Ct) values versus the logarithm of
100 the RNA copy number (Fig. 2). The amplification efficiency (E) was calculated with the slope of the standard
101 curve (S) using the formula: E= $(10^{(-1/S)})$-1, considering 100% efficiency for a value of 1. Different
102 concentrations of primers (0.25, 0.50 and 0.80 µM) and the probe (0.25, 0.4 and 0.5 µM) were assayed, and the
103 best efficiency was obtained with 0.80 µM of each primer and 0.4 µM of probe A. The standard curve showed
104 a strong linear relationship with a high determination coefficient (R^2= 0.9945) and high amplification efficiency
105 (101%). This RT-qPCR technique enable the detection of 10^3 WMV RNA copies, and quantitation was reliable
106 in the linear range covering from 10^4 to 10^{11} WMV RNA copies.

107 To quantify WMV in plant, total RNA from 0.1 g of fresh or frozen leaf tissue from WMV-infected
108 cucumber, pumpkin, watermelon, and melon was extracted with a standard protocol with
109 phenol/chloroform/isoamilic alcohol followed by ethanol precipitation (Ferriol et al., 2011). Total RNA
110 concentration was measured with a spectrophotometer NanoDrop™ 1000 and adjusted to 10 ng/ul to normalize
111 the different extractions. To test the reproducibility of this RT-qPCR method, four cucumber plants were
112 agroinoculated with an infectious clone of WMV (Aragonés et al. 2019). *Agrobacterium tumefaciens* cells,
113 strain C58C1, carrying the whole WMV genome in the binary plasmid pG35Z containing the Cauliflower
114 mosaic virus (CaMV) 35S promoter and terminator and the helper plasmid pCLEAN-S48 (Kindly provided by
115 Dr. J.A. Darós) were grown in Luria-Bertani medium (LB) containing 50 mg/l kanamycin and 5 mg/l
116 tetracycline. Individual colonies were grown shaking overnight in 5 ml of LB at 28 °C and inoculated in 50 ml
117 of induction medium (LB with 10 mM MES and 20 µM acetosyringone) that was also incubated overnight at
118 28 °C. the cells were collected by centrifugation at 6000**g** for 15 min at room temperature, resuspended in
119 infiltration medium (10 mM MgCl2, 10 mM MES, pH 5.6, and 150 µM acetosyringone), incubated at room
120 temperature for about 5 h and adjusting concentration to 1 OD600 with a spectrophotometer. Cucumber leaves
121 were infiltrated with this bacterial suspension with a syringe directly placed on the leaf surface. Also, three
122 cucumber plants were mechanically inoculated by grinding 1 g of WMV-infected cucumber leaf tissue in 5 ml
123 of 0.1 M sodium phosphate buffer (pH 7) and rubbing this preparation with carborundum (600 mesh) at the first
124 true leaf stage. Plants were kept in a growth chamber at 25 ºC and 16/8 h light/dark photoperiod. These plants
125 were analyzed by RT-qPCR in three technical replicates at 7 days post inoculation (dpi). The titer of
126 mechanically- and agro-inoculated plants was similar, $4.81 \pm 0.89 \times 10^6$ and $1.69 \pm 0.37 \times 10^6$ WMV RNA
127 copies, respectively (Table 2), so both inoculation techniques can be used to test WMV accumulation. The mean
128 coefficient of variation of Ct values between biological replicates (plants) was only 4.11 % which indicates that
129 this approach can be used for evaluation of partial resistance in breeding programs based on the accumulation
130 of WMV (Soler et al., 2015). The mean coefficient of variation of Ct values between technical replicates was
131 1.08%, which indicates a good reproducibility of this RT-qPCR technique.

132 Sixty-four plant samples collected in cucurbit fields from Spain in 2019 were analyzed by RT-qPCR by
133 using two replicates per plant and WMV was detected in 24 plants (Table 3). All negative controls (from healthy
134 plants) gave no signal. The viral titer ranged from 1.06×10^4 to 2.72×10^7 being higher in melon (3.26 ± 1.48
135 $\times 10^6$) than in pumpkin and watermelon ($1.42 \pm 0.59 \times 10^5$ and $3.81 \pm 1.26 \times 10^5$, respectively) and similar to

136 which were mechanically inoculated ($4.81 \pm 0.89 \times 10^6$)or agroinoculated ($1.69 \pm 0.37 \times 10^6$) cucumber plants
137 (Table 2). Finally, the temporal accumulation of WMV was evaluated in four WMV-agroinoculated pumpkin
138 plants by RT-qPCR of leaf samples collected at 7, 14, 21 and 28 dpi. WMV accumulated rapidly to a titer of
139 about 1.5×10^6 WMV RNA copies per ng of total RNA at 7 dpi which increased to about 1.6 x 10^7 at 14 dpi
140 and remained steady until 28 dpi (Fig. 3). Similar accumulation pattern has been observed in other plant viruses
141 such as Broad bean wilt virus 1, Cucumber mosaic virus and Pepino mosaic virus, although with higher titer
142 fluctuation (Carpino et al., 2019; Elvira-González et al., 2021). The RT-qPCR procedure developed in this work
143 can be useful for breeding programs to identify and evaluate new sources of resistance against WMV as done
144 for other plant viruses (Balaji et al., 2003; Galipienso et al., 2013; Soler et al., 2015).

145 **Author contribution**

146 LR designed the study, analyzed the data, supervised the experiments, and wrote the manuscript. LG
147 assisted with the study design, protocol development and writing and editing the manuscript. IF and AA
148 performed the surveys. KG and JR performed the experiments. All the authors read and approved this
149 manuscript

150 **Declaration of Competing Interest**

151 The authors declare that they have no competing interests

152 **Acknowledgments**

153 We thank Dr. José Antonio Daròs for the WMV infectious clone. This work was funded by grants
154 RTA2017-00061-C03-02 from Spanish Ministerio de Economia, Industria y Competitividad co-funded by the
155 European Regional Development Fund (ERDF) and 51912 from IVIA co-funded by ERDF COMUNITAT
156 VALENCIANA 2014-2020.

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239

240 **Figures and Tables**

241 **Figure 1.** Location of primers WMV-1F and WMV-1R (in bold and underlined) and probe A (in bold and in a
242 box) for RT-qPCR of Watermelon mosaic virus (WMV) on the aligned coat protein sequences of worldwide
243 WMV isolates retrieved from GenBank (Table 1). Numbers indicate nucleotide positions in the coat protein
244 gene sequence of WMV isolate Vera (GenBank accession number MH469650).

245 **Figure 2.** Standard curve prepared with 10-fold serial dilutions of *in vitro* RNA transcripts from Watermelon
246 mosaic virus (WMV) isolate Vera. Linear regression formula, coefficient of determination (R^2) and
247 amplification efficiency (E) are indicated.

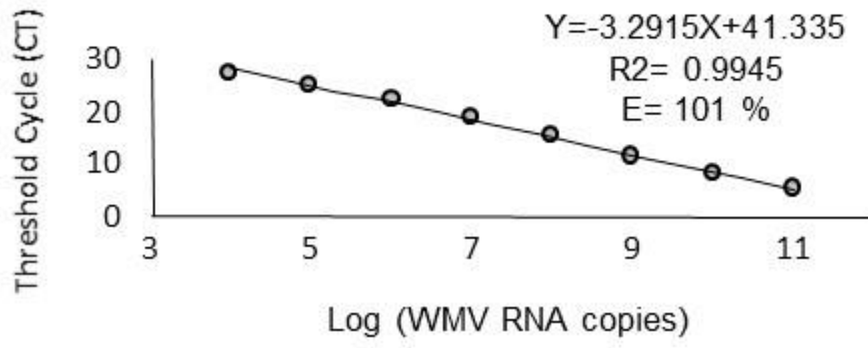
248 **Fig. 3.** Time course accumulation of Watermelon mosaic virus (WMV) RNA (Log WMV RNA copies per
249 nanogram of total RNA) in pumpkin estimated by RT-qPCR. Mean values and standard errors for four plants
250 are represented.

251

Figure 1

```
GenBank         Forward primer                                       Reverse primer
accession       WMV-1F                     Probe A                   WMV-1R
                678               698      789             808       825               844
MH469650   GGGCAAGAGAAGCAATAGCACAAA   GAGAGGCACACTGCAAGGGACGTAAATCAGAATATGCATACTTTGTTGGGTATGGGTCCAC
AB001994   ........................   ............................G.................A...........
AB693979   ........................   ............................A...............................
AJ579482   ........................   .......................G.......................A.............
AY995215   .A......................   .............................................................
DQ399708   ......................G.   .......................G........C............................
EF127832   ......................G.   .......................T..............C......................
EU660578   ........................   .......................G.......................A.............
EU660579   ........................   .......................G.....................................
EU660580   ........................   .............................................................
EU660584   ........................   ......................................C......................
EU660590   ........................   .......................G.......................A.............
FJ823122   .A......................   .......................G.....................................
HQ384216   .......G................   .......................G.G.....................A.............
JF273461   ......................G.   .......................T..............C......................
JN831648   ........................   ..A..........................................................
KP164988   ......................G.   .......................T.....................................
KU46036    ........................   .........................................................T..A
KF637299   ......................R.   .......................K........Y.........Y..................
MN854636   ......................G.   .......................G........C............................
L22907     ........................   .............................................................
AF322376   .............C..........   .......................G.....................................
```

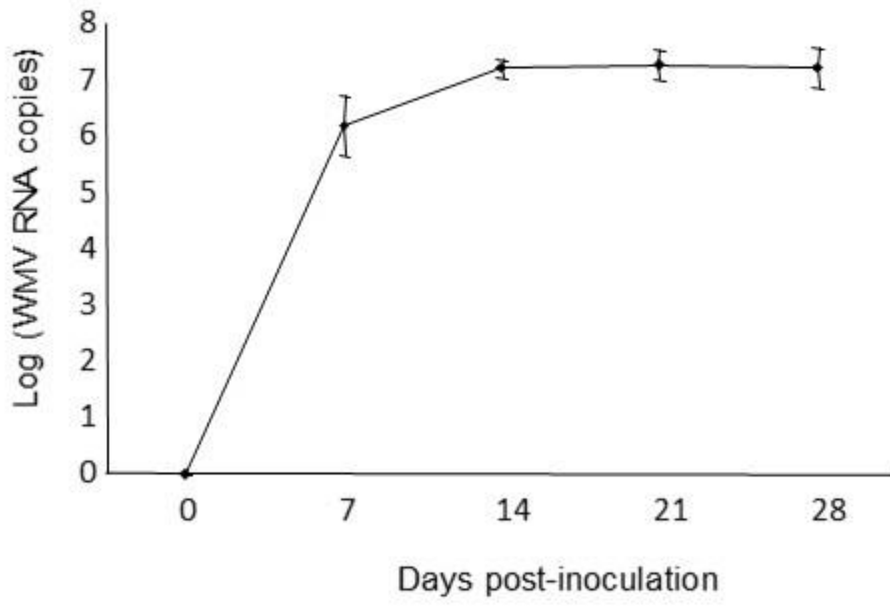
279 **Figure 2**



280

281

282 **Figure 3**



283

284

285 **Table 1**. Watermelon mosaic virus (WMV) isolates whose coat protein nucleotide sequences were used to
286 design primers and a probe for RT-qPCR

Isolate/strain	Host	Origin	Year[a]	GenBank
Vera	*Cucumis melo*	Spain: Comunidad Valenciana	2013	MH469650
Habenaria	*Habenaria radiata*	Japan: Kurashiki Okayama	1995	AB001994
S96-3	*C. sativus*	Japan: Akita	1996	AB693979
ZAR95.1	*Cucumis melo*	Spain: Aragón	1995	AJ579482
N/A[b]	N/A	New Zealand	≤ 2005[c]	AY995215
WMV-CHN	*Citrullus lanatus*	China	≤ 2006	DQ399708
Ch99/69	*Cucurbita pepo*	China	≤ 2006	EF127832
FMF00-LL2	*C. pepo*	France	2000	EU660578
TURK91	*C. pepo*	Turkey	1991	EU660579
CHI87-620	*C. pepo*	Chile	1987	EU660580
IR02-54	*C. pepo*	Iran	2002	EU660584
ITA00-G	*C. pepo*	Italy	2000	EU660590
Lecce	*C. lanatus*	Italy: Apulia	2007	FJ823122
N/A	*Dendrobium anosmum*	USA: Hawaii	2010	HQ384216
C07-349	*C. melo*	France:Saint Chaptes	2007	JF273461
RobWMV 2	*Robinia pseudoacacia*	USA: Arkansas	2009	JN831648
1SDEFF	*Curcubita moschata*	Argentina	2012	KP164988
TX29	*C. lanatus*	USA: Texas	2010	KU246036
punggi1	*Panax ginseng*	South Korea	2009	KF637299
CN-sm2-1	*Sesamum indicum*	South Korea	2019	MN854636
Tonga	*Vanilla planifolia*	Tonga	1993	L22907
N/A	N/A	Israel	≤ 2000	AF322376

287 [a]Year of collection. When it is not available, the year of publication in GenBank is indicated after "≤"

288 [b]N/A refers to non-available data

289

290 **Table 2.** Detection and quantification by RT-qPCR of cucumber (*Cucumis melo*) plants inoculated with
291 watermelon mosaic virus (WMV) isolate Vera.

Plant	Inoculation	Ct ± SE[a]	Copies ± SE[b]
C1	mechanical	15.47 ± 0.08	4.81 ± 0.28 $\times 10^6$
C2	mechanical	15.78 ± 0.01	3.87 ± 0.03 $\times 10^6$
C3	mechanical	16.96 ± 0.35	1.81 ± 0.48 $\times 10^6$
C4	agroinfiltration	16.31 ± 0.11	2.68 ± 0.20 $\times 10^6$
C5	agroinfiltration	16.86 ± 0.09	1.82 ± 0.11 $\times 10^6$
C6	agroinfiltration	17.46 ± 0.07	1.20 ± 0.05 $\times 10^6$
C7	agroinfiltration	17.77 ± 0.03	9.63 ± 0.19 $\times 10^5$

292 [a] Mean threshold cycle (Ct) and standard error (SE) obtained from three technical replicates.

293 [b] Mean number of copies of viral RNA per nanogram of total RNA from infected plants and
294 standard error (SE) obtained from three technical replicates.
295

Table 3. Detection and quantification by RT-qPCR of watermelon mosaic virus (WMV) isolates collected in Spain in 2019.

Isolate	Origin[a]	Host	Ct ± SE[b]	Copies ± SE[c]
371/19	Comunidad Valenciana	Cucurbita pepo	20.97 ± 0.02	$1.03 \pm 0.01 \times 10^{5}$
372/19	Comunidad Valenciana	C. pepo	19.88 ± 0.05	$2.21 \pm 0.08 \times 10^{5}$
410/19	Castilla la Mancha	*Citrullus lanatus*	18.84 ± 0.24	$4.62 \pm 0.77 \times 10^{5}$
411/19	Castilla la Mancha	*C. lanatus*	18.76 ± 0.12	$4.83 \pm 0.41 \times 10^{5}$
412/19	Castilla la Mancha	*C. lanatus*	24.21 ± 0.06	$1.06 \pm 0.05 \times 10^{4}$
414/19	Castilla la Mancha	*C. lanatus*	18.54 ± 0.24	$5.69 \pm 0.95 \times 10^{5}$
415/19	Castilla la Mancha	*Cucumis melo*	17.67 ± 0.14	$1.04 \pm 0.10 \times 10^{6}$
416/19	Castilla la Mancha	*C. melo*	16.07 ± 0.23	$3.21 \pm 0.50 \times 10^{6}$
417/19	Castilla la Mancha	*C. melo*	20.96 ± 0.17	$1.04 \pm 0.13 \times 10^{5}$
418/19	Castilla la Mancha	*C. melo*	17.28 ± 0.26	$1.38 \pm 0.25 \times 10^{6}$
419/19	Castilla la Mancha	*C. melo*	17.80 ± 0.17	$9.49 \pm 1.12 \times 10^{5}$
420/19	Castilla la Mancha	*C. melo*	16.17 ± 0.04	$2.95 \pm 0.08 \times 10^{6}$
422/19	Castilla la Mancha	*C. melo*	19.33 ± 0.05	$3.24 \pm 0.13 \times 10^{5}$
423/19	Castilla la Mancha	*C. melo*	17.70 ± 0.02	$1.01 \pm 0.01 \times 10^{6}$
424/19	Castilla la Mancha	*C. melo*	17.96 ± 0.11	$8.45 \pm 0.65 \times 10^{5}$
425/19	Castilla la Mancha	*C. melo*	18.07 ± 0.08	$7.81 \pm 0.44 \times 10^{5}$
426/19	Castilla la Mancha	*C. melo*	13.00 ± 0.00	$2.72 \pm 0.01 \times 10^{7}$
427/19	Castilla la Mancha	*C. melo*	16.75 ± 0.04	$1.96 \pm 0.06 \times 10^{6}$
428/19	Castilla la Mancha	*C. melo*	18.80 ± 0.00	$4.68 \pm 0.00 \times 10^{5}$
429/19	Castilla la Mancha	*C. melo*	18.38 ± 0.04	$6.30 \pm 0.15 \times 10^{5}$
430/19	Castilla la Mancha	*C. melo*	14.90 ± 0.00	$7.19 \pm 0.03 \times 10^{6}$
431/19	Castilla la Mancha	*C. melo*	20.86 ± 0.07	$1.11 \pm 0.06 \times 10^{5}$
432/19	Castilla la Mancha	*C. melo*	15.61 ± 0.03	$4.36 \pm 0.09 \times 10^{6}$
434/19	Castilla la Mancha	*C. melo*	15.70 ± 0.07	$4.10 \pm 0.20 \times 10^{6}$

[a] Samples were collected in Comunidad Valenciana (Cheste in Valencia province) and Castilla la Mancha (Tomelloso, Argamasilla de Alba y Llanos del Caudillo in Ciudad Real province).

[b] Mean threshold cycle (Ct) and standard error (SE) obtained from two technical replicates.

[c] Mean number of copies of viral RNA per nanogram of total RNA from infected plants and standard error (SE) obtained from two technical replicates.