Development and validation of a multiplex reverse transcription quantitative PCR (RT-qPCR) assay for the rapid detection of *Citrus tristeza virus*, *Citrus psorosis virus*, and *Citrus leaf blotch virus*

Fatima Osman^a, Emir Hodzic^b, Sun-Jung Kwon^{c†}, Jinbo Wang^{c§}, and Georgios Vidalakis^{c*}

^aDepartment of Plant Pathology, University of California, Davis, CA 95616, USA

^bReal-time PCR Research & Diagnostic Core Facility, School of Veterinary Medicine, University of California, Davis, CA 95616, USA

^cDepartment of Plant Pathology and Microbiology, University of California, Riverside, CA 92521, USA

[†]Present address:

National Institute of Horticultural and Herbal Science, Suwon 441-440, Korea

[§]Present address:

USDA-APHIS-PPQ-CPHST, Beltsville, MD 20705, USA

*Corresponding author:

Department of Plant Pathology and Microbiology, University of California, Riverside, CA 92521, USA. Tel: +951-827-3763; Fax: +951-827-4294. E-mail address: georgios.vidalakis@ucr.edu

ABSTRACT

A single real-time multiplex reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay for the simultaneous detection of *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV), and *Citrus leaf blotch virus* (CLBV) was developed and validated using three different fluorescently labeled minor groove binding qPCR probes. To increase the detection reliability, coat protein (CP) genes from a large number of different isolates of CTV, CPsV and CLBV were sequenced and a multiple sequence alignment was generated with corresponding CP sequences from the GenBank and a robust multiplex RT-qPCR assay was designed. The capacity of the multiplex RT-qPCR assay in detecting the viruses was compared to singleplex RT-qPCR designed specifically for each virus and was assessed using multiple virus isolates from diverse geographical regions and citrus species as well as graft-inoculated citrus plants infected with various combination of the three viruses. No significant difference in detection limits was found and specificity was not affected by the inclusion of the three assays in a multiplex RT-qPCR reaction. Comparison of the viral load for each virus using singleplex and multiplex RT-qPCR assays, revealed no significant differences between the two assays in virus detection. No significant difference in Cq values was detected when using one-step and two-step multiplex RT-qPCR detection formats. Optimizing the RNA extraction technique for citrus tissues and testing the quality of the extracted RNA using RT-qPCR targeting the cytochrome oxidase citrus gene as an RNA specific internal control proved to generate better diagnostic assays. Results showed that the developed multiplex RT-qPCR can streamline viruses testing of citrus nursery stock by replacing three separate singleplex assays, thus reducing time and labor while retaining the same sensitivity and specificity. The three targeted RNA viruses are regulated pathogens for California's mandatory "section 3701". Citrus Nursery Stock Pest Cleanliness Program".

Adopting a compatible multiplex RT-qPCR testing protocol for these viruses as well as other RNA and DNA regulated pathogens will provide a valuable alternative tool for virus detection and efficient program implementation.

Keywords: COX internal control; graft-transmissible citrus pathogens; citrus germplasm; budwood; citrus viruses; citrus nursery certification.

1. Introduction

A number of diagnostic tools have been developed for the detection of *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV), and *Citrus leaf blotch virus* (CLBV), including biological indexing (Roistacher, 1991), enzyme linked immunosorbent assay (ELISA) (Alioto et al., 1999; Bar-Joseph et al., 1979; Cambra et al., 1991, 1993, 2000; D'Onghia et al., 2001; Garcia et al., 1997; Garnsey et al., 1993; Loconsole et al., 2006; Martín et al., 2002, 2004; Nikolaeva et al., 1996; Permar et al., 1990; Potere et al., 1999; Vela et al., 1986; Vives et al., 2001), molecular hybridization with cDNA or cRNA probes (Barbarossa and Savino, 2006; Narváez et al., 2000; Rosner and Bar-Joseph, 1984) and direct tissue-blot immunoassay (DTBIA) immunoprinting or tissue print (Cambra et al., 2000; Garnsey et al., 1993). More recently, a branched signal amplification method for the detection of seven different citrus pathogens, including CTV, CPsV, and CLBV, in a single sample was also reported (Wang 2014). The disadvantages of high cost, time required to confirm infection, uneven distribution and low titer of the virus in some hosts (Galipienso et al., 2000), make biological indexing impractical for the large-scale analyses required in eradication campaigns and in extensive certification programs (Cambra et al., 2000). The use of serological or branched signal amplification tests may be limited by the low titer of the target viruses in certain periods of the year and by the uneven distribution of the viruses in infected plants (Galipienso et al., 2000; Loconsole et al., 2009; Mathews et al., 1997; Roistacher, 1993). To overcome these limitations and improve the sensitivity and detection limits of the assays, reverse-transcription polymerase chain reaction (RT-PCR) based methods have been developed, including an immunocapture phase (Nolasco et al., 1993), direct print-capture (Olmos et al., 1996), and nested PCR in a single closed tube (Olmos et al., 1999). One-step RT-PCR

(Hung et al., 2000), two-step RT-PCR (Hilf and Garnsey, 2000; Huang et al., 2004; Mehta et al., 1997) all have been adapted for the detection of CTV, CPsV (Barthe et al., 1998), and CLBV (Galipienso et al., 2004; Vives et al., 2002b) while other RT-PCR procedures have been used for the detection of variations in viral RNA, using amplification patterns with primer sets specific to virus genotypes (Ayllón et al., 1999, 2001; D'Urso et al., 2000, 2003; Hilf et al., 1999, 2005; Rubio et al., 1996; Ruiz-Ruiz et al., 2006; Sambade et al., 2002, 2003, 2007).

A valuable feature of PCR based techniques is the possibility to detect several pathogens in a single reaction, using either multiple primer pairs or a polyvalent primer pairs (Bertolini et al., 2001; Foissac et al., 2001; Gambino and Gribaudo, 2006; Ito et al., 2002; James, 1999; Roy et al., 2010; Sanchez-Navarro et al., 2005; Wang et al., 2009). For example, Roy et al., 2005, reported the development of a multiplex PCR method for the detection of multiple citrus pathogens including CPsV. In addition, the development of real time quantitative PCR (qPCR) has provided a powerful tool to analyze mixed populations in various microbial systems (Klatt and LaPara, 2003; LaPara et al., 2000; Ueno et al., 2001; Yu et al., 2006). The most popular approaches for quantitative pathogen detection using real time qPCR are either done with non-probe based fluorescent master mix such as SYBR green (Moradi et al., 2010; Ong et al., 2007; Pafundo et al., 2010; Varga and James, 2005; Yokomi et al., 2008) or with specific probes such as hydrolysis probes (Livak et al., 1995), molecular beacons (Tyagi and Kramer, 1996) and Locked Nucleic Acid (LNA) probes (Thonur et al., 2012). Minor groove binding (MGB) probes labeled with a reporter fluorophore and a non-fluorescent Black Hole Quencher® dye (BHQ) are increasingly becoming the preferred probes for qPCR detection due to their elevated specificity (De Kok et al., 2002; Doyle et al., 2005; Osman et al., 2013). This real-time qPCR technology allows the use of multiple fluorescent reporter dyes with various non-overlapping wavelengths

for the multiplex detection of several pathogens in a single reaction (Pallás et al., 1998; Loconsole et al., 2010; Saponari et al., 2013).

Real-time qPCR is known to be more sensitive, faster and relatively easier to perform compared with conventional PCR (reduced cycle times and steps, no need for electrophoresis, staining and gel documentation, reduced risk of contamination). RT-qPCR has been reported for the detection of different woody-plant infecting viruses (Marbot et al., 2003; Osman et al., 2007, 2008, 2012, 2013; Osman and Rowhani, 2006; Schneider et al., 2004; Varga and James, 2005, 2006). In citrus, RT-qPCR has been reported for the detection of CTV (Ananthakrishnan et al., 2010; Bertolini et al., 2008; Loconsole et al., 2010; Rosa et al., 2007; Ruiz-Ruiz et al., 2007, 2009b; Saponari et al., 2008, 2013; Sieburth et al., 2009; Yokomi et al., 2010), CPsV (Loconsole et al., 2010) and CLBv (Ruiz-Ruiz et al., 2009a) by different groups in separate assays. CTV, CPsV, and CLBv are regulated virus pathogens for California's Department of Food and Agriculture (CDFA) "Section 3701: Citrus Nursery Stock Pest Cleanliness Program". This program is mandatory by law (Senate Bill 140) and it has been estimated that approximately 7,000-10,000 citrus germplasm tree sources will require testing within the first 1-4 years of the program. Therefore, even though, molecular approaches using RT-qPCR assays for CTV, CPsV and CLBv have been independently developed by other researchers there is an immediate need for compatible testing of all three viruses in a multiplex RT-qPCR protocol that is flexible and high throughput in order to implement the California citrus nursery program in an efficient, economic, and effective manner.

The main goal of this paper was therefore to develop a rapid, sensitive and specific multiplex RT-qPCR assay for the detection and quantification of CTV, CPsV, and CLBv in citrus and more specifically in citrus nursery stock and germplasm tree sources. The proposed

approach can then be used as a robust diagnostic tool for citrus germplasm screening programs as well as for citrus certification programs to ensure the availability of pathogen tested materials for propagation.

2. Materials and methods

2.1. Viruses isolate and negative control sources

The virus isolates used for the evaluation of all detection methods originated from various geographical regions, genotypes, virus types, and citrus species in an effort to cover as much viral genetic diversity as possible. CTV isolates endemic to California (CA) were provided by the disease bank of the Citrus Clonal Protection Program (CCPP), University of California, Riverside, CA and the Central California Tristeza Eradication Agency (CCTEA) in Tulare, CA (Barnier, et al., 2010; Vidalakis et al., 2010a, 2010b; Wang et al. 2012 and 2013). CTV isolates, exotic to CA, were provided by the Exotic Pathogen Collection of Citrus (USDA, ARS) in Beltsville, MD and the Elizabeth Macarthur Agricultural Institute, Narellan, New South Wales, Australia (Garnsey et al., 2005). CTV isolates were maintained *in planta* in graft inoculated sweet orange (*Citrus sinensis* L.) or Mexican lime (*C. aurantifolia* Christm. Swing.). CTV genotype data of the Beltsville collection were kindly provided by Mark Hilf and Steve Garnsey, USDA-ARS. CTV genotype characterization of the CCPP collection was performed as previously described (Hilf et al., 2005; Roy et al., 2010; Roy and Bransky, 2010; Wang et al. 2013).

CPsV isolates were provided by the CCPP disease bank and were maintained in sweet orange. The psorosis type was determined by cross-protection experiments recorded in the CCPP

archives (Roistacher, 1991). CPsV isolates were also provided by the Universidad Nacional de La Plata and INTA-Estacion Experimental Agropecuaria Concordia, Argentina.

CLBV isolates were provided by the CCPP disease bank, the Elizabeth Macarthur Agricultural Institute, Narellan, New South Wales, Australia, and the Bureau of Citrus Budwood Registration, Florida Department of Agriculture and Consumer Services. CLBV isolates were maintained in sweet orange, pummelo (*C. maxima* (Burm.) Merr.), citron (*C. medica* L.), or kumquat (*Fortunella margarita* (Lour.) Swingle). All viruses isolates exotic to California were received as infected plant materials under the auspices of the USDA permits P526P-12-03953 and P526P-14-01803 or as nucleic acids.

To generate additional in house CP sequences 409 CTV (CA endemic and exotic), 15 CPsV, and 12 CLBV isolates were RT-PCR amplified and sequenced in order to cover as much as possible genomic variations of the coat protein (CP) gene. For the CTV CP, the primers CTV-RT-16201F 5'-AGAAGGCGACGATGTTGTTG-3' and CTV-RT-16809R 5'-TCCCAAGCTGCCTGACATTAG-3' located in the nucleotide position 16201-16809 of CTV GenBank accession M76485 were used. For the CPsV CP, the primers CPsV-792F1 5'-TCACAAATCAGTGAGGAATTGAGC-3' and CPsV-1352R 5'-TTGAGTCCATCAACTGTGATATTGCT-3' located in the nucleotide position 792-1352 of CPsV GenBank accession AF036926 were used. For the CLBV CP, the primers CLBV-CP-7711F 5'-TTCAAGAACTGGATTTGAATTTGC-3' and CLBV-CP-8062R 5'-TCATTTGTTGGAGGCCTTATCA-3' located in the nucleotide position 792-1352 of CLBV GenBank accession NC_003877 were used. Amplification was done in a 25 µl final volume starting with 2 µl of purified total RNA template as follows [12.39 µl dH₂O, 5 µl 5X Green GoTaq Flexi buffer, 1.25 µl 0.1M DTT, 1.25 µl each of 10 µM forward and reverse primers, 1.1

174 μ l 25 mM MgCl₂, 0.1 μ l RNasin, 0.5 μ l 10 mM dNTP mix, 0.035 μ l Superscript II RTase (200
175 U/ μ l; Invitrogen) and 0.125 μ l Go Taq DNA Polymerase]. Thermo-cycling protocol was run
176 according to Al Rwahnih et al., 2012. The amplified DNA fragments of the expected size were
177 excised from the agarose gel and extracted using the ZymoClean Gel DNA Recovery Kit (Zymo
178 Research, USA) and sequenced.

179 Non-inoculated citrus seedlings of the varieties: Washington navel and Moro blood sweet
180 orange, Mexican lime, 'Etrog' citron, Nagami kumquat, Carrizo citrange (*Poncirus trifoliata* x
181 *C. sinensis*), Eureka lemon (*C. limon* (L.) Burm.f.), Owari satsuma (*C. unshiu* (Macf.) Marc.),
182 Algerian clementine (*C. clementina* Hort.), Marsh and Ray ruby grapefruit (*C. paradisi* Macf.),
183 and Standard sour orange (*C. aurantium* L.), representing major citrus types were used as
184 negative controls.

185 2.2. Viruses sample processing and RNA extraction

187 Samples from graft-inoculated citrus were collected from phloem-rich tissues such as
188 bark from the last matured vegetative flush (approximately 12-18 months old) or petioles of fully
189 expanded leaves. Young (current flush) or older (more than 24 months) tissues were avoided
190 because they produced low quality nucleic acids as determined by spectrophotometry (i.e.
191 A260/280 and A260/230 less than 1.8) (Glasel, 1995; Manchester, 1995, 1996; Teare et al.,
192 1997).

193 To account for the possible uneven distribution of the virus within a plant, samples from
194 four to six different branches around the tree canopy were randomly collected and combined in a
195 single sample. Total RNA was extracted from citrus using a grapevine extraction and purification
196 procedure optimized for citrus phloem tissues (Osman et al., 2012). The system utilizes a freeze

drying step, the Cryo-station and Geno Grinder 2010 (SPEX SamplePrep, Metuchen, NJ) and the MagMAXTM Express-96 (Life Technologies, Grand Island, NY) along with modified 5X MME-96 Viral RNA Isolation Kit. Briefly, 250 mg of phloem rich citrus tissue (bark and/or petioles) were placed in a 2 ml eppendorf tubes and submerged in liquid N₂ for 30s (Cryo-station). The eppendorf tubes with the citrus tissue were freeze dried overnight using FreeZone[®]TriadTM 74000 (Labconco[®], Kansas City, MO). Two 5/32" stainless steel grinding balls were added in each tube and the citrus tissue was ground dry (no buffer added) for 20-30s at 1,750 rpm for 1-2 times. Subsequently the tubes were centrifuged briefly on a bench top centrifuge at maximum speed to collect the pulverized tissue at the tube bottom and 600 ml of guanidine extraction buffer was added per tube. The tubes were then placed in to the Geno Grinder 2010 cryo-blocks where the tissue was grinded with the extraction buffer for 20-30s at 1,750 rpm for 1-2 times. The crude extract was centrifuged at 4°C for 30 min on a bench top centrifuge at maximum speed. The supernatant was subjected to total RNA extraction using Applied Biosystems MagMAXTM Express-96 using the 5X MME-96 Viral RNA Isolation Kit method B as described by Osman et al, 2012. RNA was eluted in 100 µl H₂O.

Total RNA samples were used as templates for one-step multiplex RT-qPCR. RNA samples were converted to cDNA for further testing of singleplex and two-step multiplex RT-qPCR experiments according to Osman et al. (2012). The COX assay was modified from Li et al. (2006) to adjust T_m of qPCR primers and probes (Table 1). A 5 µl and 2 µl cDNA and RNA samples, respectively were analyzed by qPCR using the modified COX assay to test the integrity of the nucleic acid.

2.3. Singleplex and Multiplex qPCR assay design

The in house generated CP sequences were aligned with 668 CTV, 106 CPsV, and 29 CLBv sequences from the GenBank using Sequence Analysis and Molecular Biology Data Management software Vector NTI Advance™ 11 (Invitrogen; Life Technologies, Grand Island, NY) and subjected to BLAST analysis. These aligned CP sequences for the three viruses were used to design primers and probes used for singleplex and multiplex RT-qPCR experiments (Table 1). Five CPsV Argentinian isolates (A1-A5, GenBank accessions AM235982-AM236001) did not align with the complete sequence alignment and produced a cluster on their own (Martín et al., 2006). Therefore, a separate singleplex RT-qPCR assay (CPsVArg) was designed for their detection; CPsVArg-340 F CGATGGAAGGCCAGTGGA, CPsVArg-422p ACTGACATGTGCCATAAT and CPsVArg-483 R CCTCTCGGCCATATCAATTCTG.

The fluorophore used for CTV was TET, for CPsV was VIC and for CLBv was 6-carboxyfluorescein FAM (Table 1). In all of the three assays the 3' quencher was BHQ. All RT-qPCR assays were designed using the primer express software (Life Technologies, Grand Island, NY) following the guidelines proposed for designing multiplex RT-qPCR assays. The optimum primers' T_m was 58°C and the T_m of the qPCR probes was 10°C higher to prevent the formation of primer dimers. The amplicon length of the CTV, CPsV, and CLBv RT-qPCR assays was in the range of 135-162 bp long (Table 1). RT-qPCR assays were synthesized by Life Technologies (Grand Island, NY). The homology of the primers and qPCR probes was confirmed by BLAST searching against the non-redundant database of GenBank (NCBI).

The qPCR primer/probe mixes were prepared by mixing 20 µl each of the 100 pmol/µl forward primers, 20 µl each of 100 pmol/µl reverse primers and 4 µl each of the 80pmol/µl probes per virus in a final volume of 240 µl of water.

The singleplex qPCR reaction contained 0.42 μ l water, 0.58 μ l (final concentration of 400 nM for each primer and 80 nM for the probe), 6 μ l of commercially available PCR mastermix (TaqMan[®] Universal PCR Master Mix, Life Technologies) and 5 μ l of the cDNA in a final volume of 12 μ l. The samples were placed in a 384 well plate and amplified in a 7900HT FAST Real-time PCR system (Life Technologies, Grand Island, NY) using the manufacturer's standard amplification conditions (2 min at 50 °C, 10 min at 95 °C, then 40 cycles of 15 sec at 95 °C and 60 sec at 60 °C). A 1 μ l aliquot of each sample was qPCR analyzed using the COX qPCR assay to check the background signal and compare it with that of the negative RT control. Fluorescent signals were collected during the annealing temperature and the quantitative cycle (Cq) was calculated and exported with a threshold of 0.06 and a baseline of 3-12 for the targets of interest. The Cq was defined as the cycle in which there is a significant increase in reporter signal, above the threshold, i.e. the cycle in which fluorescence generated within a reaction crosses the fluorescence threshold. It was consequently related inversely to the initial amount of target in the sample (Bustin et al., 2009). The Cq was calculated by the qPCR machine using an algorithm with a set range of cycles at which the first detectable significant increase in fluorescence occurs; hence a Cq value below 40 indicated a positive result and a value of 40 and above indicated no amplification or a negative result.

For multiplex RT-qPCR, two different one-step multiplex RT-qPCR kits; the Path-ID Multiplex One-Step RT-PCR kit (Life Technologies, Grand Island, NY) and the QuantiFast Multiplex RT-PCR kit (Qiagen, Valencia CA) were tested and compared to the two-step multiplex RT-qPCR kit (TaqMan[®] Gene Expression Master Mix, (Life Technologies, Grand Island, NY). All kits were tested following manufacture's recommendation. For one-step multiplex RT-qPCR reactions, 2 μ l of total RNA was used as a template whereas for two-step

265 multiplex RT-qPCR, 5 µl cDNA mentioned in section 2.2 was used. The same primer probe
266 concentrations used for singleplex was used for one-step and two-step multiplex RT-qPCR.
267 Samples listed in Table 3 were used as template to test all kits.

269 2.4. Assay Validation

270 In order to assess the efficiency of multiplex RT-qPCR assay for the detection of all three
271 viruses in mixed infections across a range of virus concentrations, a series of CTV, CPsV and
272 CLBV infected biological samples collected from the field and maintained in the greenhouse
273 were used to create a dilution endpoint analysis of undiluted total RNA and tenfold dilutions of
274 up to 1:10⁵⁻⁶ of three different samples, each in triplicate. The total RNA infected samples were
275 previously tested positive by singleplex RT-qPCR for each of the three viruses with variable Cq
276 values for each of the viruses tested. In singleplex RT-qPCR mixtures, only one standard sample
277 corresponding to each virus was used and amplified. In multiplex RT-qPCR mixtures, three
278 standard samples for each virus tested were combined in a single amplification tube and qPCR
279 tested.

280 Standard curves were generated for each set of ten-fold serial dilutions of CTV, CPsV,
281 and CLBV infected samples by plotting the Cq value against the logarithm of the concentration
282 for the exponential phase of the reaction and fitting a straight line to these data by simple linear
283 regression (Bustin et al., 2009). The amplification efficiency (E) of all assays was calculated
284 from the slope of a standard curve generated on a 10-fold dilution in triplicate for every cDNA
285 sample using the formula $E = 10^{(-1/\text{slope})} - 1$. Optimal qPCR efficiency was achieved at a slope of –
286 3.32 (Bustin et al., 2009). Inter-assay and intra-assay variation of the developed multiplex RT-
287 qPCR assay was assessed within one plate and in different plates, respectively. Total RNAs from

three randomly selected CTV, CPsV, and CLBv isolates were extracted, 10 fold serial dilutions were prepared for each and tested by RT-qPCR in triplicate and their C_q values were calculated.

The intra-assay variation and inter-assay variations were calculated, by determining the percentage of coefficient of variation (CV %), which was calculated for each sample as follows: mean of the standard deviations of the duplicates divided by the grand mean of the duplicates X 100. The intra-assay and inter-assay variations were performed for singleplex and for multiplex RT-qPCR.

2.5. Testing the Viral load in singleplex and Multiplex RT-qPCR

Quantification of the amount of CTV, CPsV, and CLBv CP gene target in samples was accomplished by generating absolute standard curves, to determine the starting number of copies. The standard curves of CTV, CPsV, and CLBv RT-qPCR assays were run in singleplex and multiplex qPCR utilizing their respective fluorophores. Amplicons for CTV, CPsV, and CLBv were obtained for each primer set individually and cloned into plasmids (Eurofins MWG Operon, Huntsville, AL) in different primer probes combinations as shown in Table 1. The extracted plasmid DNA was linearized using HindIII enzyme, to increase the efficiency of dilutions. Serial tenfold dilution of plasmids carrying a known copy numbers of CTV, CPsV, or CLBv inserts were done to construct a DNA standard curve. Reactions were performed in triplicate to establish the linear response between the C_q values and the log of known copy numbers. The copy numbers for each sample were calculated as described (Leutenegger, 2001).

Statistical analysis was performed using PASW Statistics 18 software (SPSS Inc., Chicago, IL). P values < 0.05 were considered significant. The reproducibility of the assay was

tested by analysis of variance (ANOVA) comparing the quantitative cycle (Cq) values of the samples.

2.6. Assessing the Multiplex RT-qPCR assay

Infected citrus samples with broad geographical origins (Table 3) of known virus infection status were obtained from 210 CTV (Table 3; shows 54), 19 CPsV, and 14 CLBv. These samples were used to evaluate the multiplex RT-qPCR assays (one-step and two-step) and to compare with the singleplex RT-qPCR assay of each virus. The data was analyzed by comparing the Cq value of each sample using singleplex and multiplex RT-qPCR assays.

Six samples with mix infections were created via double and triple graft-inoculation of sweet orange seedlings with CTV, CLBv and CPsV. The mix-inoculated samples were processed as described in section 2.2 followed by testing using singleplex and multiplex RT-qPCR (one-step and two-step).

Negative control samples derived from non-inoculated plants and positive controls samples derived from CTV, CPsV, and CLBv graft-inoculated plants. All graft-inoculations and plant maintenance were performed as previously described (Vidalakis et al. 2004).

3. Results

3.1. Generation of in house virus sequences

A total of 436 in house CP sequences (409 CTV, 12 CLBv, and 15 CPsV) were generated. The sequences were then aligned with the corresponding CP sequences available in the GenBank for each virus. Separate CP sequence alignments were generated for each virus. For

CTV, 1077 CP sequences (668 GenBank and 409 in house sequences) and for CLB; 41 CP sequences (29 GenBank CP sequence and 12 in house sequences. For CPsV two separate CP sequence pile ups were generated; 121 CP sequences (106 GenBank and 15 in house sequences) and 20 CP sequences for the CPsV GenBank accessions AM235982-AM236001, representing five unique Argentinian isolates cluster away from the general GenBank CPsV accessions (Martín et al., 2006). RT-qPCR primer and probes were designed from CP regions showing 100% conserved sequences using the ABI primer express software. Several forward and reverse primers have been designed for CTV and CPsV to cover all sequence variations of each virus.

3.2. Assay validation

The BLAST search of the amplicon sequence for CTV, CPsV, and CLB recognized each virus sequence respectively, confirming the specificity of the designed multiplex RT-qPCR. Calculation of the average amplification efficiencies of CTV, CPsV, and CLB primer/probe multiplex RT-qPCR combinations were in the range of 92.4-104.3% which is in the range accepted by the MIQE guidelines of quantitative real-time PCR experiments (Bustin et al., 2009). All three viruses showed a linear dynamic range from 10^{5-6} copies down to < 10 copies per μ l (fig. 1).

The CV for CTV in singleplex RT-qPCR was in the range of 0.23 % - 0.612% (intra assay variation) and 0.65% - 1.4% (inter assay variation). The CV for CPsV in singleplex RT-qPCR was in the range of 0.63% - 1.494% (intra assay variation) and 1.05 - 2.31% (inter assay variation). The CV for CLB was in the range of 0.413% - 1.002% (intra assay variation) and 0.95% - 1.89% (inter assay variation). The CV for CTV in multiplex RT-qPCR was in the range of 0.86% - 3.71% (intra assay variation) and 1.13% - 3% (inter assay variation). The CV for

CPsV in multiplex RT-qPCR was in the range of 0.38% - 1.36% (inter assay variation) and 0.65% - 1.27% (intra assay variation). The CV for CLBv in multiplex RT-qPCR was in the range of 0.63% - 4.75% (intra assay variation) and 1.39% - 3.09% (inter assay variation). The CV% of multiplex RT-qPCR thus indicates low variation between different repetitions and different runs.

3.3. Testing the viral load in singleplex and multiplex qPCR

Comparing the viral load for each virus using singleplex and multiplex RT-qPCR assay showed no significant differences ($P > 0.05$) in detection (Table 2). Pathogen quantification was equally robust in mixed target assays (multiplex qPCR) versus single target assay (singleplex RT-qPCR).

3.4. Singleplex and multiplex RT-qPCR assays

The CTV, CPsV, and CLBv multiplex RT-qPCR assay was assessed to detect mixtures of all three viruses. Results presented in table 3, showed that all three viruses can be detected in mixed samples tested with singleplex and multiplex RT-qPCR assays with a Cq ratio that ranged from 0.93 to 1.02. Samples with higher ratios had higher Cq and thus correspond to having lower copy number. No amplification was detected with samples from healthy citrus or water control.

The five unique CPsV Argentinian isolates were successfully detected using the CPsVArg qPCR assay both in singleplex and multiplex format with CTV and CLBv (one-step and two-step) produced Cq values ranging from 15.09 to 28.73. The Cq value ratio between CPsVArg singleplex and multiplex was less than one.

3.5. Assessing the multiplex RT-qPCR assay

Among the two multiplex one-step RT-qPCR kits tested, the QuantiFast Multiplex RT-PCR kit (Qiagen) detected more isolates than the Path-ID Multiplex One-Step RT-PCR kit (Life Technologies) and gave the lowest Cq values (data not shown). The QuantiFast Multiplex RT-PCR kit (Qiagen) was therefore compared to the two-step multiplex RT-qPCR kit (TaqMan[®] Gene Expression Master Mix, (Life Technologies) in testing the different samples listed in Table 3. Table 4 shows the comparison between one-step and two-step multiplex RT-qPCR assays in detecting CTV, CPsV, and CLBv in infected samples across a range of different virus titer concentrations. Results revealed that one-step multiplex RT-qPCR kit was only comparable to the two-step multiplex RT-qPCR kit when using 5 µl volume of RNA template and not 2 µl when detecting different viruses with the ratio of Cq values ranging from 0.79-1.24 (Table 4).

Singleplex qPCR assay as well as the multiplex RT-qPCR (one-step and two-step) were able to detect all viruses when testing the graft-inoculated mix infected samples (Table 5). The Cq values were comparable among all the multiplex RT-qPCR kits used, with ratios less than one.

4. Discussion

Multiplex qPCR has been broadly used for the detection and quantification of a variety of microorganisms; food borne pathogens (Hyeon et al., 2010; Rodríguez et al., 2012), viruses (Fumian et al., 2010) and bacteria infecting humans (Janse et al., 2013), and viruses infecting fish (Liu et al., 2008) and other animals (Thonur et al., 2012; Wernike et al., 2013). More recently, multiplex RT-qPCR and qPCR have been reported to be as robust as singleplex RT-qPCR in detecting a broad range of different plant pathogens (Balme-Sinibaldi et al., 2006; Dai

et al., 2012; Li et al., 2009; López-Fabuel et al., 2012; Pallas et al., 2009; Price et al., 2010; Qu et al., 2011; Szemes et al., 2005; van Doorn et al., 2007), including citrus viral pathogens (Saponari et al., 2013; Loconsole et al., 2010). This paper describes the development of a multiplex RT-qPCR assay for the detection of CTV, CPsV, and CLBV using differently labeled hydrolysis probes for each virus and comparing its detection capability with singleplex RT-qPCR assays.

Genetic variation within the targeted population of a virus can potentially affect the specificity and reliability of RT-qPCR tests, leading to false negative results. Large sequence alignments derived from in house sequences as well as sequences available in GenBank were generated. As a result the RT-qPCR assays were designed from regions showing 100% consensus to increase the possibility of detecting the majority of the isolates for each of the targeted viruses. This was an important step for this study in order to increase the reliability of the new RT-qPCR assays as well as to address some of the sequence variations observed in previously developed qPCR assays after miss-alignment with the newly developed large sequence alignments (Loconsole et al., 2010, Ruiz-Ruiz et al., 2009a). Such sequence variations or miss-alignments are to be expected within a few years after an assay is developed given the speed that new virus sequences become available. Therefore, diagnostic assays need to be regularly updated.

The multiplex RT-qPCR assays developed in this study were capable of detecting each of the three targeted viruses in mixtures of infected samples as well as in single infected samples. Measuring the intra and inter assay variations confirmed the reproducibility and repeatability of the developed multiplex RT-qPCR assay. The primers/probes sequences used in the multiplex RT-qPCR were specific, and no cross-reactions among them or the non-inoculated controls were detected. The multiplex RT-qPCR detection of CTV, CPsV, and CLBV was shown to be as

robust as singleplex RT-qPCR assays, producing Cq values with concordant ratios. There was a slight increase in Cq values in multiplex RT-qPCR compared to singleplex RT-qPCR but the Cq ratio was always one or very close to one. This indicated that specificity was not lost by combining three RT-qPCR assays in a single multiplex RT-qPCR reaction. When measuring viral loads, results showed that multiplex RT-qPCR could detect minute quantities of all three viruses. Overall, the specificity and sensitivity of the citrus multiplex RT-qPCR assays were comparable to multiplex qPCR assays in potatoes (Li et al., 2009; Mumford et al., 2000) as well as other multiplex technologies targeting economically important plant pathogens, including oomycetes, fungi and nematodes (Szemes et al., 2005).

Both one-step and two-step multiplex RT-qPCR formats gave comparable Cq values with ratios close to one. However, in most cases of mixed infected samples (naturally or graft-inoculated) one-step multiplex RT-qPCR gave slightly lower Cq values and can therefore be considered as the preferred multiplex format for detecting CTV, CPsV, and CLBv in citrus germplasm programs. The Cq values were comparable among all the multiplex RT-qPCR kits used, with ratios less than one.

Singleplex qPCR assay as well as the multiplex RT-qPCR (one-step and two-step) were able to detect all viruses present in various titers in the graft-inoculated mix infected samples. The multiplex RT-qPCR assay was robust and it was able to detect lower titer viruses (e.g. CLBv: Cq 34.54 and 33.01, for two- and one-step, respectively) in the presence of higher titer viruses (e.g. CTV and CPsV: Cq 20.41 and 30.79 and 16.36 and 24.02, for two- and one-step, respectively).

Multiplex RT-qPCR could effectively replace three currently available molecular detection systems (i.e. conventional RT-PCR, multiplex RT-PCR and singleplex RT-qPCR) in

routine citrus diagnostics. Multiplex RT-qPCR can simultaneously identify and quantify a broad spectrum of citrus viruses by combining multiple primers and probes. These primers/probes combinations add flexibility to the multiplex RT-qPCR assays, as it was demonstrated by the detection of a unique sub-population of one of the targeted viruses in this study. Multiplex RT-qPCR combined with high throughput nucleic acid extraction can streamline the detection of multiple citrus viruses by reducing testing time and subsequently reduce labor and reagent costs. Such high throughput systems can allow monitoring of the overall sanitary status of citrus trees (e.g. nurseries, commercial orchards, or dooryards), thus enhance different citrus programs (e.g. disease management, germplasm, certification, or eradication) around the world (Anonymous, 2010; Belasque Jr. et al., 2010; Navarro, 1986; Santos Filho et al., 2000; Vidalakis et al., 2010a, 2010b; Von Broembsen and Lee, 1988).

Acknowledgements

This research was funded by the California Citrus Nursery Board project “The Future of the Cooperative Registration Program of Nursery Owned Citrus Source Trees” awarded to GV and it was supported in part by the Citrus Research Board (project 6100), the USDA National Institute of Food and Agriculture, Hatch project 233744 and the National Clean Plant Network (12-8100-1544-CA). We would like to thank Cindy Thomas, Jill Barnier, and Subhas Hajeri, CCTEA, Tulare, CA, John Hartung and Cristina Paul, Citrus Quarantine Unit, USDA-ARS, Beltsville, MD, Peggy Sieburth, Florida Department of Agriculture and Consumer Services, Winter Haven, FL, Nerida Donovan and Grant Chambers, Elizabeth Macarthur Agricultural Institute, Narellan, New South Wales, Australia, Luis Navarro, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain, Maria Laura Garcia and Agustina De Francesco,

Universidad Nacional de La Plata, and Maria Ines Plata Tamayo and Norma Beatriz Costa, INTA-Estacion Experimental Agropecuaria Concordia, Argentina for the supply of citrus viruses isolates. We would like also to thank Mark Hilf USDA-ARS, Ft. Pierce, FL and Steve Garnsey USDA-ARS retired, for sharing their data on the CTV genotypes. Finally, we would like to acknowledge Tavia Rucker, Tyler Dang, and the CCPP personnel at UC Riverside as well as Samantha Mapes from the Real-time PCR Research & Diagnostic Core Facility at UC Davis for their excellent technical assistance. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the authors or their institutions.

References

- Alioto, D., Gangemi, M., Deaglio, S., Sposato, P., Noris, E., Luisoni, E., Milne, R.G., 1999. Improved detection of citrus psorosis virus using polyclonal and monoclonal antibodies. *Plant Pathol.* 48, 735-741.
- Ananthakrishnan, G., Venkataprasanna, T., Roy, A., Brlansky, R.H., 2010. Characterization of the mixture of genotypes of a Citrus tristeza virus isolate by reverse transcription-quantitative real-time PCR. *J. Virol. Methods* 164, 75-82.
- Anonymous, 2010. Research Board aggressively responds to unprecedented threat. *Citrograph* 1, 12-14.
- Ayllón, M.A., Rubio, L., Moya, A., Guerri, J., Moreno, P., 1999. The haplotype distribution of two genes of citrus tristeza virus is altered after host change or aphid transmission. *Virology* 255, 32-39.
- Ayllón, M.A., López, C., Navas-Castillo, J., Garnsey, S.M., Guerri, J., Flores, R. Moreno, P., 2001. Polymorphism of the 5' terminal region of Citrus tristeza virus (CTV) RNA: incidence of three sequence types in isolates of different origin and pathogenicity. *Arch. Virol.* 146, 27-40.
- Balme-Sinibaldi, V., Tribodet, M., Croizat, F., Lefeuvre, P., Kerlan, C., Jacquot, E., 2006. Improvement of Potato virus Y (PVY) detection and quantitation using PVYN- and PVYO-specific real-time RT-PCR assays. *J. Virol. Methods* 134, 261-266.
- Barbarossa, L., Savino, V., 2006. Sensitive and Specific Digoxigenin-labelled RNA Probes for Routine Detection of Citrus tristeza virus by Dot-blot Hybridization. *J. Phytopathol.* 154, 329-335.

- Bar-Joseph, M., Garnsey, S.M., Gonsalves, D., Moscovitz, M., Purcifull, D.E., Clark, M.F., Loebenstein, G., 1979. The use of enzyme-linked immunosorbent assay for detection of citrus tristeza virus. *Phytopathology* 69, 190-194.
- Barnier, J., Grafton-Cardwell, B., Polek, M., 2010. Citrus tristeza virus (CTV): Then and now. *Citrograph* Nov./Dec, 16-23.
- Barthe, G.A., Ceccardi, T.L., Manjunath, K.L., Derrick, K.S., 1998. Citrus psorosis virus: nucleotide sequencing of the coat protein gene and detection by hybridization and RT-PCR. *J. Gen. Virology* 79, 1531-1537.
- Belasque Jr., J., Bassanezi, R.B., Yamamoto, P.T., Ayres, A.J., Tachibana, A., Violante, A.R., Tank Jr, A., Di Giorgi, F., Tersi, F.E.A., Menezes, G.M., 2010. Lessons from huanglongbing management in São Paulo State, Brazil. *J. Plant Pathol.* 92, 285-302.
- Bertolini, E., Olmos, A., Martínez, M.C., Gorris, M.T., Cambra, M., 2001. Single-step multiplex RT-PCR for simultaneous and colourimetric detection of six RNA viruses in olive trees. *J. Virol. Methods* 96, 33-41.
- Bertolini, E., Moreno, A., Capote, N., Olmos, A., de Luis, A., Vidal, E., Pérez-Panadés, J., Cambra, M., 2008. Quantitative detection of Citrus tristeza virus in plant tissues and single aphids by real-time RT-PCR. *Eur. J. Plant Pathol.* 120, 177-188.
- Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55, 611-622.
- Cambra, M., Camarasa, E., Gorris, M.T., Garnsey, S.M., Carbonell, E., 1991. Comparison of different immunosorbent assays for citrus tristeza virus (CTV) using CTV-specific monoclonal and polyclonal antibodies. *Proceedings of the 11th Conference of the International Organization of Citrus Virologists*, 38-45.
- Cambra, M., Camarasa, E., Gorris, M.T., Garnsey, S.M., Gumpf, D.J., Tsai, M.C., 1993. Epitope diversity of citrus tristeza virus isolates in Spain. *Proc 12th Conf IOCV*, Riverside, California, USA, 33-38.
- Cambra, M., Gorris, M.T., Roman, M.P., Terrada, E., Garnsey, S.M., Camarasa, E., Olmos, A., Colomer, M., 2000. Routine detection of Citrus tristeza virus by direct immunoprinting-ELISA method using specific monoclonal and recombinant antibodies. *14th Conference of the International Organization of Citrus Virologist. da GRAÇA*, JV, 34-41.
- D'Onghia, A.M., Djelouah, K., Frasher, D., Potere, O., 2001. Detection of Citrus psorosis virus by direct tissue blot immunoassay. *J. Plant Pathol.* 83, 139-142.
- D'Urso, F., Ayllón, M.A., Rubio, L., Sambade, A., Hermoso de Mendoza, A., Guerri, J., Moreno, P., 2000. Contribution of uneven distribution of genomic RNA variants of citrus tristeza virus (CTV) within the plant to changes in the viral population following aphid transmission. *Plant Pathol.* 49, 288-294.
- D'Urso, F., Sambade, A., Moya, A., Guerri, J., Moreno, P., 2003. Variation of haplotype distributions of two genomic regions of Citrus tristeza virus populations from eastern Spain. *Mol. Ecology* 12, 517-526.
- Dai, J., Peng, H., Chen, W., Cheng, J., Wu, Y., 2012. Development of multiplex real-time PCR for simultaneous detection of three Potyviruses in tobacco plants. *J. Appl. Microbiology* 114, 502-508.

- De Kok, J.B., Wiegerinck, E.T.G., Giesendorf, B.A.J., Swinkels, D.W., 2002. Rapid genotyping of single nucleotide polymorphisms using novel minor groove binding DNA oligonucleotides (MGB probes). *Human Mutation* 19, 554-559.
- Doyle, C.K., Labruna, M.B., Breitschwerdt, E.B., Tang, Y.W., Corstvet, R.E., Hegarty, B.C., Bloch, K.C., Li, P., Walker, D.H., McBride, J.W., 2005. Detection of Medically Important *Ehrlichia* by Quantitative Multicolor TaqMan Real-Time Polymerase Chain Reaction of the *dsb* Gene. *The J. Mol. Diagnostics* 7, 504-510.
- Foissac, X., Svanella-Dumas, L., Dulucq, M.J., Candresse, T., Gentit, P., 2001. Polyvalent detection of fruit tree tricho, capillo and foveaviruses by nested rt-pcr using degenerated and inosine containing primers (PDO RT-PCR). *Acta Hort. (ISHS)* 550, 37-44.
- Fumian, T.M., Leite, J.P.G., Castello, A.A., Gaggero, A., Caillou, M.S.L.d., Miagostovich, M.P., 2010. Detection of rotavirus A in sewage samples using multiplex qPCR and an evaluation of the ultracentrifugation and adsorption-elution methods for virus concentration. *J. Virol. Methods* 170, 42-46.
- Galipienso, L., Navarro, L., Ballester-Olmos, J.F., Pina, J.A., Moreno, P., Guerri, J., 2000. Host range and symptomatology of a graft-transmissible pathogen causing bud union crease of citrus on trifoliate rootstocks. *Plant Pathol.* 49, 308-314.
- Galipienso, L., Vives, M.C., Navarro, L., Moreno, P., Guerri, J., 2004. Detection of citrus leaf blotch virus using digoxigenin-labeled cDNA probes and RT-PCR. *Eur. J. Plant Pathol.* 110, 175-181.
- Gambino, G., Gribaudo, I., 2006. Simultaneous detection of nine grapevine viruses by multiplex reverse transcription-polymerase chain reaction with coamplification of a plant RNA as internal control. *Phytopathology* 96, 1223-1229.
- Garcia, M.L., Sánchez De La Torre, M.E., Dal Bo, E., Djelouah, K., Rouag, N., Luisoni, E., Milne, R.G., Grau, O., 1997. Detection of citrus psorosis-ringspot virus using RT-PCR and DAS-ELISA. *Plant Pathol.* 46, 830-836.
- Garnsey, S.M., Permar, T.A., Cambra, M., Henderson, C.T., 1993. Direct tissue blot immunoassay (DTBIA) for detection of citrus tristeza virus (CTV). 12th Conference of International Organization of Citrus Virologist. MORENO, P, 39-50.
- Garnsey, S., Civerolo, E., Gumpf, D., Paul, C., Hilf, M., Lee, R., Brlansky, R., Yokomi, R., Hartung, J., 2005. Biological characterization of an international collection of Citrus tristeza virus (CTV) isolates. *Int. Organ. Citrus Virol.* 16, 75-93.
- Glasel, J., 1995. Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. *Biotechniques* 18, 62-63.
- Hajeri, S., Ramadugu, C., Keremane, M., Vidalakis, G., Lee, R., 2010. Nucleotide sequence and genome organization of Dweet mottle virus and its relationship to members of the family Betaflexiviridae. *Arch. Virol.* 155, 1523-7.
- Hilf, M.E., Karasev, A.V., Albiach-Marti, M.R., Dawson, W.O., Garnsey, S.M., 1999. Two paths of sequence divergence in the citrus tristeza virus complex. *Phytopathology* 89, 336-342.
- Hilf, M.E., Garnsey, S.M., 2000. Characterization and classification of citrus tristeza virus isolates by amplification of multiple molecular markers. *Proceedings of the 14th Conference of International Organization of Citrus Virologists*, 18-27.
- Hilf, M.E., Mavrodieva, V.A. Garnsey, S.M., 2005. Genetic marker analysis of a global collection of isolates of Citrus tristeza virus: characterization and distribution of CTV genotypes and association with symptoms. *Phytopathology* 95, 909-917.

593 Huang, Z., Rundell, P.A., Guan, X., Powell, C.A., 2004. Detection and isolate differentiation of
594 Citrus tristeza virus in infected field trees based on reverse transcription-polymerase
595 chain reaction. *Plant Dis.* 88, 625-629.

596 Hung, T.H., Wu, M.L., Su, H.J., 2000. A rapid method based on the one-step reverse
597 transcriptase-polymerase chain reaction (RT-PCR) technique for detection of different
598 strains of citrus tristeza virus. *J. Phytopathol.* 148, 469-475.

599 Hyeon, J.Y., Park, C., Choi, I.S., Holt, P.S., Seo, K.H., 2010. Development of multiplex real-
600 time PCR with Internal amplification control for simultaneous detection of Salmonella
601 and Cronobacter in powdered infant formula. *Int. J. Food Microbiol.* 144, 177-181.

602 Ito, T., Ieki, H., Ozaki, K., 2002. Simultaneous detection of six citrus viroids and Apple stem
603 grooving virus from citrus plants by multiplex reverse transcription polymerase chain
604 reaction. *J. Virol. Methods* 106, 235-239.

605 James, D., 1999. A simple and reliable protocol for the detection of apple stem grooving virus by
606 RT-PCR and in a multiplex PCR assay. *J. Virol. Methods* 83, 1-9.

607 Janse, I., Hamidjaja, R.A., Hendriks, A.C.A., van Rotterdam, B.J., 2013. Multiplex qPCR for
608 reliable detection and differentiation of *Burkholderia mallei* and *Burkholderia*
609 *pseudomallei*. *BMC Inf. Dis.* 13, 86.

610 Klatt, C.G., LaPara, T.M., 2003. Aerobic biological treatment of synthetic municipal wastewater
611 in membrane-coupled bioreactors. *Biotechnology and bioengineering* 82, 313-320.

612 LaPara, T.M., Nakatsu, C.H., Pantea, L., Alleman, J.E., 2000. Phylogenetic analysis of bacterial
613 communities in mesophilic and thermophilic bioreactors treating pharmaceutical
614 wastewater. *Appl. Environ. Microbiol.* 66, 3951-3959.

615 Leutenegger, C.M., 2001. The real-time TaqMan PCR and applications in veterinary medicine.
616 *Vet Sci Tomorrow* 1, 1-15.

617 Li, W., Hartung, J.S., Levy, L., 2006. Quantitative real-time PCR for detection and identification
618 of *Candidatus Liberibacter* species associated with citrus huanglongbing. *J. Microbiol.*
619 *Methods* 66, 104-115.

620 Li, W., Abad, J.A., French-Monar, R.D., Rascoe, J., Wen, A., Gudmestad, N.C., Secor, G.A.,
621 Lee, I.M., Duan, Y., Levy, L., 2009. Multiplex real-time PCR for detection, identification
622 and quantification of '*Candidatus Liberibacter solanacearum*' in potato plants with zebra
623 chip. *J. Microbiol. Methods* 78, 59-65.

624 Liu, Z., Teng, Y., Liu, H., Jiang, Y., Xie, X., Li, H., Lv, J., Gao, L., He, J., Shi, X., 2008.
625 Simultaneous detection of three fish rhabdoviruses using multiplex real-time quantitative
626 RT-PCR assay. *J. Virol. Methods* 149, 103-109.

627 Livak, K.J., Flood, S.J., Marmaro, J., Giusti, W., Deetz, K., 1995. Oligonucleotides with
628 fluorescent dyes at opposite ends provide a quenched probe system useful for detecting
629 PCR product and nucleic acid hybridization. *Genome Res.* 4, 357-362.

630 Loconsole, G., Castellano, M.A., Dell'Orco, M., Boscia, D., Savino, V., 2006. Serological
631 detection of Citrus psorosis virus using a polyclonal antiserum to recombinant virus coat
632 protein. *J. Plant Pathol.* 88, 171-177.

633 Loconsole, G., Fatone, M.T., Savino, V., 2009. Specific digoxigenin-labeled riboprobes for
634 detection of Citrus psorosis virus and Citrus variegation virus by molecular hybridization.
635 *J. Plant Pathol.* 91, 311-319.

636 Loconsole, G., Saponari, M., Savino, V., 2010. Development of real-time PCR based assays for
637 simultaneous and improved detection of citrus viruses. *Eur. J. Plant Pathol.* 128, 251-259.

- López-Fabuel, I., Wetzel, T., Bertolini, E., Bassler, A., Vidal, E., Torres, L.B., Yuste, A., Olmos, A., 2012. Real-time multiplex RT-PCR for the simultaneous detection of the five main grapevine viruses. *J. Virol. Methods* 188, 21-24.
- Manchester, K.L., 1995. Value of A260/A280 ratios for measurement of purity of nucleic acids. *Biotechniques* 19, 208.
- Manchester, K., 1996. Use of UV methods for measurement of protein and nucleic acid concentrations. *Biotechniques* 20, 968.
- Marbot, S., Salmon, M., Vendrame, M., Huwaert, A., Kummert, J., Dutrecq, O., Lepoivre, P., 2003. Development of real-time RT-PCR assay for detection of *Prunus* necrotic ringspot virus in fruit trees. *Plant Dis.* 87, 1344-1348.
- Martín, S., Alioto, D., Milne, R.G., Guerri, J., Moreno, P., 2002. Detection of Citrus psorosis virus in field trees by direct tissue blot immunoassay in comparison with ELISA, symptomatology, biological indexing and cross-protection tests. *Plant Pathol.* 51, 134-141.
- Martín, S., Alioto, D., Milne, R.G., Garnsey, S.M., García, M.L., Grau, O., Guerri, J., Moreno, P., 2004. Detection of Citrus psorosis virus by ELISA, molecular hybridization, RT-PCR and immunosorbent electron microscopy and its association with citrus psorosis disease. *Eur. J. Plant Pathol.* 110, 747-757.
- Martín, S., García, M.L., Troisi, A., Rubio, L., Legarreta, G., Grau, O., Alioto, D., Moreno, P., Guerri, J., 2006. Genetic variation of populations of Citrus psorosis virus. *J. Gen. Virol.* 87, 3097-3102.
- Mathews, D.M., Riley, K., Dodds, J.A., 1997. Comparison of detection methods for citrus tristeza virus in field trees during months of nonoptimal titer. *Plant Dis.* 81, 525-529.
- Mehta, P., Brlansky, R.H., Gowda, S., Yokomi, R.K., 1997. Reverse-transcription polymerase chain reaction detection of Citrus tristeza virus in aphids. *Plant Dis.* 81, 1066-1069.
- Moradi, M., Oerke, E.C., Steiner, U., Tesfaye, D., Schellander, K., Dehne, H.W., 2010. Microbiological and SYBR green real-time PCR detection of major *Fusarium* head blight pathogens on wheat ears. *Microbiology* 79, 646-654.
- Mumford, R.A., Walsh, K., Barker, I., Boonham, N., 2000. Detection of Potato mop top virus and Tobacco rattle virus using a multiplex real-time fluorescent reverse-transcription polymerase chain reaction assay. *Phytopathology* 90, 448-453.
- Narváez, G., Slimane Skander, B., Ayllón, M.a.A., Rubio, L., Guerri, J., Moreno, P., 2000. A new procedure to differentiate Citrus tristeza virus isolates by hybridization with digoxigenin-labeled cDNA probes. *J. Virol. Methods* 85, 83-92.
- Navarro, L., 1986. Citrus certification in Mediterranean countries1. *EPPO Bulletin* 16, 227-238.
- Nikolaeva, O.V., Karasev, A.V., Powell, C.A., Gumpf, D.J., Garnsey, S.M., Lee, R.F., 1996. Mapping of epitopes for citrus tristeza virus-specific monoclonal antibodies using bacterially expressed coat protein fragments. *Phytopathology* 86, 974-979.
- Nolasco, G., De Blas, C., Torres, V., Ponz, F., 1993. A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pathogens. *J. Virol. Methods* 45, 201-218.
- Olmos, A., Dasí, M.A., Candresse, T., Cambra, M., 1996. Print-capture PCR: a simple and highly sensitive method for the detection of Plum pox virus (PPV) in plant tissues. *Nucleic Acids Res.* 24, 2192-2193.

682 Olmos, A., Cambra, M., Esteban, O., Gorris, M.T., Terrada, E., 1999. New device and method
683 for capture, reverse transcription and nested PCR in a single closed-tube. *Nucleic Acids*
684 *Res.* 27, 1564-1565.

685 Ong, W.T., Omar, A.R., Ideris, A., Hassan, S.S., 2007. Development of a multiplex real-time
686 PCR assay using SYBR Green 1 chemistry for simultaneous detection and subtyping of
687 H9N2 influenza virus type A. *J. Virol. Methods* 144, 57-64.

688 Osman, F., Rowhani, A., 2006. Application of a spotting sample preparation technique for the
689 detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). *J.*
690 *Virol. Methods* 133, 130-136.

691 Osman, F., Leutenegger, C., Golino, D., Rowhani, A., 2007. Real-time RT-PCR (TaqMan®)
692 assays for the detection of Grapevine Leafroll associated viruses 1–5 and 9. *J. Virol.*
693 *Methods* 141, 22-29.

694 Osman, F., Leutenegger, C., Golino, D., Rowhani, A., 2008. Comparison of low-density arrays,
695 RT-PCR and real-time TaqMan® RT-PCR in detection of grapevine viruses. *J. Virol.*
696 *Methods* 149, 292-299.

697 Osman, F., Olineka, T., Hodzic, E., Golino, D., Rowhani, A., 2012. Comparative procedures for
698 sample processing and quantitative PCR detection of grapevine viruses. *J. Virol. Methods*
699 179, 303-310.

700 Osman, F., Hodzic, E., Alicja Omanska-Klusek, Olineka, T., Rowhani, A., 2013. Development
701 and validation of a multiplex quantitative PCR assay for the rapid detection of Grapevine
702 virus A, B and D. *J. Virol. Methods* 194, 138-145.

703 Pafundo, S., Gulli, M., Marmioli, N., 2010. Multiplex real-time PCR using SYBR® GreenER™
704 for the detection of DNA allergens in food. *Anal. and Bioanal. Chemistry* 396, 1831-
705 1839.

706 Pallás, V., Más, P., Sánchez-Navarro, J.A. 1998. Detection of plant RNA viruses by nonisotopic
707 dot-blot hybridization, *Plant Virology Protocols*, Springer, pp. 461-468.

708 Pallás, V., Sanchez-Navarro, J., Varga, A., Aparicio, F., James, D. 2009. Multiplex polymerase
709 chain reaction (PCR) and real-time multiplex PCR for the simultaneous detection of plant
710 viruses, *Plant Pathology*, Springer, pp. 193-208.

711 Permar, T.A., Garnsey, S.M., Gumpf, D.J., Lee, R.F., 1990. A monoclonal antibody that
712 discriminates strains of citrus tristeza virus. *Phytopathology* 80, 224-228.

713 Potere, O., Boscia, D., Djelouah, K., Elicio, V., Savino, V., 1999. Use of monoclonal antibodies
714 to citrus psorosis virus for diagnosis. *J. Plant Pathol.* 81, 209-212.

715 Price, J.A., Smith, J., Simmons, A., Fellers, J., Rush, C.M., 2010. Multiplex real-time RT-PCR
716 for detection of Wheat streak mosaic virus and Triticum mosaic virus. *J. Virol. Methods*
717 165, 198-201.

718 Qu, X.S., Wanner, L.A., Christ, B.J., 2011. Multiplex real-time PCR (TaqMan) assay for the
719 simultaneous detection and discrimination of potato powdery and common scab diseases
720 and pathogens. *J. Appl. Microbiol.* 110, 769-777.

721 Rodríguez, A., Rodríguez, M., Andrade, M.J., Córdoba, J.J., 2012. Development of a multiplex
722 real-time PCR to quantify aflatoxin, ochratoxin A and patulin producing molds in foods.
723 *Int. J. Food Microbiol.* 155, 10-18.

724 Roistacher, C.N., 1991. Graft-transmissible diseases of citrus: handbook for detection and
725 diagnosis. Food & Agriculture Org.

726 Roistacher, C.N., 1993. Psorosis-a review. *Proceedings of the 12th Conference of the*
727 *International Organization of Citrus Virologists*, Vol. 1992, 139-154.

- Rosa, C., Polek, M., Falk, B.W., Rowhani, A., 2007. Improved efficiency for quantitative and qualitative indexing for Citrus tristeza virus and Citrus psorosis virus. *Plant Dis.* 91, 1089-1095.
- Rosner, A., Bar-Joseph, M., 1984. Diversity of citrus tristeza virus strains indicated by hybridization with cloned cDNA sequences. *Virology* 139, 189-193.
- Roy, A., Fayad, A., Barthe, G., Brlansky, R.H., 2005. A multiplex polymerase chain reaction method for reliable, sensitive and simultaneous detection of multiple viruses in citrus trees. *J. Virol. Methods* 129, 47-55.
- Roy, A., Ananthakrishnan, G., Hartung, J.S., Brlansky, R.H., 2010. Development and application of a multiplex reverse-transcription polymerase chain reaction assay for screening a global collection of Citrus tristeza virus isolates. *Phytopathology* 100, 1077-1088.
- Roy, A., Brlansky, R.H., 2010. Genome analysis of an orange stem pitting citrus tristeza virus isolate reveals a novel recombinant genotype. *Virus Res.* 151, 118-30.
- Rubio, L., Ayllon, M., Guerri, J., Pappu, H., Niblet, C., Moreno, P., 1996. Differentiation of citrus tristeza closterovirus (CTV) isolates by single-strand conformation polymorphism analysis of the coat protein gene. *Ann. Appl. Biol.* 129, 479-489.
- Ruiz-Ruiz, S., Moreno, P., Guerri, J., Ambros, S., 2006. The complete nucleotide sequence of a severe stem pitting isolate of Citrus tristeza virus from Spain: comparison with isolates from different origins. *Arch. Virol.* 151, 387-398.
- Ruiz-Ruiz, S., Moreno, P., Guerri, J., Ambrós, S., 2007. A real-time RT-PCR assay for detection and absolute quantitation of Citrus tristeza virus in different plant tissues. *J. Virol. Methods* 145, 96-105.
- Ruiz-Ruiz, S., Ambrós, S., Vives, M.d.C., Navarro, L., Moreno, P., Guerri, J., 2009a. Detection and quantitation of Citrus leaf blotch virus by TaqMan real-time RT-PCR. *J. Virol. Methods* 160, 57-62.
- Ruiz-Ruiz, S., Moreno, P., Guerri, J., Ambrós, S., 2009b. Discrimination between mild and severe Citrus tristeza virus isolates with a rapid and highly specific real-time reverse transcription-polymerase chain reaction method using TaqMan LNA probes. *Phytopathology* 99, 307-315.
- Sambade, A., Rubio, L., Garnsey, S.M., Costa, N., Müller, G.W., Peyrou, M., Guerri, J., Moreno, P., 2002. Comparison of viral RNA populations of pathogenically distinct isolates of Citrus tristeza virus: application to monitoring cross-protection. *Plant Pathol.* 51, 257-265.
- Sambade, A., López, C., Rubio, L., Flores, R., Guerri, J., Moreno, P., 2003. Polymorphism of a specific region in gene p23 of Citrus tristeza virus allows discrimination between mild and severe isolates. *Arch. Virol.* 148, 2325-2340.
- Sambade, A., Ambrós, S., López, C., Ruiz-Ruiz, S., de Mendoza, A.H., Flores, R., Guerri, J., Moreno, P., 2007. Preferential accumulation of severe variants of Citrus tristeza virus in plants co-inoculated with mild and severe variants. *Arch. Virol.* 152, 1115-1126.
- Sanchez-Navarro, J.A., Aparicio, F., Herranz, M.C., Minafra, A., Myrta, A., Pallas, V., 2005. Simultaneous detection and identification of eight stone fruit viruses by one-step RT-PCR. *Eur. J. Plant Pathol.* 111, 77-84.
- Santos Filho, H.P., Passos, O.S., da Cunha Sobrinho, A.P., Barbosa, C.d.J., Nickel, O., 2000. The citrus variety improvement program in northeastern Brazil after 15 Years. *Proc. 14th Conf. Int. Org. Citrus Virol.* P. Moreno, JV da Graça, and LW Timmer, eds. IOCV, University of California, Riverside, 403-404.

- Saponari, M., Manjunath, K., Yokomi, R.K., 2008. Quantitative detection of Citrus tristeza virus in citrus and aphids by real-time reverse transcription-PCR (TaqMan®). *J. Virol. Methods* 147, 43-53.
- Saponari, M., Loconsole, G., Liao HH., Jiang, B., Savino, V., Yokomi, R.K. 2013. Validation of high-throughput real time polymerase chain reaction assays for simultaneous detection of invasive citrus pathogens. *J. Virol. Methods* 193, 478-486.
- Schneider, W.L., Sherman, D.J., Stone, A.L., Damsteegt, V.D., Frederick, R.D., 2004. Specific detection and quantification of Plum pox virus by real-time fluorescent reverse transcription-PCR. *J. Virol. Methods* 120, 97-105.
- Sieburth, P.J., Nolan, K.G., Alderman, S.M., Dexter, R.J., 2009. Increased Efficiency and Sensitivity for Identifying Citrus Greening and Citrus Tristeza Virus using Real-time PCR Testing. *Proc. Fla. State Hort. Soc.*, Vol. 122, 141-146.
- Szemes, M., Bonants, P., de Weerd, M., Baner, J., Landegren, U., Schoen, C.D., 2005. Diagnostic application of padlock probes—multiplex detection of plant pathogens using universal microarrays. *Nucleic Acids Res.* 33, Art No. e70.
- Teare, J., Islam, R., Flanagan, R., Gallagher, S., Davies, M., Grabau, C., 1997. Measurement of nucleic acid concentrations using the DyNA Quant and the GeneQuant. *Biotechniques* 22, 1170-1174.
- Thonur, L., Maley, M., Gilray, J., Crook, T., Laming, E., Turnbull, D., Nath, M., Willoughby, K., 2012. One-step multiplex real time RT-PCR for the detection of bovine respiratory syncytial virus, bovine herpesvirus 1 and bovine parainfluenza virus 3. *BMC Vet. Res.* 8, 37.
- Tyagi, S., Kramer, F.R., 1996. Molecular beacons: probes that fluoresce upon hybridization. *Nature biotechnol.* 14, 303-308.
- Ueno, Y., Haruta, S., Ishii, M., Igarashi, Y., 2001. Changes in product formation and bacterial community by dilution rate on carbohydrate fermentation by methanogenic microflora in continuous flow stirred tank reactor. *Appl. Microbiol. Biotechnol.* 57, 65-73.
- Van Doorn, R., Szemes, M., Bonants, P., Kowalchuk, G.A., Salles, J.F., Ortenberg, E. Schoen, C.D., 2007. Quantitative multiplex detection of plant pathogens using a novel ligation probe-based system coupled with universal, high-throughput real-time PCR on Open Arrays™. *BMC Genomics* 8, 276.
- Varga, A., James, D., 2005. Detection and differentiation of Plum pox virus using real-time multiplex PCR with SYBR Green and melting curve analysis: a rapid method for strain typing. *J. Virol. Methods* 123, 213-220.
- Varga, A., James, D., 2006. Real-time RT-PCR and SYBR Green I melting curve analysis for the identification of Plum pox virus strains C, EA, and W: Effect of amplicon size, melt rate, and dye translocation. *J. Virol. Methods* 132, 146-153.
- Vela, C., Cambra, M., Cortés, E., Moreno, P., Miguét, J.G., de San Román, C.P., Sanz, A., 1986. Production and characterization of monoclonal antibodies specific for citrus tristeza virus and their use for diagnosis. *J. Gen. Virol.* 67, 91-96.
- Vidalakis, G., Garnsey, S.M., Bash, J.A., Greer, G.D., Gumpf, D.J., 2004. Efficacy of bioindexing for graft-transmissible citrus pathogens in mixed infections. *Plant Dis.* 88, 1328-1334.
- Vidalakis, G., Da Graca, J.V., Dixon, W.N., Ferrin, D., Kesinger, M., Krueger, R., Lee, R.R., Melzer, M.J., Olive, J., Polek, M., Sieburth, P.J., Williams, L.L., Wright, G.C., 2010a. Citrus quarantine, sanitary and certification programs in the USA. *Prevention of*

- introduction and distribution of citrus diseases. Part 1- Citrus quarantine and introduction programs. . Citrograph 1, 26-35.
- Vidalakis, G., Da Graca, J.V., Dixon, W.N., Ferrin, D., Kesinger, M., Krueger, R., Lee, R.R., Melzer, M.J., Olive, J., Polek, M., Sieburth, P.J., Williams, L.L., Wright, G.C., 2010b. Citrus quarantine, sanitary and certification programs in the USA. Prevention of introduction and distribution of citrus diseases. Part 2-Certification schemes and national programs. Highlighs of new California citrus nursery regulations. . Citrograph 1, 27-39.
- Vives, M.C., Galipienso, L., Navarro, L., Moreno, P., Guerri, J., 2001. The nucleotide sequence and genomic organization of citrus leaf blotch virus: candidate type species for a new virus genus. *Virology* 287, 225-233.
- Vives, M.a.C., Rubio, L., Galipienso, L., Navarro, L., Moreno, P., Guerri, J., 2002a. Low genetic variation between isolates of Citrus leaf blotch virus from different host species and of different geographical origins. *J. Gen. Virol.* 83, 2587-2591.
- Vives, M.C., Galipienso, L., Navarro, L., Moreno, P., Guerri, J., 2002b. Citrus leaf blotch virus: a new citrus virus associated with bud union crease on trifoliolate rootstocks. *Proceedings of the 15th Conference of the International Organization of Citrus Virologists, IOCV, Riverside 2002*, 205-212.
- Von Broembsen, L., Lee, A.T.C., 1988. South Africa's citrus improvement programme. *Proceedings of the 10th Conference of the International Organization of Citrus Virologists* (LW Timmer, SM Garnsey and L. Navarro, Eds.), 407-416.
- Wang, X., Zhou, C., Tang, K., Zhou, Y., Li, Z., 2009. A rapid one-step multiplex RT-PCR assay for the simultaneous detection of five citrus viroids in China. *Eur. J. Plant Pathol.* 124, 175-180.
- Wang, J., Bozan, O., Kwon, S., Rucker, T., Thomas, C., Yokomi, R.K., Lee, R.F., Folimonova, S., Vidalakis, G., 2012. Molecular diversity of Citrus tristeza virus (CTV) strains collected over the past 50 years and maintained in CTV collections in California. *International Citrus Congress Proceedings. S14P06*, p.240.
- Wang J., Bozan, O., Kwon, S-J., Dang, T., Rucker, T., Yokomi, R.K., Lee, R.F., Folimonova, S.Y., Krueger, R.R., Bash, J., Greer, G., Diaz, J., Serna, R., Vidalakis, G. 2013. Past and future of a century old Citrus tristeza virus collection: a California citrus germplasm tale. *Frontiers in Microbiology* 4, 1-13.
- Wang, J. 2014. Multiplex assay for the detection of citrus pathogens. United States Patent Application Publication No.: US 2014/0030698 A1.
- Wernike, K., Hoffmann, B., Beer, M., 2013. Single-Tube Multiplexed Molecular Detection of Endemic Porcine Viruses in Combination with Background Screening for Transboundary Diseases. *J. Clin. Microbiol.* 51, 938-944.
- Yokomi, R.K., Mello, A.F.S., Saponari, M., Fletcher, J., 2008. Polymerase chain reaction-based detection of *Spiroplasma citri* associated with citrus stubborn disease. *Plant Dis.* 92, 253-260.
- Yokomi, R.K., Saponari, M., Sieburth, P.J., 2010. Rapid differentiation and identification of potential severe strains of Citrus tristeza virus by real-time reverse transcription-polymerase chain reaction assays. *Phytopathology* 100, 319-327.
- Yu, Y., Kim, J., Hwang, S., 2006. Use of real-time PCR for group-specific quantification of acetivlastic methanogens in anaerobic processes: Population dynamics and community structures. *Biotechnology and bioengineering* 93, 424-433.

Figure captions

Figure 1. Standard curve analysis of qPCR sensitivity in singleplex RT-qPCR and multiplex RT-qPCR format. The X-axis displays the log copies/well and the Y-axis represents the C_q value. RT-qPCR efficiency is determined by using a 10-fold serial dilutions of total RNA samples. CTV: *Citrus tristeza virus*. CPsV: *Citrus psorosis virus*. CLBv: *Citrus leaf blotch virus*.

Table 1. Oligonucleotide primers and qPCR probes used for singleplex and multiplex RT-qPCR

Primers/Probes	Sequence 5'-3'	Nucleotide position	Amplicon size (bp)	Primer/Probe combinations
COX_multi-32 F	AATCTGACCTTCTTTCCCATGC	32-53	162	COX_multi-32 F
COX_multi-194 R	AAGTGATTGTTACGACCACGAAGA	194-171		COX_multi-194 R
COX_multi-96 p-FAM	ATCCAGATGCTTACGCTGG	96-114		COX_multi-96 p-FAM
CTV CA-CP-16678 F	TGTGTGCAGATTTCTTGACCG	16617-16637	135	CTV CA-CP-16678 F
CTV CA-CP-16679 F2	TGTGTGCGGATTTCTTGACTG	16617-16637		CTV CA-CP-16813 R
CTV CA-CP-16813 R	TTCCCAAGCTGCCTGACATT	16753-16733		CTV CA-CP-16763 p-TET
CTV CA-CP-16763 p-TET	AAGCGAGGGGCTGAT	16702-16717		CTV CA-CP-16679 F2
				CTV CA-CP-16813 R
				CTV CA-CP-16763 p-TET
CPsV-792 F1	TCACAAATCAGTGAGGAATTGAGC	792-816	154	CPsV-792 F1
CPsV-791 F2	CACAAATCAGTGATGAATTGAGCC	793-817		CPsV-946 R1
CPsV-946 R1	GCAAACCCAGCATATCTCACAG	947-925		CPsV-851 p-VIC
CPsV-946 R2	CGCAAACCCAGCATATCTTACAG	948-925		CPsV-792 F1
CPsV-851 p-VIC	TCTCAAGATTGATATAGACAAC	851-873		CPsV-946 R2
				CPsV-851 p-VIC
				CPsV-791 F2
				CPsV-946 R1
				CPsV-851 p-VIC
				CPsV-791 F2
				CPsV-946 R2
				CPsV-851 p-VIC
CLBV CP-7711 F	TTCAAGAACTGGATTTGAATTGTC	7711-7735	161	CLBV CP-7711 F
CLBV CP-7872 R	TGCACAGAATTGCCTCACAGT	7874-7853		CLBV CP-7872 R
CLBV CP-7738 p-FAM	AAGTTGTGGATCAAGAAG	7738-7756		CLBV CP-7738 p-FAM

COX: Cytochrome oxidase gene of host plants used as positive internal control. COX nucleotide position GenBank CX297817. CTV: *Citrus tristeza virus*. CTV nucleotide position GenBank M76485. CPsV: *Citrus psorosis virus*. CPsV nucleotide position GenBank AF036926. CLBV: *Citrus leaf blotch virus*. CLBV nucleotide position GenBank NC_003877. CP: Coat protein, F: forward primer, R: Reverse primer, and p: RT-qPCR probe.

Table 2. Comparison between singleplex and multiplex RT-qPCR assays in detecting CTV, CLB V and CPsV. Viral Load/1 mg is shown.

RT-qPCR	CTV		CPsV		CLBV	
	Singleplex Cq	Multiplex Cq	Singleplex Cq	Multiplex Cq	Singleplex Cq	Multiplex Cq
Viral load mean (n= 3)	1.44E+06	1.19E+06	7.26E+04	5.31E+04	3.84E+0.5	2.51E+05
Standard deviation	1.66E+06	1.42+06	8.30E+04	6.14E+04	4.79+0.5	3.05+05
<i>p</i> value	0.142054		0.234670		0.213125	

CTV: *Citrus tristeza virus*, CPsV: *Citrus psorosis virus*, CLB V: *Citrus leaf blotch virus*.

Statistical analysis was performed using PASW Statistics 18 software (SPSS Inc., Chicago, IL). The reproducibility of the assay was tested by analysis of variance (ANOVA) comparing the quantitative cycle (Cq) values of the samples.

Table 3. Comparison between singleplex and multiplex RT-qPCR assays in detecting CTV (A), CPsV (B), and CLBV (C) in infected samples across a range of different virus titer concentrations.

A. <i>Citrus tristeza virus</i> (CTV)						B. <i>Citrus psorosis virus</i> (CPsV)					
Isolate	Origin/Genotype	RT-qPCR-Cq Values			S/M Ratio	Isolate	Origin/Type	RT-qPCR-Cq Values			S/M Ratio
		COX	Singleplex (S)	Multiplex (M)				COX	Singleplex (S)	Multiplex (M)	
B7	Africa/T36+T30+VT	22.29	17.90	18.50	0.97	P200A	China/A	23.43	26.00	26.77	0.97
B14	Brazil/T36+T30+T3+VT	23.02	26.90	27.03	0.99	P201*	USA, CA/A	22.03	23.89	24.43	0.98
B28	USA, FL/B165/T68	20.61	19.26	20.88	0.92	P202	Thailand/A	22.61	24.94	25.23	0.99
B53 [#]	Spain	18.94	19.22	19.50	0.99	P203A	USA, CA/B	21.19	27.06	27.85	0.97
B162 [#]	Spain/T30	21.96	20.20	21.61	0.93	P205*	USA, CA/A	21.76	22.98	23.43	0.98
B163 [#]	Spain	22.25	20.99	22.34	0.94	P208	USA, CA/B	22.27	25.97	26.38	0.98
B165	India/B165/T68	19.78	21.32	24.00	0.89	P212	USA, CA/A	21.18	24.05	24.56	0.98
B215	Japan/T30	14.08	18.23	19.00	0.96	P213 ^s	USA, CA/A	21.34	22.05	22.43	0.98
B227	India/T30+VT	22.08	18.90	19.07	0.99	P215	USA, CA/A	20.70	22.85	23.07	0.99
B233R	Taiwan/VT	23.02	26.90	27.03	1.00	P250*	USA, CA/A	23.79	27.03	27.72	0.98
B237R	Taiwan	22.03	19.60	20.06	0.98	P379	USA, CA/A	19.72	22.12	22.84	0.97
B249	Venezuela/T3+VT	23.34	27.80	28.09	0.99	P546	USA, CA/A	22.08	27.99	28.12	1.00
B256	Indonesia	21.87	21.80	22.14	0.98	P1365 ^s	USA, CA/A	21.21	23.97	24.49	0.98
B288	Colombia	22.44	25.90	26.05	0.99	P1405	USA, CA/A	22.17	26.99	27.84	0.97
B296	Dominican Republic /T30+VT	22.09	19.80	20.18	0.98	P1407	USA, CA/B	23.72	28.00	28.55	0.98
B300	Puerto Rico/VT	21.63	22.80	23.09	0.99	B53 [#]	Spain	18.94	26.55	28.74	0.92
B301	Puerto Rico	21.88	20.60	21.14	0.97	B162 [#]	Spain	21.96	23.43	22.58	0.93
B302	Jamaica	23.34	27.80	28.09	0.99	B163 [#]	Spain	22.25	25.30	24.96	0.94
B303	Cuba	21.96	21.70	22.12	0.98	B421 [#]	Peru	19.61	23.95	23.96	1.00
B304	Cuba	22.25	21.60	22.07	0.98						
B305	Cuba/T30	21.87	22.90	23.01	1.00	C. <i>Citrus leaf blotch virus</i> (CLBV)					
B308	Cuba	21.75	18.80	19.09	0.98	RT-qPCR-Cq Values					
B313	Cuba	21.87	21.80	22.14	0.98	Isolate	Origin	COX	Singleplex (S)	Multiplex (M)	S/M Ratio
B317	Taiwan	23.31	17.90	18.14	0.99	FL-053 ^s	USA, FL	22.73	26.98	27.12	0.99
B331	Dominican Republic	21.25	17.80	18.10	0.98	FL-193 ^s	USA, FL	24.47	25.48	26.15	0.97
B334	USA, CA	21.35	19.60	20.03	0.98	FS-642 ^s	USA, FL	24.86	20.57	21.14	0.97
B338	Trinidad	22.27	29.80	30.10	0.99	FS-668 ^s	USA, FL	24.54	20.48	21.30	0.96
B339	Trinidad	21.63	22.80	23.09	0.99	FS-693	USA, FL	24.76	29.94	29.41	1.02
B340	Trinidad	21.87	22.90	23.01	1.00	FS-694	USA, FL	24.49	26.95	27.39	0.98
B343	Guatemala	22.09	19.80	20.18	0.98	FS-712	USA, FL	25.49	30.15	30.21	1.00
B344	Dominican Republic	22.08	18.90	19.07	0.99	DMV930.1*	USA, CA	20.33	22.13	22.76	0.97
B370 [@]	Australia/T30+VT	22.27	29.80	30.10	0.99	DMV930.2	USA, CA	21.20	25.27	26.08	0.97
B421 [#]	Peru/T3	19.61	19.03	19.94	0.95	DMV931*	USA, CA	20.50	22.14	22.86	0.97
SY550	China/VT	20.40	21.85	22.21	0.98	DMV932*	USA, CA	22.39	22.91	23.25	0.99
SY553*	USA, CA/VT+T36	17.90	16.78	17.04	0.98	CLBV-3069-1	Spain	22.99	21.49	22.15	0.97
SY555	USA, CA/VT	21.09	24.90	25.01	1.00	CLBV	Australia	21.05	21.03	23.98	0.88
SY558	Hawaii/T30+VT+T36	16.99	17.99	18.25	0.99	B370 [@]	Australia	22.27	24.56	27.54	0.89
SY567*	USA, CA/T30+VT	18.90	15.88	16.00	0.99						
SY568	USA, CA/T30+VT	18.90	20.66	21.01	0.98	D. Non-Inoculated Negative Controls					
SY582	USA	17.54	17.90	18.50	0.97	RT-qPCR-Cq Values					
T508*	USA, CA/T30	19.08	17.90	18.60	0.96	Citrus Variety/Type	COX				
T514	USA, CA/T30	20.01	19.20	19.90	0.96	Washington navel orange	21.64				
T519	USA, CA/T30+VT	15.02	21.56	21.90	0.98	Moro blood orange	23.67				
T521	USA, CA/T30+VT	18.03	16.90	17.22	0.98	Mexican lime	23.05				
T528	USA, CA/T30	20.87	18.33	19.01	0.96	‘Etrog’ citron	23.26				
T535	Japan/T30+VT+T36	17.90	20.80	21.01	0.99	Nagami kumquat	23.21				
PB61	Australia	20.87	23.56	25.02	0.94	Carrizo citrange	22.58				
PB80	Australia	20.99	18.54	20.20	0.92	Eureka lemon	21.80				
P213 ^s	USA, CA/A	21.34	20.45	20.30	1.00	Owari satsuma	22.62				
P1365 ^s	USA, CA/A	21.21	28.32	28.98	0.98	Algerian clementine	23.20				
FL-053 ^s	USA, FL	22.73	17.81	18.73	0.95	Marsh grapefruit	21.96				
FL-193 ^s	USA, FL	24.47	19.14	20.18	0.95	Ray ruby grapefruit	21.85				
FS-642 ^s	USA, FL	24.86	19.47	20.37	0.96	Standard sour orange	21.57				
FS-668 ^s	USA, FL	24.54	19.81	20.50	0.97						

COX: Cytochrome oxidase gene of host plants used as positive internal control. From the 210 CTV isolates tested results for 54 are presented here. Cq values of the 210 CTV singleplex, and multiplex RT-qPCR ranged between 15.88-27.90 and 16.00-28.90, respectively. Cq values for COX ranged between 14.08-23.98 for all CTV isolates tested. Dweet mottle virus (DMV) is synonymous to CLBV (Hajeri et al., 2010). Virus isolates noted with (*) where used for the inter- and intra-assay validation. Naturally mix infected samples are noted for CTV with (\$), CPsV with (#), and CLBV with (@). In singleplex RT-qPCR mixtures, only one standard cDNA sample corresponding to each virus was used and amplified. In multiplex RT-qPCR mixtures, three standard cDNA samples for each virus tested were combined and tested. Ratio between singleplex and multiplex RT-qPCR was in concordance. Ratio ≤ 1.02 .

Table 4. Comparison between one-step multiplex RT-qPCR and two-step multiplex qPCR assays in detecting CTV (A), CPsV (B), and CLB (C) in infected samples across a range of different virus titer concentrations.

A. <i>Citrus tristeza virus</i> (CTV)				B. <i>Citrus psorosis virus</i> (CPsV)			
Isolate	Multiplex RT-qPCR-Cq Values		1/2 Ratio	Isolate	Multiplex RT-qPCR-Cq Values		1/2 Ratio
	one-step (1)	two-step (2)			one-step (1)	two-step (2)	
B7	19.01	18.50	1.03	P200A	26.01	26.77	0.97
B14	23.92	27.03	0.88	P201	22.91	24.43	0.94
B28	18.89	20.88	0.90	P202	26.19	25.23	1.04
B53 [#]	18.65	19.50	0.96	P203A	28.01	27.85	1.01
B162 [#]	23.98	21.61	1.11	P205	24.11	23.43	1.03
B163 [#]	23.91	22.34	1.07	P208	25.45	26.38	0.96
B165	23.00	24.00	0.96	P212	22.63	24.56	0.92
B215	19.13	19.00	1.01	P213 ^s	23.27	22.43	1.04
B227	19.64	19.07	1.03	P215	24.54	23.07	1.06
B233R	30.04	27.03	1.11	P250	27.88	27.72	1.01
B237R	24.41	20.06	1.22	P379	24.08	22.84	1.05
B249	22.96	28.09	0.82	P546	29.34	28.12	1.04
B256	24.13	22.14	1.09	P1365 ^s	24.06	24.49	0.98
B288	25.96	26.05	1.00	P1405	23.25	27.84	0.84
B296	20.91	20.18	1.04	P1407	25.21	28.55	0.88
B300	22.99	23.09	1.00	B53 [#]	24.51	28.74	0.85
B301	24.27	21.14	1.15	B162 [#]	26.43	22.58	1.17
B302	26.58	28.09	0.95	B163 [#]	26.30	24.96	1.05
B303	19.07	22.12	0.86	B421 [#]	22.52	23.96	0.94
B304	22.02	22.07	1.00	C. <i>Citrus leaf blotch virus</i> (CLBV)			
B305	24.10	23.01	1.05	Multiplex RT-qPCR-Cq Values			
B308	22.11	19.09	1.16	Isolate	one-step (1)	two-step (2)	1/2 Ratio
B313	25.88	22.14	1.17				
B317	20.73	18.14	1.14	FL-053 ^s	24.28	27.12	0.90
B331	22.41	18.10	1.24	FL-193 ^s	22.42	26.15	0.86
B334	22.83	20.03	1.14	FS-642 ^s	21.48	21.14	1.02
B338	29.55	30.10	0.98	FS-668 ^s	18.57	21.30	0.87
B339	24.29	23.09	1.05	FS-693	28.90	29.41	0.98
B340	26.49	23.01	1.15	FS-694	27.80	27.39	1.01
B343	23.39	20.18	1.16	FS-712	30.01	30.21	0.99
B344	23.97	19.07	1.26	DMV930.1	23.75	22.76	1.04
B370 [@]	25.57	30.10	0.85	DMV930.2	27.21	26.08	1.04
B421 [#]	20.01	19.94	1.00	DMV931	21.83	22.86	0.95
SY550	22.05	22.21	0.99	DMV932	22.02	23.25	0.95
SY553	18.63	17.04	1.09	CLBV-3069-1	22.89	22.15	1.03
SY555	30.49	25.01	1.22	CLBV	21.18	23.98	0.89
SY558	18.66	18.25	1.02	B370 [@]	25.96	27.54	0.94
SY567	19.27	16.00	1.20				
SY568	18.38	21.01	0.87				
SY582	19.05	18.50	1.03				
T508	23.37	18.60	1.26				
T514	21.98	19.90	1.10				
T519	25.99	21.90	1.19				
T521	17.88	17.22	1.04				
T528	20.22	19.01	1.06				
T535	22.97	21.01	1.09				
PB61	23.63	25.02	0.94				
PB80	18.22	20.20	0.90				
P213 ^s	23.07	20.30	1.14				
P1365 ^s	29.41	28.98	1.01				
FL-053 ^s	18.07	18.73	0.96				
FL-193 ^s	17.27	20.18	0.86				
FS-642 ^s	17.53	20.37	0.86				
FS-668 ^s	16.22	20.50	0.79				

Dweet mottle virus (DMV) is synonymous to CLB (Hajeri et al., 2010). Naturally mix infected isolates are noted for CTV with (\$), CPsV with (#), and CLB with (@). Total RNA was used as template for one-step multiplex RT-qPCR, using the QuantiFast Multiplex RT-PCR Kit (Qiagen) and cDNA derived from the total RNA was used as a template for two-step multiplex qPCR, using TaqMan[®] Gene Expression Master Mix, (Life Technologies). In multiplex RT-qPCR mixtures, three standard cDNA samples for each virus tested were combined and tested. Ratio between one-step and two-step multiplex RT-qPCR was in concordance. Ratio ≤ 1.17.

Table 5. Comparison between singleplex and multiplex RT-qPCR assays in detecting *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV), and *Citrus leaf blotch* (CLBV) virus in mix infected citrus trees.

Triple mix infected samples	RT-qPCR-Cq Values												
	COX	Multiplex									1/2 Ratio		
		Singleplex				one-step (1)							
						two-step (2)							
CTV	CPsV	CLBV	CTV	CPsV	CLBV	CTV	CPsV	CLBV	CTV	CPsV	CLBV		
SY558+P250+CLBV-3069-1	21.07	16.74	27.82	28.42	16.17	28.13	28.92	19.76	29.92	30.31	0.82	0.94	0.95
SY558+P203A+CLBV-3069-1	21.86	16.93	27.79	32.52	16.07	26.78	31.98	20.41	30.79	34.54	0.79	0.87	0.93
SY568+P203A+P250+CLBV-3069-1	20.12	17.46	23.97	32.31	16.36	24.02	33.01	19.14	25.98	34.12	0.91	0.92	0.97
P213 ^{\$} +CLBV-3069-1	21.43	19.24	26.65	20.68	17.80	23.50	20.16	20.85	26.86	21.00	0.85	0.87	0.96
P1365 ^{\$} +CLBV-3069-1	19.89	19.70	25.15	29.20	19.74	22.32	28.49	22.58	25.80	30.12	0.87	0.87	0.95
P1365 ^{\$} +DMV930.1	20.75	19.54	24.95	28.38	19.12	22.15	26.62	22.45	26.78	29.64	0.85	0.83	0.90
Virus RNA mix (CTV:CPsV:CLBV, 1:1:1)	24.69	22.38	27.35	27.14	23.17	25.76	22.03	24.55	28.33	29.73	0.94	0.91	0.74

COX: Cytochrome oxidase gene of host plants used as positive internal control. Virus isolates: CTV (SY558 and SY568), CPsV (P250, P203A, P213 and P1365), and CLBV (CLBV-3069-1 and DMV930.1). RNA and cDNA samples were used as positive control for one-step and two-step multiplex RT-qPCR, respectively. In singleplex RT-qPCR mixtures, only one standard cDNA sample corresponding to each virus was used and amplified. In multiplex RT-qPCR mixtures, three standard cDNA samples for each virus tested were combined and tested. CPsV isolates P213 and P1365 are naturally mix infected with CTV (\$). For information on viruses isolates see Table 2.