CALIFORNIA CITRUS NURSERY BOARD
The Future of the Cooperative Registration Program of Nursery Owned Citrus Source Trees
Final Report (year 4 of 4)
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Objectives

Development, validation, and employment of broad range/universal molecular diagnostic techniques for citrus pathogens of regulatory importance:

1. Reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR) protocols for the RNA pathogens:
    a. Citrus viroids (exocortis, cachexia, and other abnormalities)
    b. *Citrus tristeza virus* (quick decline, stem pitting, and seedling yellows)
    c. *Citrus psorosis virus* (seed transmission)
    d. *Citrus leaf blotch virus* (dweet mottle virus-seed transmission)
2. qPCR protocols for the DNA pathogens:
    a. *Candidatus* Liberibacter sp. (huanglongbing)
    b. *Spiroplasma citri* (stubborn)

The development of high throughput, reliable, sensitive and simple methods for the detection of citrus pathogens is important for the routine testing of citrus propagative material. We made significant progress towards the overall goal for a unified, high throughput, and cost, time and labor effective RT-qPCR and qPCR, universal and multiplex detection system for regulated pathogens of the §3701 CDFA Citrus Nursery Stock Pest Cleanliness Program.

- We developed, fine-tuned and used extensively (more than 7000 nursery samples) a nucleic acid extraction and purification procedure optimized for citrus tissue using the semi-automated high throughput systems Geno Grinder 2010 and MagMAX^{TM} Express-96. The high throughput automation system has been enhanced by the addition of a robotic liquid handler Beckman Coulter Biomek 4000 for standardization and optimization of the method.
- An RT-qPCR protocol for the universal detection of all known citrus viroids was developed and approved for use by the CDFA in September, 2012.
- A multiplex RT-qPCR protocol for the detection of three regulated viruses (CTV, CPsV and CLBV) was written and submitted to CDFA for approval on October, 2013.
- A multiplex qPCR protocol for the detection of two regulated bacterial pathogens (*S. citri* and *C.* Liberibacter) has been developed and validated in several laboratories around the world. We expect to submit this protocol for CDFA approval in the following months.

1. Development of RT-qPCR protocols for RNA citrus pathogens

a. Citrus viroids RT-qPCR assay

Seven distinct viroid species representing four genera of the Pospiviroidae family have been identified in citrus. Citrus exocortis viroid (CEVd, genus Pospiviroid), Hop stunt viroid (HSVd, genus Hostuviroid), Citrus bark cracking viroid (CBCVd, genus Cocadviroid) and Citrus bent leaf viroid (CBLVd), Citrus dwarfing viroid (CDVd), Citrus viroid V (CVd-V) and CVd-VI of the genus Apscaviroid cause various citrus diseases and abnormalities. We developed two sets of degenerate primers named "Apsca-Group" and "Non-Apsca-Group" and their respective SYBR Green RT-qPCR protocols capable of detecting all known citrus viroids in two reactions.

In September 2012, CDFA approved the use of the "Real Time SYBR Green Reverse Transcription Quantitative Polymerase Chain Reaction (SYBR RT-qPCR) for Universal Detection of Citrus Viroids" in the Citrus Nursery Stock Pest Cleanliness Program (PERMIT NO. QC 1354). A patent application (U.S. 61/556634) has been submitted and communication with a diagnostics company has begun for the commercialization of the method (http://www.freepatentsonline.com/y2013/0115591.html). The peer reviewed publication for the method is currently under preparation.

Two sets of primers have been developed, namely "Apsca-Group" and "Non-Apsca-Group", and their respective SYBR Green RT-qPCR protocols capable of detecting all known citrus viroids in two reactions.

The "Apsca-Group" set of primers was developed for the detection of CBLVd, CDVd, CVd-V, and CVd-VI of the Apscaviroid genus (Fig. 1 and Table 2). The "Non-Apsca-Group" set of primers was developed for the detection of CEVd, HSVd, and CBCVd, of the Pospiviroid, Hostuviroid, and Cocadviroid genus, respectively (Fig. 2 and Table 2). Both set of primers are detecting the viroids related variants (Table 1).

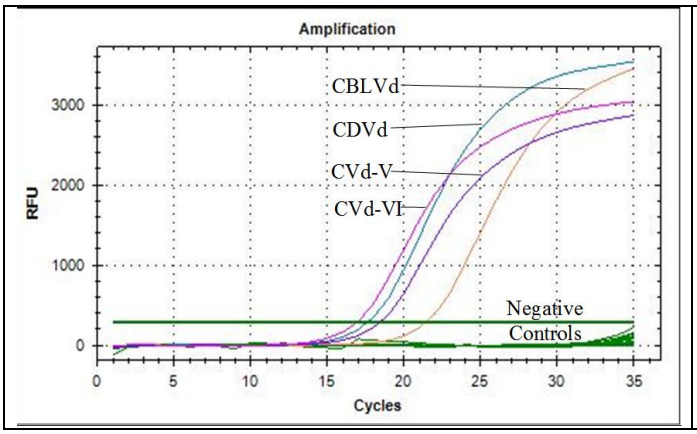


Fig. 1. RT-qPCR amplification of *Citrus bent leaf viroid* (CBLVd-orange), *Citrus dwarfing viroid* (CDVd-blue), *Citrus viroid V* (CVd-V-purple) and CVd-VI (pink), using the newly developed primers "Apsca-Group".

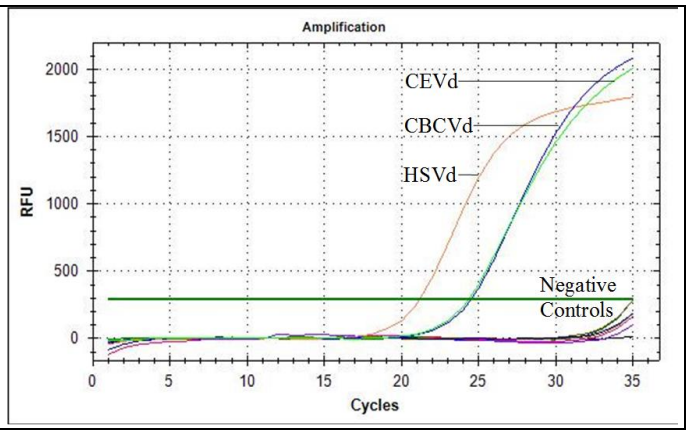
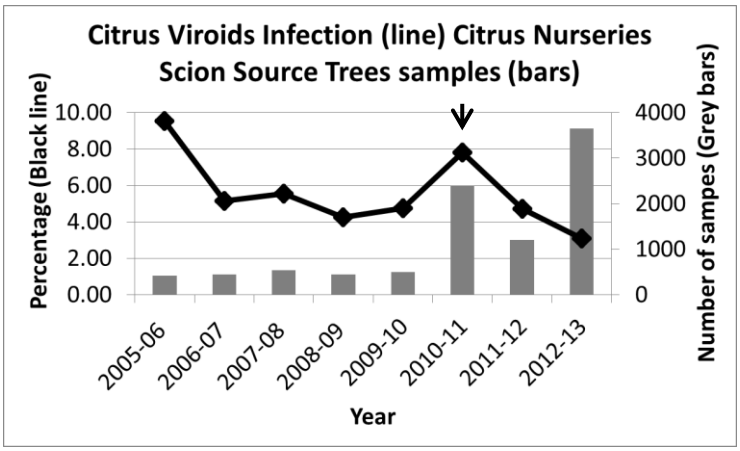


Fig. 2. RT-qPCR amplification of *Citrus exocortis viroid* (CEVd-green), *Citrus bark cracking viroid* (CBCVd-blue) and *Hop stunt viroid* (HSVd-orange), using the newly developed primers "Non-Apsca-Group".

Table 2. RT-qPCR cycles (Ct) values with the two newly developed set of primers for universal detection of citrus viroids.

	RT-qPCR Ct Values	
Target	Apsca-Group	Non-Apsca-Group
Apscaviroids (Fig. 1)		
Citrus bent leaf viroid	21.39-orange	-
Citrus dwarfing viroid	17.59-blue	-
Citrus viroid V	18.40-purple	-
Citrus viroid VI	16.93-pink	-
Non-Apscaviroids (Fig. 2)		
Citrus exocortis viroid	-	24.32- green
Citrus bark cracking viroid	-	24.48-blue
Hop stunt viroid	-	21.16-orange
Healthy and non-Template Controls		
Etrog C*itron*	-	-
Trifoliate	-	-
Valencia	-	-
Tangelo	-	-
Grapefruit	-	-
Mandarin	-	-
Water Control	-	-
No RNA template	-	-

For RT-qPCR primers the recommended or optimum values for the reaction efficiency (E) and regression coefficient (R^2) are 90-110% and approximately 0.98, respectively. The Apsca-Group primers had E values 92.56-107.09% and R^2 0.9857-0.9937 for the four different targeted viroid species. Similarly, the E and R^2 of the Non-Apsca-Group primers in the detection of three different citrus viroid species were within the recommended limits with E= 94.17-108.70% and R^2= 0.9885-0.9988.



The benefits to the registration program from the use of just one of the newly developed methods i.e**.** universal detection of citrus viroids (Vidalakis and Wang 2013) since 2001-11 (arrow in figure) are already visible. The testing capacity of the registration program has increased dramatically (grey bars). As a result a trend of reduced viroid infection is developing (black line). In other words, during the years of biological indexing (2005-2010) the number of samples tested was limited. As a result the program was always behind the curve and the viroid infection was maintained stable. From the 2010 forward and with the number of samples tested dramatically increased the program is getting ahead of the curve and the viroid infection appears to move downwards. If the trend continues and the combination with best management nursery practices the frequency and eventually the cost for viroids testing can be reduced. When all three high throughput methods are harmonized and streamlined the benefits to the industry will be maximized.

b. <u>Multiplex RT-qPCR assay for the detection of CTV, CPsV and CLBV</u>

Our experience with the viroid RT-qPCR protocol shows that qPCR-based citrus pathogen detection techniques, such as the one presented here for the simultaneous detection of three regulated citrus viruses, will create the necessary testing capacity for a successful testing program that could be evolved to a nursery certification program. In addition, the qPCR-based techniques have been proven to be high throughput, robust, simple in their use, rapid, sensitive, cost-effective especially in comparison with the currently approved diagnostic protocols of ELISA (CTV) and bioindexing (CPsV and CLBV).

A single real-time multiplex quantitative polymerase chain reaction (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 (Fig.1).

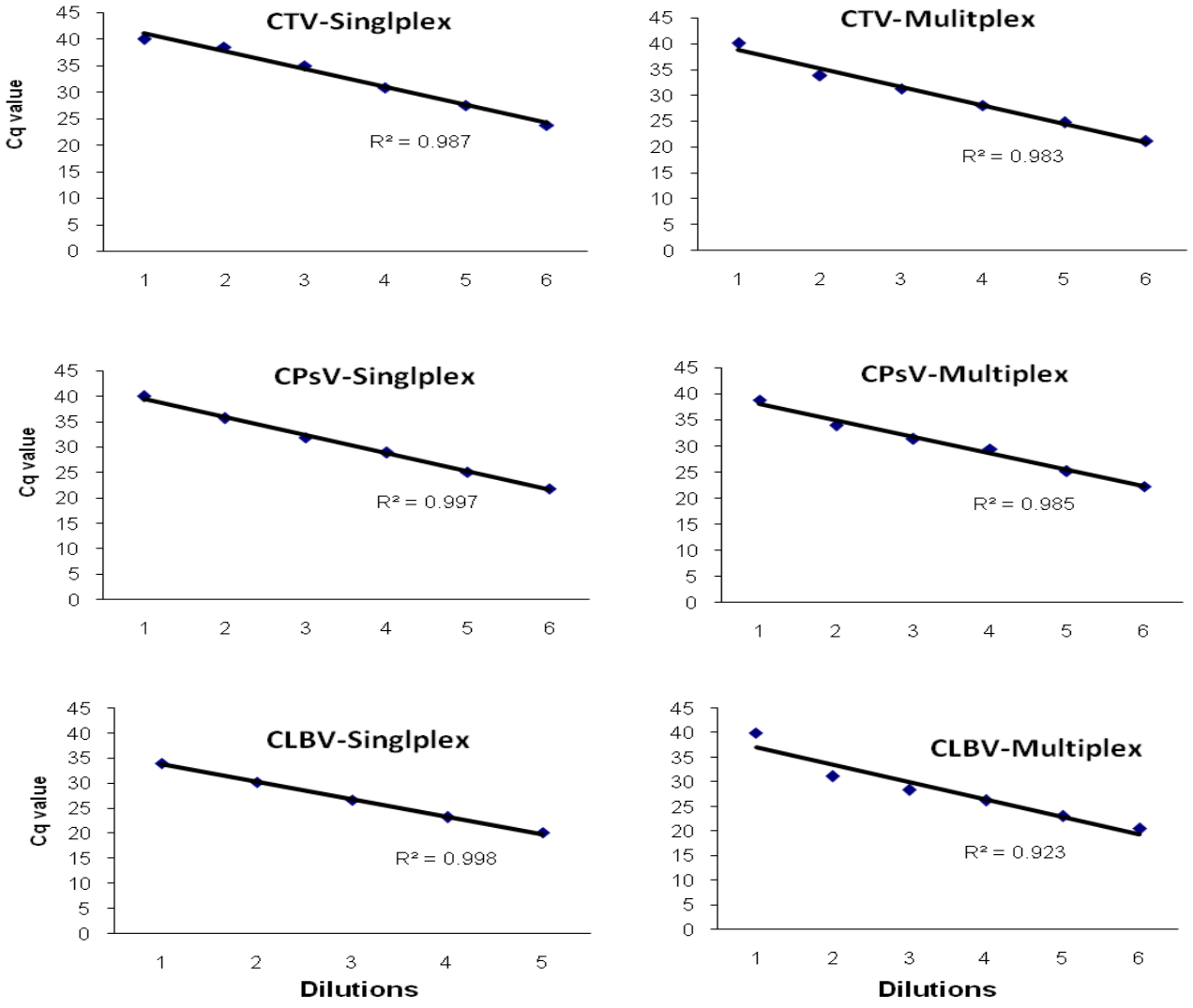


Figure 1. Standard curve analysis of qPCR sensitivity in singleplex and multiplex RT-qPCR format. The X-axis displays the log copies/well and the Y-axis represents the Cq value. RT-qPCR efficiency is determined by using a 10-fold serial dilutions of total RNA extracts. CTV: *Citrus tristeza virus*. CPsV: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

To increase the detection reliability, coat protein (CP) genes from a large number of different isolates of CTV (409 CTV (CA endemic and exotic), CPsV (15 isolates) and CLBV (12 isolates) were sequenced and piled up with corresponding CP sequences from the GenBank and robust multiplexed RT-qPCR assays were designed.

Three RT-qPCR assays have been designed (using the designated primer and probe sequences listed in Table 1) and multiplexed.

Table 1

Primers/probes	Sequence 5'- 3'	Position[1]	Amplicon size (bp)
CTV CA-CP-16678 F	TGTGTGCAGATTTCTTGACCG	16617-16637	136
CTV CA-CP-16679 F2	TGTGTGCGGATTTCTTGACTG	16617-16637	
CTV CA-CP-16813 R	TTCCCAAGCTGCCTGACATT	16753-16733	
CTV CA-CP-16763 **p-TET**	AAGCGAGGGGCTGAT	16702-16717	
CLBV CP 7711 F	TTCAAGAACTGGATTTGAATTTGC	7711-7735	163
CLBV CP 7872 R	TGCACAGAATTGCCTCACAGT	7874-7853	
CLBV CP 7738 **p-FAM**	AAGTTGTGGATCAAGAAG	7738-7756	
CPsV -792 F1	TCACAAATCAGTGAGGAATTGAGC	792-816	156
CPsV-791 F2	CACAAATCAGTGATGAATTGAGCC	793-817	
CPsV 946 R1	GCAAACCCAGCATATCTCACAG	947-925	
CPsV 946 R2	CGCAAACCCAGCATATCTTACAG	948-925	
CPsV-851 **p-VIC**	TCTCAAGATTGATATAGACAAC	851-873	

The newly developed multiplex reverse transcription qPCR (RT-qPCR) was compared to singleplex RT-qPCR designed specifically for each virus. To assess the capacity of the multiplex RT-qPCR assay in detecting CTV, CPsV, and CLBV in mixed infections from graft-inoculated hosts, a series of cDNA derived from virus isolates from diverse geographical regions, virus types, and citrus species were subjected to singleplex and multiplex RT-qPCR **(Table 2).**

Table 2. 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 concentration. Samples with variable Cq values for each of the viruses tested were used as template. Ratio between singleplex (S) and multiplex (M) RT-qPCR was in concordance (S/M ratio ≤ 1.02).

A-*Citrus tristeza virus* (CTV)

		RT-qPCR			
Isolate	Origin/Genotype	Cox	Singleplex (S)	Multilex (M)	S/M Ratio
B7	Africa/T36+T30+VT	22.29	17.90	18.50	0.97
B14	Brazil/T36+T30+T3+VT	23.02	26.90	27.03	0.99
B53[#]	Spain	18.94	19.22	19.50	0.99
B162[#]	Spain/T30	21.96	20.20	21.61	0.93
B163[#]	Spain	22.25	20.99	22.34	0.94
B215	Japan/T30	14.08	18.23	19.00	0.96
B227	India/T30+VT	22.08	18.90	19.07	0.99
B233R	Taiwan/VT	23.02	26.90	27.03	0.99
B237R	Taiwan	22.03	19.60	20.06	0.98
B249	Venezuela/T3+VT	23.34	27.80	28.09	0.99
B256	Indonesia	21.87	21.80	22.14	0.98
B288	Colombia	22.44	25.90	26.05	1.00
B296	Dominican R. /T30+VT	22.09	19.80	20.18	0.98
B300	Puerto Rico/VT	21.63	22.80	23.09	0.99
B301	Puerto Rico	21.88	20.60	21.14	0.97
B302	Jamaica	23.34	27.80	28.09	0.99
B303	Cuba	21.96	21.70	22.12	0.98
B304	Cuba	22.25	21.60	22.07	0.98
B305	Cuba/T30	21.87	22.90	23.01	1.00
B308	Cuba	21.75	18.80	19.09	0.98
B313	Cuba	21.87	21.80	22.14	0.98
B317	Taiwan	23.31	17.90	18.14	0.99
B331	Dominican R.	21.25	17.80	18.10	0.98
B334	USA, CA	21.35	19.60	20.03	0.98
B338	Trinidad	22.27	29.80	30.10	0.99
B339	Trinidad	21.63	22.80	23.09	0.99
B340	Trinidad	21.87	22.90	23.01	1.00
B343	Guatemala	22.09	19.80	20.18	0.98
B344	Dominican R.	22.08	18.90	19.07	0.99
B370[@]	Australia/T30+VT	22.27	29.80	30.10	0.99
B421[#]	Peru/T3	19.61	19.03	19.94	0.95
SY550	China/VT	20.40	21.85	22.21	0.98
SY553*	USA, CA/VT+T36	17.90	16.78	17.04	0.98
SY555	USA, CA/VT	21.09	24.90	25.01	1.00
SY558	Hawaii/T30+VT+T36	16.99	17.99	18.25	0.99
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
SY582	USA	17.54	17.90	18.50	0.97
T508*	USA/T30	19.08	17.90	18.60	0.96
T514	USA/T30	20.01	19.20	19.90	0.96
T519	USA, CA/T30+VT	15.02	21.56	21.90	0.98
T521	USA, CA/T30+VT	18.03	16.90	17.22	0.98
T528	USA, CA/T30	20.87	18.33	19.01	0.96
T535	Japan/T30+VT+T36	17.90	20.80	21.01	0.99
P213	USA, CA/A	21.34	20.45	20.30	1.00
P1365	USA, CA/A	21.21	28.32	28.98	0.98
FL-053	USA, FL	22.73	17.81	18.73	0.95
FL-193	USA, FL	24.47	19.14	20.18	0.95
FS-642	USA, FL	24.86	19.47	20.37	0.96
FS-668	USA, FL	24.54	19.81	20.50	0.97

B-*Citrus psorosis virus* (CPsV)

		RT-qPCR			
Isolate	Origin/Type	Cox	Singleplex	Multiplex	S/M Ratio
P200A	China/A	23.43	26.00	26.77	0.97
P201*	USA, CA/A	22.03	23.89	24.43	0.98
P202	Thailand/A	22.61	24.94	25.23	0.99
P203A	USA, CA/B	21.19	27.06	27.85	0.97
P205*	USA, CA/A	21.76	22.98	23.43	0.98
P208	USA, CA/B	22.27	25.97	26.38	0.98
P212	USA, CA/A	21.18	24.05	24.56	0.98
P213[$]	USA, CA/A	21.34	22.05	22.43	0.98
P215	USA, CA/A	20.70	22.85	23.07	0.99
P250*	USA, CA/A	23.79	27.03	27.72	0.98
P379	USA, CA/A	19.72	22.12	22.84	0.97
P546	USA, CA/A	22.08	27.99	28.12	1.00
P1365[$]	USA, CA/A	21.21	23.97	24.49	0.98
P1405	USA, CA/A	22.17	26.99	27.84	0.97
P1407	USA, CA/B	23.72	28.00	28.55	0.98
B53	Spain	18.94	26.55	28.74	0.92
B162	Spain	21.96	23.43	22.58	0.93
B163	Spain	22.25	25.30	24.96	0.94
B421	Peru	19.61	23.95	23.96	1.00

C-*Citrus leaf blotch virus* (CLBV)

		RT-qPCR			
Isolate	Origin	Cox	Singleplex	Multiplex	S/M Ratio
FL-053[$]	USA, FL	22.73	26.98	27.12	0.99
FL-193[$]	USA, FL	24.47	25.48	26.15	0.97
FS-642[$]	USA, FL	24.86	20.57	21.14	0.97
FS-712	USA, FL	25.49	30.15	30.21	1.00
FS-694	USA, FL	24.49	26.95	27.39	0.98
FS-693	USA, FL	24.76	29.94	29.41	1.02
FS-668[$]	USA, FL	24.54	20.48	21.30	0.96
DMV930*	USA, CA	20.33	22.13	22.76	0.97
DMV931*	USA, CA	20.50	22.14	22.86	0.97
DMV932*	USA, CA	22.39	22.91	23.25	0.99
DMV930	USA, CA	21.20	25.27	26.08	0.97
CLBV3069	Spain	22.99	21.49	22.15	0.97
B370	Australia	22.27	24.56	27.54	0.89

CTV isolates B and their genotypes were provided by the Exotic Pathogen Collection, USDA-ARS, Beltsville, MD. CTV isolates SY and T and their genotypes were provided by the Citrus Clonal Protection Program (CCPP), University of California, Riverside. From the 209 CTV isolates tested results for 50 are presented here. Cq values of the 209 CTV singleplex, and multiplex RT-qPCRs 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. CPsV P isolates and their types were provided by the CCPP. CLBV isolates FL and FS were provided by the Bureau of Citrus Budwood Registration, Florida Department of Agriculture and Consumer Services and isolates of Dweet mottle virus (DMV, syn. CLBV) (Hajeri et al., 2010) and CLBV were provided by the CCPP. Virus isolates noted with (*) where used for the inter- and intra-assay validation. Mixed infected samples are noted for CTV with ($), CPsV with (#), and CLBV with (@).

No significant differences in detection limits were found between singleplex and multiplex RT-qPCR and specificity was not affected by the inclusion of the three assays in a multiplex RT-qPCR reaction.

Our results show that the newly developed multiplex RT-qPCR assay is a cost-effective diagnostic tool that streamlines the testing of citrus viruses, replacing three separate singleplex RT-qPCR assays for CTV, CPsV, and CLBV with a single multiplex RT-qPCR assay, thus reducing time and labor while retaining the same sensitivity and specificity. 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. The targeted viruses were effectively identified effectively in infected citrus by the newly developed multiplex RT-qPCR and proved to be as sensitive as the singleplex tests, thus providing a valuable alternative tool for virus detection.

As a result of this study, the developed protocols were submitted for CDFA approval on October 2013 and a manuscript was submitted to Journal of Virological Methods (Re-submitted after incorporating reviewer's comments-Dec 5, 2013). Osman, F., Hodzic, E., Kwon, S-J, Wang, J., and Vidalakis, G. "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*"

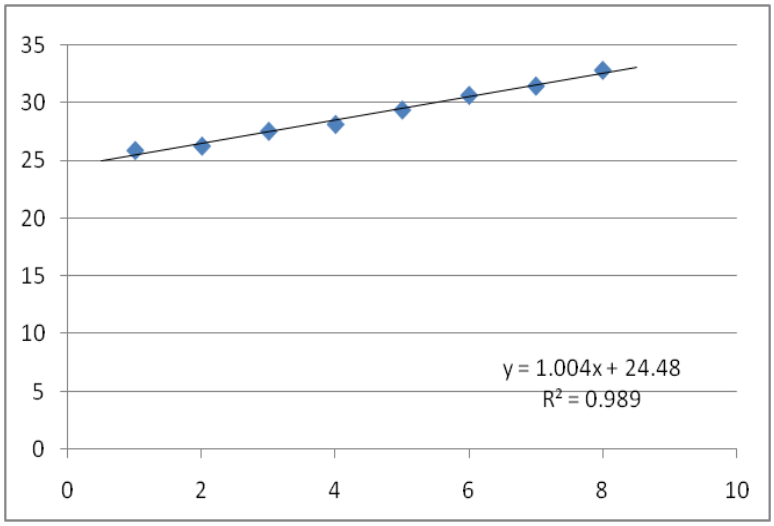
2.Development of multiplex qPCR protocols for DNA citrus pathogens (Candidatus Liberibacter sp. And S. citri)

a. Candidatus Liberibacter sp. qPCR assay

- GenBank Sequences of three Candidatus Liberibacter species associated with huanglongbing were piled up.
- Three universal qPCR assays (Can. L, Can. L2 and HLB new) were developed on the 16 S rRNA sequence pile up of the three *C.* Liberibacter species (Las, Laf, and Lam). Several samples with known low Cq values were chosen to validate the three *C.* Liberibacter qPCR assays (Can. L, Can. L2 designed by F.O. and the HLB new qPCR assay which has been modified from Li et al., 2006 qPCR assay. The HLB new qPCR assay has been validated and was shown to be more sensitive in detecting the three C. Liberibacter species. This universal HLB new qPCR assay was tested against the three Las, Laf and Lam species kindly provided from Florida, South Africa, and Brazil (Fig. 6 and Table 6).
- A general sequence pile up of the three species (Las, Laf and Lam) for the 16 S rRNA sequences is created. The sequence was much conserved and the assay designed by Li et al., 2006 has been updated to take into the consideration the melting temperature of the qPCR primers and probes as dictated by Primer Express. This assay was able to detect all three *C.* Liberibacter species.

The following HLB new qPCR Assay is:

C.lib. New # 3	
Primers/probes	Sequence 5'- 3'
HLBas.2a	CGAGCGCGTATGCAATACG
HLBas.2b	CGAGCGCGTATGCGATACG
HLBaf.2	CGAGCGCGTATTTTATACGAGC
HLBam.2	GAGCGAGTACGCAAGTACTAGCG
HLBr.2	GCGTTATCCCGTAGAAAAAGGTAG
HLBp (MGB)	AGACGGGTGAGTAACG



Slope: 1 Efficiency: 100

Collaborators kindly supplied *C.* Liberibacter nucleic acids used in the validation experiments. Samples have been sent by: Svetlana Folimonova-Florida, Gerhard Pietersen-South Africa, Nelson Wulff-Brazil and Cristina Paul-Beltsville. We would like also to acknowledge Dr. MaryLou Polek and Dr. Cynthia LeVesque of the CRB for providing the USDA-APHIS-PPQ permit and laboratory facilities for the importation of Las infected lyophilized tissue from Florida in order to test the newly developed high throughput robotic nucleic acid extraction protocol approved for use in the registration testing program.

This universal *C.* Liberibacter HLB new qPCR assay was capable of detecting all three *C.* Liberibacter species (Lam, Las and Laf) and was validated against many C. Liberibacter samples representing the three spp. (Asiaticus, Americanus and Africanus). This universal C.L. HLB new was capable of successful in detecting all samples representing the three spp. Most importantly this HLB new qPCR assay was able to detect HLB-CA isolate that was found in the backyard of a residence March/April of 2012 in Hacienda Heights of LA County, CA (as shown in Table 3).

Table 3. Testing Can L HLB new qPCR assay against Can. Liberibacter samples derived from Florida and Beltsville Maryland (asiaticus), South Africa (africanus) and Brazil (americanus).

#	Sample Type	Comments	Candidatus L. spp.	HLB
1	Unknown	DNA sent by G. Pietersen U. of Pretoria	C. L. africanus	19.04
2	Unknown	DNA sent by G. Nelson Wulff, Fundecitrus, SP Brazil	C. L. americanus	24.77
3	Unknown	DNA sent by G. Nelson Wulff, Fundecitrus, SP Brazil	C. L. americanus	31.32
4	Unknown	DNA sent by G. Nelson Wulff, Fundecitrus, SP Brazil	C. L. americanus	24.83
1	Valencia/Volk	DNA sent by Svetlana U.of Florida	C.L. asiaticus	21.76
2	SCS/Volk	DNA sent by Svetlana U.of Florida	C.L. asiaticus	21.34
3	Valencia/Volk	DNA sent by Svetlana U.of Florida	C.L. asiaticus	21.64
4	M. Vinous	DNA sent by Svetlana U.of Florida	C.L. asiaticus	21.35
5	Psyllid	DNA sent by Svetlana U.of Florida	C.L. asiaticus	23.21
6	Valencia/Volk	DNA sent by Svetlana U.of Florida	C.L. asiaticus	21.47
7	Unknown	DNA sent by Svetlana U.of Florida	C.L. asiaticus	18.93
8	CA-Hacienga isolate	DNA sent by Christina Paul -Beltsville	C.L. asiaticus	30.65
9	Unknown	DNA sent by Christina Paul -Beltsville	C.L. asiaticus	25.79
		+ve control mix of HLB (Las, Lam and Laf)		23.94

The entire collection of HLB samples (C. l. asiaticus, americanus and africanus) has been tested using the HLB3 qPCR

Attached are the results in the excel sheet (Annex 1)

b. Spiroplasma citri qPCR assay

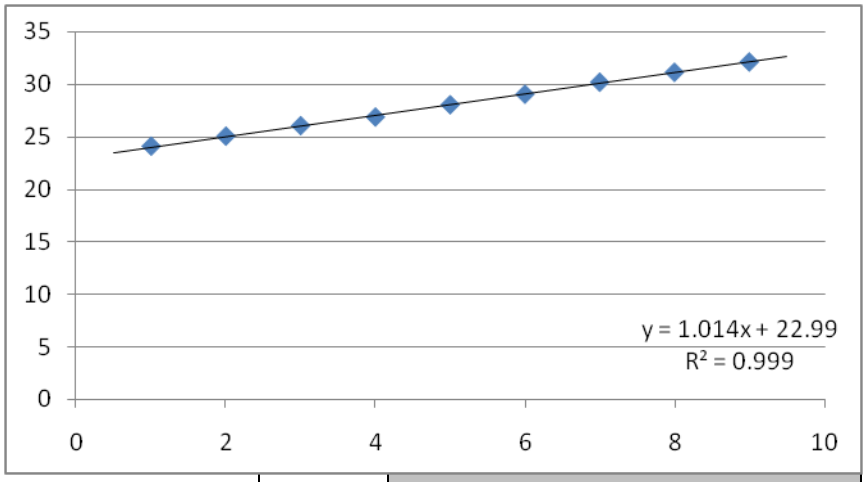
All *S. citri* GenBank accessions were sorted by the DNAstar software and several contigs containing all possible genome sequences were generated. These contig sequences pertaining to different locations within the *S. citri* genome were piled up.

Six *S. citri* qPCR assays were designed in different regions of the *S. citri* genome, namely *S. citri* contig 29, contig 26, contig 13, contig 4, Spiralin, and P58. Additionally, the qPCR assay designed by Yokomi *et al.*, 2008 was altered by designing an internal qPCR probe between the forward and the reverse primers and generated two additional qPCR assays (S. citri Yokomi C2 and C4). The *S. citri*-contig 29 was shown to produce the best Cq results as shown in Table 4.

Table 4. Validation-Efficiency of the *S. citri* qPCR assays using representatives *S. citri* isolates.

	***S. citri* Isolates and Cq Values**				**Validation**
***S. citri* qPCR Assay**	**C189**	**S600**	**S616**	**H2O**	
Contig 29	25.61	29.56	23.94	-	98.6%
Contig 26	32.92	39.26	33.88	-	
Contig 13-Spiralin	27.59	32.9	26.96	-	105.9%
Contig 4	-	-	39.17	-	
Spiralin	30.85	35.46	30.38	-	
P58	36.89	-	34.47	-	
Yokomi C2	28.6	-	26.87	-	
Yokomi C4	31.9	31.6	28.48	-	

S. citri-Contig 29 qPCR assays was validated with an efficiency of 98.6 % using 10 fold serial dilutions of a positive control (Fig. 7; Table 7).



slope	Efficiency
1.01	98.6

Oligonucleotide primers and probes used as for multiplex qPCR assays of COX, C. Liberibacter and S. citri

Primers/probes	Sequence 5'- 3'
Cox Internal Control	
Cox_multi-32 F	AATCTGACCTTCTTTCCCATGC
Cox_multi-194 R	AAGTGATTGTTACGACCACGAAGA
Cox_multi-96 p FAM	ATCCAGATGCTTACGCTGG
Candidatus Liberibacter Sp.	
HLBas.2 (extra G)	CGAGCGCGTATGCGATACG
HLBas.2	CGAGCGCGTATGCAATACG
HLBaf.2	CGAGCGCGTATTTTATACGAGC
HLBam.2	GAGCGAGTACGCAAGTACTAGCG
HLBr.2	GCGTTATCCCGTAGAAAAAGGTAG
HLBp (MGB) FAM	AGACGGGTGAGTAACG
Spriroplasma citri	
S. citri-Contig 29	
S. citri-C.29-31594 F	ACCTTGGGCGGCTTCCT
S. citri-C.29-31665 R	ACGTTCTCGGGTCTTGTACACA
S. citri-C.29-31650 p (MGB)	TCATGGTGTGACGGGC

Cox: the cytochrome oxidase gene of host plants for positive internal control. Cox (GenBank accession CX297817). HLB; Candidatus Liberibacter . Spiroplasma citri F: forward primer. R: Reverse primer. p: qPCR probe.

We would like to thank the CCNB for their support and we would like to express our commitment on working with citrus nurseries for a productive, profitable, and sustainable industry.