

PROTOCOL FOR THE DIAGNOSIS OF QUARANTINE ORGANISM

Citrus tristeza virus

Name:	<i>Citrus tristeza virus</i> (CTV)
Taxonomic position:	<i>Closteroviridae</i> , Genus <i>Closterovirus</i>
Common names:	Tristeza disease
Vector:	Aphids
Quarantine status:	EPPO A2 list, EU Annex II/AII European isolates (EU Annex II/AI non- European isolates)

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DIAGNOSTIC PROTOCOLS FOR ORGANISMS HARMFUL TO PLANTS. SMT PROJECT SMT4-CT98-2252

Diagnostic protocol for *Citrus tristeza virus* (CTV)

Introduction

Citrus tristeza virus (CTV) causes one of the most damaging diseases of citrus (Bar-Joseph and Lee, 1989) and is the most economically important viral pathogen of this crop (Lee and Bar-Joseph, 2000). The tristeza virus probably originated in Asia and has been disseminated to practically all citrus growing countries by movement of infected plant material. Subsequent spread by aphid vectors has created major epidemics. Epidemics of tree losses on sour orange rootstock were first reported from South Africa in the early part of the 20th century, and in Argentina and Brazil in the 1930s following the importation of CTV-infected plants and the efficient aphid vector *Toxoptera citricida*. More than 80 million of trees grafted on sour orange (*Citrus aurantium*) rootstock have been killed or rendered unproductive by CTV-induced decline. The losses caused in Argentina (more than 10 million trees), Brazil (more than 6 million trees) and USA (more than 3 million trees) have been reported by Bar-Joseph et al. (1989). Only in Spain more than 40 million trees, mainly sweet orange (*Citrus sinensis*) and mandarin (*Citrus reticulata*) grafted on sour orange, have declined progressively (Cambra et al., 2000a). In addition, CTV may cause stem pitting in some citrus varieties regardless the rootstock used, that is responsible of important losses of fruit quality and yield.

CTV is easily transmitted by graft and in a semi- persistent manner by the main aphid species visiting citrus: *T. citricida*, *Aphis gossypii*, *A. spiraecola* and *T. aurantii*. *T. citricida* (not yet present in continental Europe and on the Mediterranean Basin) is a much more efficient vector than *A. gossypii*, but epidemic spread has occurred in Spain when *A. gossypii* was the predominant aphid species (Cambra et al., 2000a). *A. spiraecola* is not an efficient vector, but since its populations can become so high it may be a significant factor in CTV spread in some areas. *T. aurantii* apparently transmits only certain CTV isolates (Lee and Bar-Joseph, 2000). Eight aphid species (*T. citricida* not included) have been assayed as vectors of different Mediterranean CTV isolates (Hermoso de Mendoza et al., 1984, 1988). *A. gossypii* was always the most efficient vector and transmission efficiencies were up to 78%, whereas *A. spiraecola* and *T. aurantii* had very low efficiencies (0 to 6%). The spatial and temporal spread of tristeza disease have been studied in European citrus orchards (Cambra et al., 1988, 1990a; Gottwald et al., 1996, 1997; Cambra et al., 2000a). A long time may elapse between the introduction of a primary source of inoculum and the development of a disease epidemic (Garnsey and Lee, 1988).

CTV is a member of the *Closterovirus* genus (Karasev et al., 1995). The virions are flexuous (2000 x 11 nm in size) and contain a non -segmented, positive-sense, single stranded RNA genome. The sequence of the CTV genome contains 12 open reading frames (ORFs), potentially encoding at least 17 proteins. The ORFs 7 and 8 encode proteins with estimated molecular weights of 27.4 (P27) and 24.9 kDa that have been identified as the capsid proteins. The complete sequence of several CTV isolates has been reported (Pappu et al., 1994; Karasev et al., 1995; Mawassi et al., 1996; Vives et al., 1999; Yang et al., 1999; Albiach-Marti et al., 2000; Suastika et al., 2001), including the sequence of a typical mild Spanish CTV isolate (Vives et al., 1999).

Field CTV isolates may vary in pathogenicity and may contain multiple genomic virus variants that can be separated by aphids or graft transmission to different citrus host species. The sub-isolates segregated in this way can be differentiated by pathogenicity tests in different hosts, by dsRNA patterns (Moreno et al., 1993) or serologically using specific monoclonal antibodies (Cambra et al., 1993). The monoclonal antibody MCA13 (Permar et al., 1990) was

described in Florida (USA) as specific for severe and CTV decline-inducing isolates. Reactions with MCA13 is not necessarily correlated with the decline of trees in the Mediterranean Basin, but constitute a good indication of potential aggressiveness of an isolate. There are no molecular methods allowing reliable typing of CTV isolates according to their aggressiveness. It has been demonstrated that the haplotype distribution of two CTV genes can be altered after host change or aphid transmission (Ayllón et al., 1999). Molecular hybridisation (Albiach et al., 1995) and single-strand conformation polymorphisms analysis of the coat protein gene (Rubio et al., 1996), have been used to differentiate Mediterranean CTV isolates.

The classic identification procedure for CTV is to graft-inoculate indicator seedlings of Mexican lime (Wallace and Drake, 1951) and observe them for vein clearing, leaf cupping, and stem pitting. Electron and light microscopy can be used to identify CTV particles and inclusions, but DAS-ELISA (Bar-Joseph et al., 1979; Cambra et al., 1979) revolutionised diagnosis, making it feasible to test many samples during surveys of large citrus areas, for CTV control in nurseries and for epidemiological studies.

Polyclonal antibodies from antisera were used from 1978 to 1983 for routine ELISA tests. The production of monoclonal antibodies specific to CTV (Vela et al., 1986; Permar et al., 1990) and others reported by Nikolaeva et al. (1996) solved the problems of specificity and increased sensitivity of ELISA tests. A mixture of two monoclonal antibodies (3DF1 and 3CA5) or their recombinant versions (Terrada et al., 2000) recognise all CTV isolates tested from different international collections (Cambra et al., 1990b). A detailed description and characterisation of these monoclonal antibodies has been summarised (Cambra et al., 2000a).

The development of Tissue print-ELISA (Garnsey et al., 1993; Cambra et al., 2000b) for CTV detection in imprinted sections of plant material on nitrocellulose membranes, allowed the sensitive indexing of thousands of samples simply and without the need to prepare extracts.

PCR-based assays have been developed based on immunocapture (Nolasco et al., 1993) or print or squash capture (Olmos et al., 1996; Cambra et al., 2000c). A simple procedure has been described to perform nested-PCR in a single closed tube (Olmos et al., 1999) which allowed CTV detection in single aphids and in plant tissues. A Co-operational PCR system (Co-PCR) using a universal probe for hybridisation with PCR products (Olmos et al., 2002) has been described, supplying similar sensitivity than nested PCR.

Principal hosts

Most species of *Citrus* and some species in other genera of the family *Rutaceae* (*Aegle marmelos*, *Aeglopsis chevalieri*, *Afraegle paniculata*, *Citropsis gilletiana*, *Microcitrus australis*, and *Pamburus missionis*), in addition to *Passiflora gracilis*, *P. caerulea*, *P. incense* and *P. incarnata*, have been reported as hosts for CTV. Most trifoliate orange clones and many of their hybrids are resistant to infection.

Protoplast of *Nicotiana benthamiana* have been experimentally infected by CTV.

Symptoms

Symptom expression in citrus hosts is highly variable and affected by environmental conditions, host species and the aggressiveness of the CTV isolate. Some CTV isolates are mild and produce no noticeable effect on most commercial citrus varieties. In general, mandarins are especially tolerant to CTV infection. Sweet orange, sour orange, rough lemon (*C. jambhiri*) and Rangpur lime (*C. limonia*) are usually symptomless but may react to some aggressive isolates. Reactive hosts include limes, grapefruit (*C. paradisi*), some pummelos (*C. grandis*), alemow (*C. macrophylla*), some sweet oranges, some citrus hybrids and some citrus relatives above mentioned. Stunting, leaf cupping, vein clearing and chlorosis, stem pitting, and reduced fruit size are common symptoms of susceptible hosts.

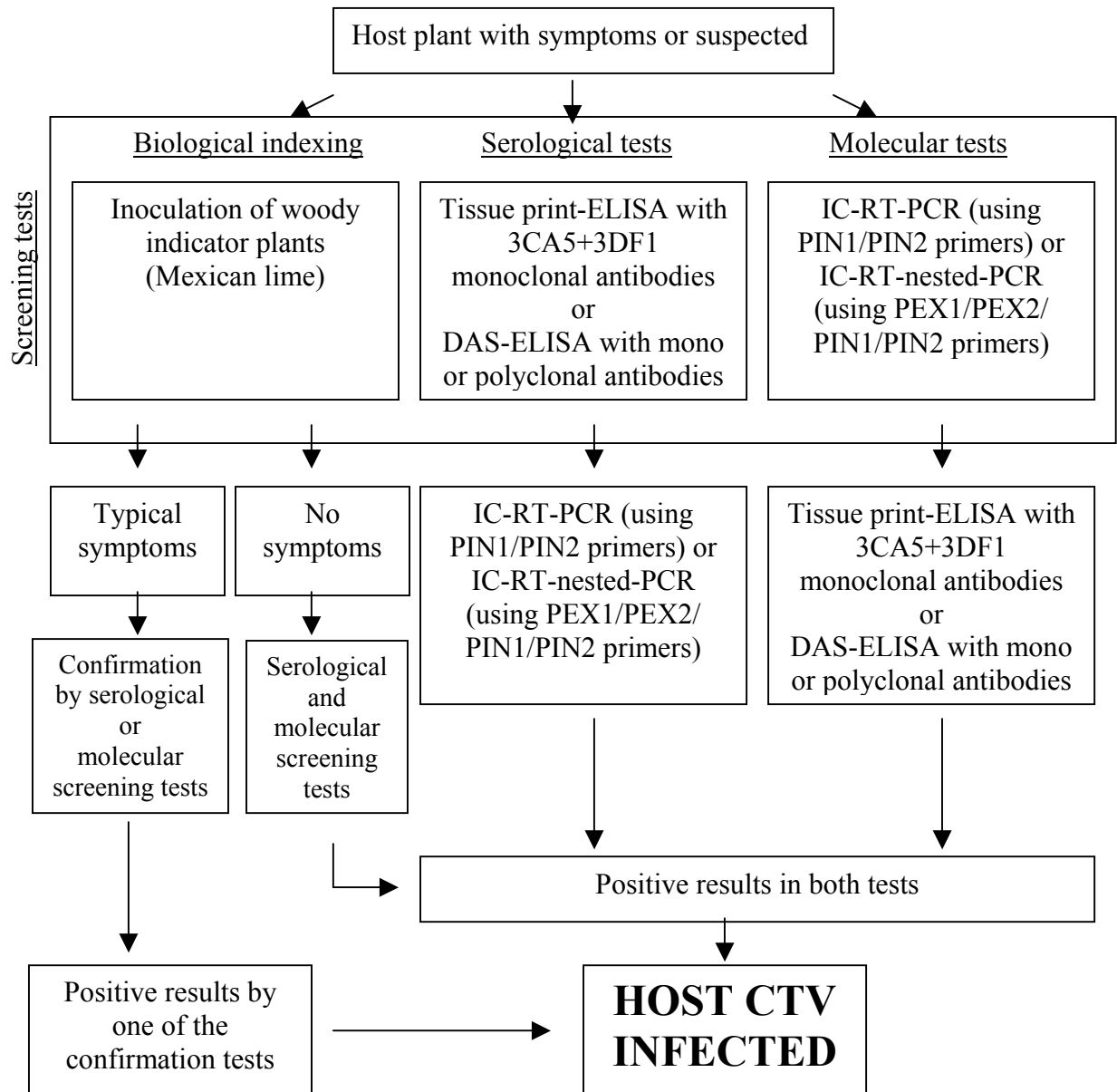
One of the most economically significant symptoms of the tristeza disease is the decline of trees grafted on sour orange. Sweet orange, mandarins and grapefruits on sour orange rootstock become stunted, chlorotic and often die after a period of several months or years (slow decline), or some days after the first symptoms (quick decline). The decline results from viral effects on the phloem of the sour orange rootstock just below the bud union. Trees that decline slowly, generally have a bulge above the bud union, and inverse pinhole pitting (honey combing) on the inner face of the sour orange bark. Some isolates of the virus do not induce decline symptoms, even in trees on sour orange, for many years.

Aggressive CTV isolates can severely affect trees inducing stem pitting on the trunk and branches of limes, grapefruits and sweet oranges. Stem pitting may sometimes cause a bumpy or ropy appearance of the trunks and limbs of adult trees. Deep pits in the wood are present under depressed areas of the bark. Fruit quality and yield are greatly reduced in trees with severe stem pitting. Nevertheless, most CTV isolates are able to cause stem pitting in *C. macrophylla* rootstocks and reduce tree vigour.



A: Chlorotic and declining sweet orange trees grafted on sour orange rootstock infected by CTV, compared with a looking-healthy tree in the middle (picture from Dr. M. Cambra, IVIA, Spain), B: Tristeza-induced quick decline of a sweet orange tree on sour orange rootstock in the middle, surrounded by trees in different states of slow decline (picture from Dr. M. Cambra, IVIA, Spain), C and D: Bud-union of sweet orange CTV-infected tree grafted on sour orange rootstock, and pin-holing or honeycombing in the inner face of the bark of the sour orange rootstock below the bud union of the tristeza-infected tree (pictures kindly provided by Drs. L. Navarro and P. Moreno, IVIA, Spain), E, F and G: Tristeza aggressive isolate-induced small fruits (compared with a normal fruit on the hand) and stem pitting in branches and trunk of a grapefruit tree in Uruguay (pictures from Dr. M. Cambra, IVIA, Spain).

Flow Diagram for Diagnosis



Sampling

Appropriate sampling is critical for serological or molecular CTV detection. The standard sampling for adult trees involves 5 young shoots (from the last flush) or fruit peduncles, or 10 fully expanded leaves, or 5 flowers or fruits, collected around the canopy of each individual tree from each scaffold branch. Samples (shoots or fully expanded leaves and peduncles) could be used at any time of year from sweet orange, mandarins; lemons and grapefruits, in Mediterranean areas, but springtime give the highest CTV titres. A reduced CTV titre is observed in Satsuma mandarins during summer. Consequently, the recommended period for sampling would include all vegetative seasons avoiding summer (July-August in the Mediterranean Basin). Flowers or fruit (when available) are also suitable materials for analysis (Cambra *et al.*, 2002).

Standard sampling for nursery plants involves 2 young shoots or 4 leaves.

Samples (shoots, leaf petioles, fruit peduncles and flowers) can be stored at 4°C for not more than 7 days before processing. Fruits can be stored for 1 month at 4°C.

Detection

Sample preparation

Preparation of tissue prints for testing

Make clean cuts on tender shoots, leaf petioles, fruit peduncles or flowers. Carefully press the fresh cut sections against a nitrocellulose membrane (0.45 mm). Let the trace or the print dry for a few minutes.

For routine testing perform at least two printings per selected shoot or peduncle and one per leaf petiole or flower (see sampling).

Printed membranes can be kept for several years in a dry place.

Preparation of plant extracts for testing

Weight approximately 1 g of plant material. Cut it in small pieces and place in a suitable tube or plastic bag for processing.

Add approximately 20 volumes of extraction buffer and homogenise the sample in tubes using a Polytron (Kinematica) or similar, or homogenise the sample into plastic bags using Homex 6 machine (Bioreba) or any manual roller, hammer, or similar.

Extraction buffer Phosphate buffer saline (PBS) pH 7.2-7.4 (see Appendix 1) supplemented with 0.2% sodium diethyl dithiocarbamate (DIECA) or 0.2% mercaptoethanol.

Samples for serological testing can be prepared in tubes or in plastic bags. Samples for molecular testing must be prepared in appropriate individual plastic bags.

Screening tests

Biological indexing

The object of indexing is to detect the presence of CTV in plant accessions or selections, or in samples whose sanitary status is to be assessed, and to estimate the aggressiveness

of the isolate on *Citrus aurantifolia* (Mexican lime). The indicator will be graft-inoculated according to conventional methods and held under standard conditions (Roistacher, 1991). Make 4-6 repetitions and compare symptom onset with positive and negative control plants.

Serological tests

Tissue print-ELISA

Tissue print or immunoprinting-ELISA or direct tissue blot immunoassay (DTBIA) will be performed according to Garnsey et al. (1993) and Cambra et al. (2000b) using the detailed protocol described in Annexe I and materials described in Appendix 1.

Double Antibody Sandwich-ELISA (DAS-ELISA)

Conventional or biotin/streptavidin system of DAS-ELISA will be performed according to Garnsey and Cambra (1991) using the detailed protocol described in Annexe I and materials described in Appendix 1.

Molecular tests

Immunocapture RT-PCR (IC-RT-PCR)

Immunocapture phase and the RT-PCR will be performed according to Wetzel et al. (1992), Nolasco et al. (1993) and Rosner et al. (1998) using the detailed protocol described in Annexe II and materials described in Appendix 1 (antibodies) and Appendix 2 (oligonucleotide primer sequences and buffers).

Immunocapture nested RT-PCR in a single closed tube

The method will be performed according to Olmos et al. (1999) using the detailed protocol described in Annexe II and materials described in Appendix 1 (antibodies) and Appendix 2 (oligonucleotide primer sequences and buffers).

Requirements for a positive identification

When CTV is diagnosed for the first time, or in critical cases (import/export) the following should be performed and provided:

- The original sample (with labels, if applicable) should be kept under proper conditions as long as possible. Sample extract and PCR amplification product should be kept at - 80 °C for 3 months (or longer for legal purposes). Printed tissue sections on nitrocellulose (see sample preparation) and the developed membrane after reading should be kept at room temperature for six months.
- A combination of two different screening methods based on biological indexing (inoculation of Mexican lime); serological or molecular detection (with the validated protocols and reagents) will be required to officially underwrite a positive CTV detection.

Report on the diagnosis

A report on the execution of the protocol should include: information and documentation on the origin of the plant material, comments on the certainty or doubts about identification and the results obtained with the screening tests.

Contact point for protocol

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Standard CTV-infected and healthy citrus controls, CTV specific monoclonal antibodies (in addition to the commercially available-Appendix 1) and CTV specific oligonucleotide primer sequences are available for non-profit institutions at IVIA (above indicated).

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Annexe I

Detailed protocols for serological tests

Tissue print-ELISA (Garnsey et al., 1993; Cambra et al., 2000)

1. Preparation of plant tissue prints (see sample preparation above described).
2. Membrane blocking: Prepare 1-% solution of bovine serum albumin (BSA) in distilled water. Place the membranes (recommended size about 7x13 cm) in an appropriate container (tray, hermetic container, and plastic bag...). Pour, covering them, the albumin solution and incubate for 1 h at room temperature, or overnight at 4°C. Slight agitation is recommended over this step. Discard the albumin solution and keep the membranes in the same container.
3. Addition of monoclonal antibodies alkaline phosphatase linked or recombinant antibodies alkaline phosphatase-fused: Prepare a solution of CTV specific 3DF1+3CA5 monoclonal antibodies linked to alkaline phosphatase (about 0.1µg/ml each monoclonal antibody in PBS) (see Appendix 1) or of 3DF1 scFv-AP/S+3CA5 scFv-AP/S fusion proteins expressed in *E.coli* (appropriate dilution in PBS). Pour the solution on the membranes, covering them and incubate for 2-3 h at room temperature, then discard the conjugate solution.
4. Washing of membranes: Rinse the membranes and the container with washing buffer (see Appendix 1). Wash by shaking (manually or mechanically) for 5 min. Discard the washing buffer and repeat twice the process.
5. Membrane development: Pour the alkaline phosphatase substrate buffer over the membranes (see Appendix 1) and let them incubate until a purple-violet colour appears in positive controls (about 10-15 min). Stop the reaction by washing the membranes with tap water. Spread the membranes on absorbent paper and let them dry.
6. Membranes reading: Observe the printings by using a low power magnification (X10-X20). Presence of purple-violet precipitates in the vascular region of plant material, reveals the presence of citrus tristeza virus.

DAS-ELISA (Garnsey and Cambra, 1991) conventional or biotin/streptavidin system

- 1.-Plate coating.
Prepare an appropriate dilution of polyclonal antibodies or monoclonal antibodies 3DF1 + 3CA5 (see Appendix 1) (usually between 1-2 µg/ml) in carbonate buffer pH 9.6 (see Appendix 1).
Add 200 µl to each well.
Incubate at 37°C for 4 h or at 4°C for 16 h.
- 2.-Washing.
Wash the wells three times with PBS-Tween (washing buffer) (see Appendix 1)
- 3.-Adding sample.

Add 200 µl per well of the plant extract (see sample preparation). Use two wells of the plate for each sample or positive controls and at least two wells for negative controls. Incubate at 4°C for 16 h.

4.-Washing.

Proceed as step 2.

5.-Adding specific polyclonal or monoclonal antibodies (3DF1 + 3CA5) linked with alkaline phosphatase or biotin (see Appendix 1).

Prepare an appropriate dilution of the conjugated antibodies (approx. 0.1 µg/ml in PBS with 0.5% bovine serum albumin-BSA added). Add 200 µl to each well. Incubate at 37°C for 3 hours.

6.-Washing.

Proceed as step 2.

7.-Developing and read the results.

When antibodies are linked with biotin, use an appropriate dilution of streptavidin-alkaline phosphatase conjugated (see Appendix 1). Add 200 µl to each well. Incubate at 37°C for 30 min and wash the plates as in step 2.

For both methods (conventional or biotin/streptavidin) prepare 1 mg/ml alkaline phosphatase solution (p-nitrophenyl phosphate) in substrate buffer. Add 200 µl to each well. Incubate at room temperature and read at 405 nm after 30, 60 and 90 min.

The ELISA test is negative if the absorbance of the sample is less than two times the absorbance of the healthy control. The ELISA test is positive if the absorbance of the sample is equal or greater than two times the absorbance of the healthy control.

Annexe II

Detailed protocols for molecular tests

IC-RT-PCR

A.-) Immunocapture phase (IC) (Wetzel ET al., 1992; Nolasco ET al., 1993; Rosner ET al., 1998):

1. Preparation of coated eppendorf tubes:
 - 1.1.- Prepare a dilution (1µg/ml) of polyclonal antibodies CTV specific or a dilution (0.5µg/ml + 0.5µg/ml) of monoclonal antibodies (3DF1+3CA5) in carbonate buffer pH 9.6 (see Appendix 2).
 - 1.2.- Dispense 100 µl of the diluted antibodies into the eppendorf tubes.
 - 1.3.- Incubate at 37°C or on ice for 3 hours.
 - 1.4.- Wash two times the tubes with 150 µl of sterile washing buffer (see Appendix 2).
2. Clarify 100 µl plant extract previously obtained (see extract preparation) by centrifugation (5 min at 13,000 rpm), and submit sample to an Immunocapture phase for 2 hours on ice (Rosner *et al.*, 1998) or alternatively at 37°C (Wetzel ET al., 1992), in coated Eppendorf tubes.
3. After immunocapture phase, wash three times eppendorf tubes with 150 µl of sterile washing buffer.

B.-) Amplification by RT-PCR

B.1.-) CTV detection (PIN1-PIN2 primers; Olmos ET al., 1999) (see Appendix 2)

PIN1: 5'-3' GGT TCA CGC ATA CGT TAA GCC TCA CTT
PIN2: 5'-3' TAT CAC TAG ACA ATA ACC GGA TGG GTA

Cocktail reaction

Ingredient	(µl)
H ₂ O	14.30
10X-Taq Polymerase Buffer	2.5
25 mM MgCl ₂	1.5 (1.5 mM)
5 mM dNTPs	1.25 (250 µM)
4 % Triton X-100	2 (0.3 %)
25 µM primer PIN1	1 (1 µM)
25 µM primer PIN2	1 (1 µM)
DMSO	1.25 (5%)
10 U/µl AMV	0.1
5 U /µl Taq Polymerase	0.1

Total volume 25 µl
Add directly to the washed tubes, 25 µl of the cocktail reaction.

Conditions for RT-PCR:

42°C for 45 minutes
92°C for 2 minutes

40 cycles		92°C for 30 seconds
		60°C for 30 seconds
		72°C for 1 minute

72°C for 10 minutes

4°C hold

IC nested RT-PCR IN A SINGLE CLOSED TUBE (Olmos ET al., 1999)

A. -) Immunocapture phase (IC) (Wetzel *et al.*, 1992; Nolasco *et al.*, 1993; Rosner *et al.*, 1998):

Proceed as above described for IC-RT-PCR.

B.-) Amplification by nested RT-PCR

B.1. -) CTV detection (PEX1, PEX2, PIN1, PIN2 primers) (Olmos *et al.*, 1999) (see Appendix 2).

PEX1: 5'-3' TAA ACA ACA CAC ACT CTA AGG

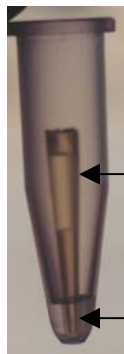
PEX2: 5'-3' CAT CTG ATT GAA GTG GAC

PIN1: 5'-3' GGT TCA CGC ATA CGT TAA GCC TCA CTT

PIN2: 5'-3' TAT CAC TAG ACA ATA ACC GGA TGG GTA

Device for compartmentalisation of a 0.5 ml Eppendorf tube for nested RT-PCR in a single closed tube, according Olmos *et al.* (1999).

0.5 ml Eppendorf tube



Cocktail B (into the end of a pipette tip cone)

Cocktail A (into the bottom of the Eppendorf tube)

Cocktail A (to be dropped in the bottom of the Eppendorf tube):

Ingredient	volume (μl)
H ₂ O	15.8
10X-Taq Polymerase Buffer	3
25 mM MgCl ₂	3.6 (3mM)
5 mM dNTPs	2 (300μM)
4 % Triton X-100	2.2 (0.3%)
25 μM primer PEX1	0.6 (0.5μM)
25 μM primer PEX2	0.6 (0.5μM)
DMSO	1.5 (5%)
10 U/μl AMV	0.2
5 U /μl Taq Polymerase	0.5
Total volume	30 μl

Cocktail B (to be placed into the cone):

Ingredient	volume (μl)
H ₂ O	2.6
10X-Taq Polymerase Buffer	1
25 μM primer PIN1	3.2 (8 μM)
25 μM primer PIN2	3.2 (8 μM)
Total volume	10 μl

Conditions for RT-PCR:

42°C for 45 minutes
92°C for 2 minutes

25 cycles	92°C for 30 seconds
	45°C for 30 seconds
	72°C for 1 minute

After this first step, vortex the tube and centrifuge (6000g x 5 sec) to mix cocktail B with products of first amplification. Place the tubes on the thermal cycler and proceed as follows:

Conditions for nested PCR

40 cycles	92°C for 30 seconds
	60°C for 30 seconds
	72°C for 1 minute

72°C for 10 min

ELECTROPHORESIS OF PCR PRODUCTS

Prepare 2% agarose gel in TAE buffer 0.5 x (see Appendix 2). Place ca. 3 µl droplets of loading buffer (see Appendix 2) on parafilm, mix 20 µl of PCR product by gentle aspiration with the pipette before loading. Load wells of gel and include positive and negative controls. Include DNA marker 100 bp in the first well of the gel.

Run the gel for 20 min at 120 V (medium gel tray: 15x10 cm) or 40 min at 160 V (big gel tray or electrophoresis tank: 15x25 cm). Soak the gel in ethidium bromide solution for 20 minutes.

Visualise the amplified DNA fragments by UV transillumination. Observe specific amplicons of 131 bp.

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Appendix 1

MATERIALS FOR DETECTION OF CTV IN PLANT TISSUES BY SEROLOGICAL TESTS

Standard CTV-infected and healthy citrus controls and CTV specific monoclonal antibodies (in addition to the commercially available below indicated) are available for non-profit purposes at Instituto Valenciano de Investigaciones Agrarias (IVIA). Carretera de Moncada-Náquera km 5. 46113 Moncada (Valencia). Spain.

Tissue print-ELISA kit based on 3DF1+3CA5 CTV specific monoclonal antibodies:

PLANT PRINT Diagnòstics, S.L. (validated in ring tests)

A complete kit including pre-printed membranes with positive and negative controls and all reagents, buffers and substrate is commercially available.

De la Mar 36, Bajo

46512 Faura, Valencia. Spain

E-mail: plantprint@wanadoo.es

DAS-ELISA kits (conventional or biotin/streptavidin system) for CTV detection:

Complete kits based on 3DF1 + 3CA5 specific monoclonal antibodies to CTV are commercially available from:

DAS-ELISA biotin/streptavidin system

INGENASA (validated in ring tests)

Hermanos García Noblejas 41, 2ª planta

28037 Madrid. Spain

<http://www.ingenasa.es>

REAL

CE Durviz S.L.

Parque Tecnológico de Valencia

Leonardo Da Vinci, 10

46980 Paterna (Valencia). Spain

<http://www.durviz.com>

DAS-ELISA conventional

Agdia Incorporated

30380 County Road 6

46514 Elkart. USA

<http://www.agdia.com>

Complete kits based on polyclonal antibodies to CTV are commercially available from:

Adgen Limited.

Nellies Gate. Anchincruive

Ayr KA6 5HW. United Kingdom

<http://www.adgen.co.uk>

BIORAD Laboratories-SANOFI
Rue Raimond Poincaré 3-BD
92430 Marnes La Coquette. France
<http://www.bio-rad.com>

Bioreba
Chr. Merian-Ring 7
CH 4153 Reinach BL1. Switzerland
<http://www.bioreba.ch>

Streptavidin alkaline phosphatase linked. Cat No. 1089 161 - Roche Diagnostics GmbH
(Mannheim), Germany

Buffers:

PBS, pH 7.2-7.4:

NaCl	8 g
KCl	0.2 g
Na ₂ HPO ₄ ·12H ₂ O	2.9 g
KH ₂ PO ₄	0.2 g
Distilled water	1 l

Carbonate buffer pH 9.6

Na ₂ CO ₃	1.59 g
NaHCO ₃	2.93 g
Distilled water	1 l

Washing buffer (PBS, pH 7.2-7.4 supplemented with 0.05% Tween 20)

NaCl	8 g
KCl	0.2 g
Na ₂ HPO ₄ ·12H ₂ O	2.9 g
KH ₂ PO ₄	0.2 g
Tween 20	500 µl
Distilled water	1 l

Colorimetric substrate buffer for alkaline phosphatase

Diethanolamine 97 ml
Dilute in 800 ml of distilled water

Adjust pH 9.8 with concentrated HCl
Adjust at 1000 ml with distilled water

Precipitating substrate buffer for alkaline phosphatase

Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (BCIP-NBT) –
Cat No. –B-5655 – Sigma Aldrich GmbH (Stenheim), Germany

Appendix 2

MATERIALS FOR DETECTION OF CTV IN PLANT TISSUES BY MOLECULAR TESTS

Standard CTV-infected and healthy citrus controls are available for non-profit purposes at Instituto Valenciano de Investigaciones Agrarias (IVIA). Carretera de Moncada-Náquera km 5. 46113 Moncada (Valencia). Spain.

Oligonucleotide primer sequences (validated in ring-test)

PEX1: 5'-3' TAA ACA ACA CAC ACT CTA AGG

PEX2: 5'-3' CAT CTG ATT GAA GTG GAC

PIN1: 5'-3' GGT TCA CGC ATA CGT TAA GCC TCA CTT

PIN2: 5'-3' TAT CAC TAG ACA ATA ACC GGA TGG GTA

Buffers

Carbonate buffer pH 9.6

Na ₂ CO ₃	1.59 g
NaHCO ₃	2.93 g
Distilled water	1 l

Washing buffer (PBS, pH 7.2-7.4 supplemented with 0.05% Tween 20)

NaCl	8 g
KCl	0.2 g
Na ₂ HPO ₄ ·12H ₂ O	2.9 g
KH ₂ PO ₄	0.2 g
Tween 20	500 µl
Distilled water	1 l

50X TAE buffer

Tris	242 g
0.5 M Na ₂ EDTA pH 8.0	100 ml
Glacial acetic acid	57.1 ml
Distilled water	Adjust volume to 1 l

Loading buffer

0.25% bromophenol blue
30% glycerol in H₂O