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EPPO STANDARD ON DIAGNOSTICS

PM 7/31 (2) Citrus tristeza virus

Specific scope: This Standard describes a diagnostic protocol for citrus tristeza virus.[1]
This Standard should be used in conjunction with PM 7/76 Use of EPPO diagnostic protocols.
Specific approval and amendment: First approved in 2003–09. First revision approved in 2022–11.
This revision was initially prepared to align the EPPO Diagnostic Protocol to the IPPC Diagnostic Protocol adopted in 2016 (Annex 15 of ISPM 27. Citrus tristeza virus (FAO, 2016)). However, it also includes other tests evaluated in the framework of the EU funded project VALITEST.

1 | INTRODUCTION

Citrus tristeza virus (CTV) causes one of the most damaging diseases of citrus, devastating epidemics of which have changed the course of the citrus industry (Moreno et al., 2008). The term 'tristeza', refers to the decline seen in many citrus species when grafted on *Citrus aurantium* (sour orange) or *Citrus limon* (lemon) rootstocks. Although tristeza is predominantly a bud union disease (Román et al., 2004), some CTV isolates induce other syndromes, including stem pitting, stunting, reduced productivity and impaired fruit quality of many commercial cultivars, even when they are grafted on CTV tolerant rootstocks.

CTV probably originated from South-East Asia, the putative area of origin of citrus, and it has been disseminated to almost all citrus-growing countries through the movement of infected plant material. Subsequent local spread by aphid vector species has created major epidemics.

Tree losses on sour orange rootstock were first reported in South Africa in the early twentieth century, and in Argentina and Brazil in the 1930s. CTV-induced tree decline has killed or rendered unproductive trees grafted on sour orange rootstock (Bar-Joseph et al., 1989; Cambra et al., 2000). CTV outbreaks have been observed in the United States, some Caribbean countries and some Mediterranean countries (especially Italy and Morocco). Detailed information on the distribution of CTV can be found in EPPO Global Database (EPPO, 2021a).

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

Like all viruses, CTV is a quasi-species, which implies that infected plants contain a population of different genotypes. In the case of CTV, these genotypes can even belong to different phylogenetic groups, which hampers the establishment of an unambiguous relationship between genotype and pathogenic characteristics (Harper, 2013). Moreover, establishing such relation can be further complicated by recombination.

It should also be noted that the term 'strain' has been used in literature both as a synonym for 'isolate' and to group isolates on their molecular and/or biological properties (EFSA, 2017). Therefore, in this protocol the Panel on Diagnostics in Virology and Phytoplasmology decided to use the concept of phylogenetic group in relation to genetic characteristics and strain in relation to pathogenic characteristics. To date, six major CTV phylogenetic groups have been described: T36 (Karasev et al., 1995), T3 (Hilf et al., unpublished), VT (Mawassi et al., 1996), T30 (Albiach-Marti et al., 2000), RB (Harper et al., 2010) and T68 (Harper, 2013) based on their genomic features. In the EPPO region, three of the six major phylogenetic groups are either absent (T68) and/or have a limited distribution (RB & T36) (Cevik et al., 2013; Ghosh et al., 2022). This implies that CTV isolates present in the EPPO region represent only a fraction of the biological and genetic diversity present in CTV isolates throughout the world. Consequently, introduction and further spread of genotypes belonging to these three 'foreign' phylogenetic groups will increase the genetic diversity and may affect the impact of the virus. Therefore, it is important to be able to identify these isolates at phylogenetic-group level in order to prevent their introduction and/or further spread within the region. Although sequence variants genetically similar to those of the stem pitting-inducing non-European CTV isolates have been detected in Europe (i.e. VT, T3) and have even been involved in outbreaks with severe tristeza decline symptoms, stem pitting symptoms in sweet orange have not been observed in surveys. Biological indexing of these isolates resulted in rare occurrence of inconspicuous symptoms on indicator plants. Outside of Europe, in the main citrus producing countries of the world, CTV isolates causing stem pitting appear to be present (EFSA, 2017).

CTV is naturally transmitted by some aphid species in a semi-persistent manner. Worldwide, the most efficient

vector of CTV is *Toxoptera citricida* (Kirkaldy). *Aphis gossypii* Glover is the main vector in Spain, Israel, some citrus growing areas in California (United States) and areas where *T. citricida* is absent (Cambra et al., 2000; Marroquín et al., 2004; Yokomi et al., 1989). Other aphid species have also been described as CTV vectors (Moreno et al., 2008), including *Aphis spiraecola* Patch, *Aphis aurantii* (Boyer de Fonsicolombe), *Myzus persicae* (Sulzer), *Aphis craccivora* Koch and *Uroleucon jaceae* (Linnaeus). Information on CTV vectors is available in the EPPO datasheet (EPPO, 2022).

CTV is also graft-transmitted, but not transmitted through seed.

Under natural conditions, CTV readily infects most species of *Citrus* and *Fortunella* and some species in genera known as citrus-relatives of the family Rutaceae. A list of CTV host species can be found in the Global Database (EPPO, 2021a).

Routine testing for CTV focusses on plant material and testing of vectors is not covered in this Standard.

A flow diagram describing the diagnostic procedure for citrus tristeza virus in plant material is presented in Figure 1.

2 | IDENTITY

Preferred name: Citrus tristeza virus
Other names: Citrus tristeza closterovirus
Acronym: CTV
Taxonomic position: Viruses, *Riboviria, Closteroviridae*, *Closterovirus*
EPPO code: CTV000
Phytosanitary categorization: EPPO A2 list n°93, EU A1 Quarantine pest (Annex II) A for non EU isolates, EU PZ Quarantine pest (Annex III) EU isolates, EU RNQP (Annex IV) EU isolates

Note on the phytosanitary categorization: CTV non-EU isolates are able to cause severe symptoms on a range of citrus crops that EU isolates do not induce. For this reason, non-EU CTV isolates have been evaluated as Union quarantine pests (EFSA, 2017). Throughout this document, CTV isolates that can cause severe symptoms are referred to as 'severe isolates'.

Note Virus nomenclature in Diagnostic protocols is based on the latest release of the official classification by the International Committee on Taxonomy of Viruses (ICTV, Release 2021, https://talk.ictvonline.org/

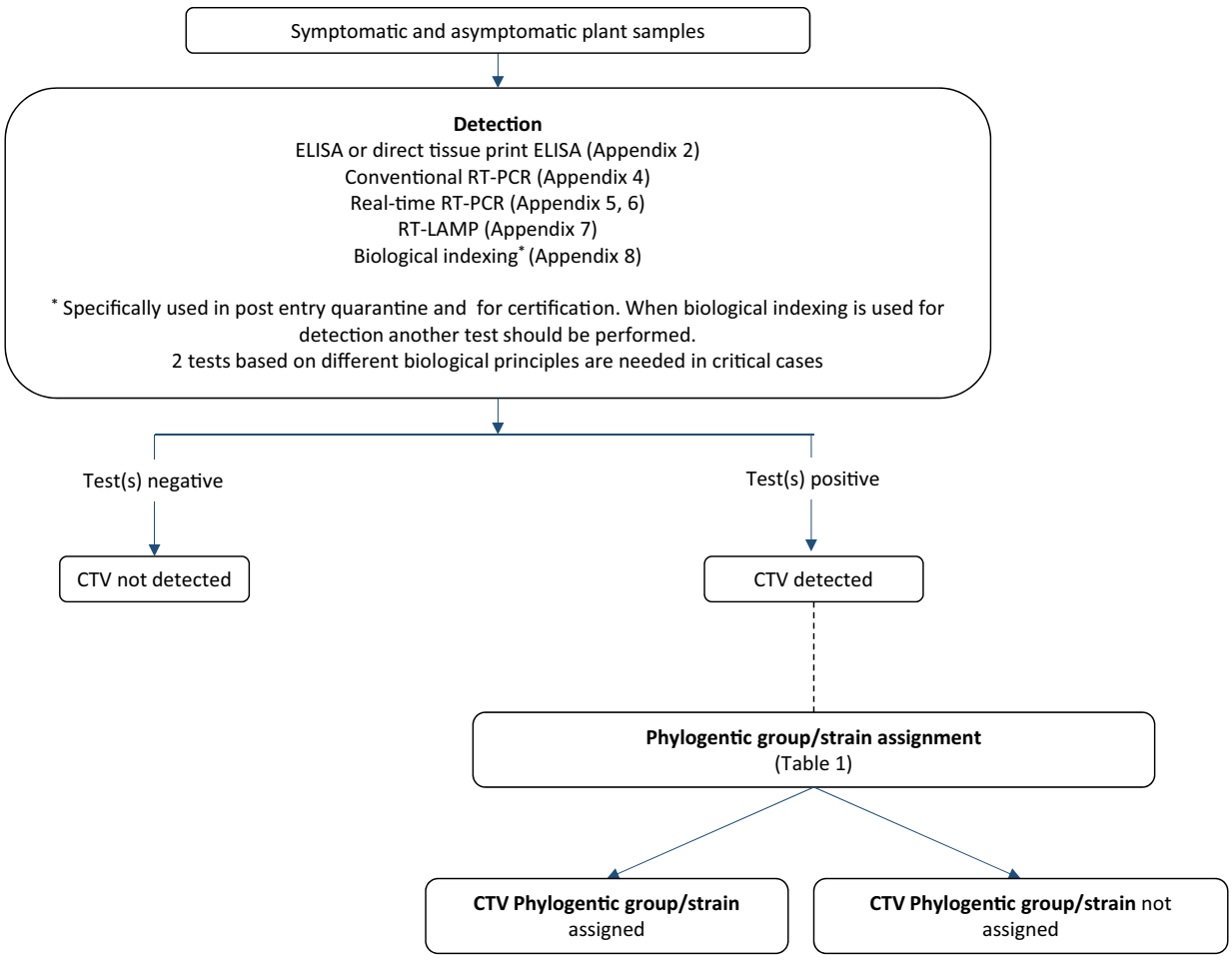

FIGURE 1 Flow diagram describing the diagnostic procedure for citrus tristeza virus in plant samples.

taxonomy/). Accepted species names are italicized when used in their taxonomic context, whereas virus names are not, corresponding to ICTV instructions. The integration of the genus name within the name of the species is currently not consistently adopted by ICTV working groups and, therefore species names in diagnostic protocols do not include the genus name. Names of viruses not included in the official ICTV classification are based on first reports. Transfer to a binomial nomenclature is in progress (ICTV website) and will be implemented gradually in EPPO Diagnostic Protocols.

3 | DETECTION

3.1 | Symptoms

Symptoms and symptom expression in CTV-infected citrus hosts is highly variable and is influenced by environmental conditions, host species and isolate. In general, *Citrus reticulata* (mandarin) plants infected with CTV do not show symptoms. *Citrus sinensis* (sweet orange), *C. aurantium* (sour orange, as a seedling and not as grafted rootstock), *C. jambhiri* (rough lemon) and *C. limonia* (mandarin lime) are usually symptomless, but symptoms can be observed when infected by some severe CTV isolates. Citrus hosts that develop symptoms include *C. aurantiifolia* (lime), *C. macrophylla* (alemow), *C. paradisi* (grapefruit and some cultivars of pomelo), some citrus hybrids and some citrus relatives of the family Rutaceae.

Depending on the CTV isolate and citrus species or rootstock/scion combination, the virus may cause no symptoms, tristeza, stem pitting or seedling yellows (Dawson et al., 2013; Moreno & Garnsey, 2010).

Typically, mild CTV isolates belonging to the T30 or RB phylogenetic groups produce no noticeable symptoms on most commercial citrus species, and citrus species grafted on *C. aurantium* remain symptomless for many years.

Types of symptoms and pathogenicity associated with the six major phylogenetic groups are presented in Table 1.

It is also important to note that in areas where CTV and '*Candidatus* Liberibacter' spp. (huanglongbing) are present, co-infection by both pathogens is common and can lead to increased disease severity due to synergism (Fu et al., 2017). The same stands for other pathogens [e.g. *Phytophthora* spp., citrus psorosis virus (CPsV) in Argentina, citrus sudden death associated virus (CSDaV) in Brazil, citrus exocortis viroid (CEVd) on sensitive rootstocks] and environmental conditions. In the Mediterranean basin in particular, drought is greatly contributing to the tristeza syndrome on infected trees grafted on sour orange rootstock (M. Cambra, pers. comm.).

3.1.1 | Tristeza (decline syndrome)

Tristeza is a bud union disease that develops only in susceptible rootstocks-scion combinations. The vast majority of CTV isolates cause a decline syndrome in different citrus species such as *C. sinensis*, *C. reticulata*, *C. paradisi*, *Fortunella* spp. and *C. aurantiifolia* when grafted on rootstocks of *C. aurantium* or *C. limon*.

The decline can be extremely rapid ('quick decline'), with wilting and death of trees occurring within a few days or weeks, or it can be a slower process ('slow decline'), with no symptoms or symptoms appearing over months or even years (EFSA, 2014).

Decline symptoms resemble those caused by root injury. These symptoms include thinning of foliage, twig defoliation and dieback, delayed growth and possibly tree collapse. Trees that decline slowly generally have a bulge above the bud union, a brown line just at the point of bud union, and inverse pinhole pitting (honeycombing) on the inner face of sour orange rootstock bark. On susceptible hosts, stunting, leaf cupping, vein clearing, chlorotic leaves, stem pitting and reduced fruit size are symptoms commonly observed.

3.1.2 | Stem pitting

Stem pitting syndrome (caused by severe isolates within the T3, T68 and occasionally VT phylogenetic groups) occurs in susceptible species regardless of the rootstock used and can affect both rootstock and grafted varieties (Moreno et al., 2008). Severe CTV isolates can seriously affect trees, inducing stem pitting on the trunk and branches of lime, grapefruit and sweet orange. However, it should be noted that most CTV isolates seriously affect rootstocks of *Citrus macrophylla* by causing stem pitting that results in reduced tree vigour.

The stem pitting syndrome on inoculated *C. paradisi* and/or *C. sinensis* seedlings may sometimes cause a bumpy or ropy appearance of the trunks and limbs of adult trees, deep pits in the wood under depressed areas of the bark, and a reduction in fruit quality and yield.

TABLE 1 Symptoms and pathogenicity associated with the six major phylogenetic groups.

Phylogenetic group	T36	T68	RB	T3	VT	T30
Type of symptoms currently described in the field	SY, QD, SP	SP	NN[a]	SY, SP	SP, SY, QD	SD
Pathogenicity	Mild and severe	Severe	Mild	Severe	Mild and severe	Mild

Abbreviations: NN, generally no noticeable symptoms; QD, quick decline; SD, slow decline; SP, stem pitting; SY, seedling yellows.

[a]The RB isolate present in the island of Crete is inducing slow decline in trees grafted on sour orange.

FIGURE 2 Decline: Leaf chlorosis (a, b) and twig defoliation (b on top) of CTV infected sweet orange trees grafted on sour orange rootstock. (a) healthy tree on the left. (b) healthy trees on the right. Courtesy: Varveri C, BPI, Greece.

FIGURE 3 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 CTV-infected tree. Courtesy: Navarro L and Moreno P, IVIA, Spain.

3.1.3 | Seedling yellows

The seedling yellows syndrome (caused by isolates within the T36, T3, VT phylogenetic groups) is observed in young plants of sour orange, grapefruit and lemon, most notably under greenhouse conditions (20–26°C) rather than in field situations.

The seedling yellows syndrome is characterized by stunting, production of chlorotic or pale leaves, development of a reduced root system, and stops the growth of the trees grafted on *C. aurantium*, and also stops the growth of *C. aurantium*, *C. limon* and *C. paradisi* seedlings.

Figures 2–12 show the main symptoms caused by CTV.

3.2 | Test sample requirements

General guidance on sampling methodologies is described in ISPM 31*Methodologies for sampling of consignments*[2] and in Cambra et al. (2002) specifically for CTV sampling. Procedures for sample preparation are described in Appendix 1.

[2]ISPM 31 provides information on the number of units to be sampled, which is considered useful to determine sample sizes for both consignments and places of production.

FIGURE 4 Seedlings of Duncan grapefruit inoculated with a CTV strain inducing seedling yellows syndrome. Healthy seedling on the right. Courtesy: Yokomi R, ARS-USDA Parlier, Parlier, USA.

3.2.1 | Plant material

Collection of plant material by hand is recommended to avoid mechanical contamination (e.g. by using scissors). Samples (shoots or fully expanded leaves and peduncles) can be taken all year round from grapefruit, lemon, mandarin and sweet orange in temperate Mediterranean climates. Spring and autumn are the optimal sampling periods because the highest CTV titres are observed in the plant during these seasons. During summer, reduced CTV titres are observed when temperatures rise above 35°C.

The decision to test samples (e.g., shoots, leaves, petioles) from individual or multiple plants by serological

FIGURE 5 Seedling of sour orange inoculated with a CTV strain inducing seedling yellows syndrome. Courtesy: Yokomi R, ARS-USDA Parlier, Parlier, USA.

FIGURE 6 Seedling yellows symptoms in Duncan grapefruit. Courtesy: Harper SJ, Washington State University, US.

or molecular methods depends on the expected virus concentration in the plants, the prevalence of CTV in the area (Vidal et al., 2012), and the level of confidence required by the NPPO. Specific examples of testing of multiple plants are given below.

Samples usually consist of leaves and shoots. Fruits and flowers can also be tested.

Samples can be stored at 4°C for up to 7 days before processing. Fruits can be stored for 1 month at 4°C. Samples can also be stored at −20°C for up to 3 months and at −80°C for longer periods.

FIGURE 7 Seedling yellows symptoms in Mexican lime. Courtesy: Harper SJ, Washington State University, US.

FIGURE 8 Seedling yellows symptoms in sour orange. Courtesy: Harper SJ, Washington State University, US.

3.2.1.1 | *Leaves*

The best tissue for testing is the main leaf vein and the petiole.

In orchards, the standard sample for adult trees consists of ten fully expanded leaves collected throughout the canopy of an individual tree including different scaffold branches.

In Spain, leaf material from up to 5 trees are pooled in one sample when using molecular tests. In Greece, leaf material from up to 4 trees is pooled when using ELISA and up to 25 trees when using molecular tests (Sambade et al., 2002).

For nursery plants, the standard sample is composed of four leaves per plant. Experience with serological tests shows that samples can be prepared by pooling leaves from up to five nursery plants.

3.2.1.2 | *Shoots*

In orchards, the standard sample for an adult tree is five young shoots collected throughout the canopy including different scaffold branches.

For nursery plants, the standard sample is composed of two young shoots per plant. Shoots from up to 10 nursery plants can be pooled when serological methods are used for detection.

From woody shoots phloem scrapings are taken.

3.2.1.3 | *Fruits*

A standard sample for an adult tree consists of five fruits or fruit peduncles collected throughout the canopy

FIGURE 9 Severe CTV 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. Courtesy: Cambra M, IVIA, ES.

FIGURE 10 Leaf cupping symptoms in Mexican lime. Courtesy Harper SJ, Washington State University (US).

FIGURE 11 Vein clearing symptoms in Mexican lime. Courtesy: Harper SJ Washington State University (US)

FIGURE 12 Severe stem pitting symptoms in alemow. Courtesy: P Moreno (formerly IVIA, ES)

including different scaffold branches. Tissue from the fruit peduncle (taken at the junction between the peduncle and the fruit), or from the columella (Figure 13) is the best tissue for testing.

3.2.1.4 | *Flowers*

The standard sample for an adult tree consists of five flowers collected throughout the canopy including different scaffold branches.

3.3 | Screening tests

3.3.1 | Serological tests

Polyclonal and monoclonal antibodies are available and can be used in double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), double antibody sandwich indirect (DASI) or triple-antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) and direct tissue print ELISA. A commercial kit Immunostrip Flashkit is available from Agdia. Instructions on how to perform an ELISA test are described in the EPPO Standard PM 7/125 *ELISA tests for viruses* (EPPO, 2015) and further information is provided in Appendix 2.

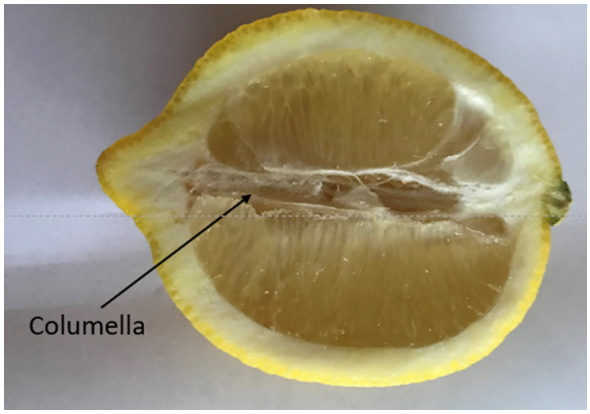

FIGURE 13 Columella (courtesy Petter F, EPPO)

3.3.2 | Molecular tests

Procedures for RNA extraction are described in Appendix 3.

The following molecular tests which have been evaluated in the framework of the EU funded VALITEST project (https://www.valitest.eu/) are recommended to detect CTV:

- Conventional reverse transcription PCR (RT-PCR) (Olmos et al., 1999), see Appendix 4.
- Real-time RT-PCR (Saponari et al., 2008), see Appendix 5
- Real-time RT-PCR kit from Ipadlab, based on Bertolini et al. (2008), see Appendix 6.
- RT-LAMP (Wang et al., 2013), see Appendix 7.

'Immunocapture (IC)-RT-PCR' and 'IC-nested RT-PCR in a single closed tube' are not recommended in this protocol as they are no longer commonly used.

An in silico analysis (19 isolates) showed that the tests of Saponari et al. (2008) and Wang et al. (2013) were able to detect all phylogenetic groups/isolates included, unlike the protocols of Bertolini et al. (2008) and Olmos et al. (1999) which missed one isolate (GenBank acc. no MF595989) (Varveri, pers. comm.).

The real-time RT-PCR (Saponari et al., 2008), the real-time RT-PCR kit developed by Ipadlab based on Bertolini et al. (2008) and the RT-LAMP (Wang et al., 2013), were also evaluated with tissue prints (Cambra et al., 2019) (see respective appendices).

3.3.3 | Biological indexing

Biological indexing (see Appendix 8) is commonly used in the framework of certification programmes or post entry quarantine for *Citrus* fruit trees. It is considered a sensitive and reliable method for the detection and characterization of new and/or unusual isolates. However, it has some disadvantages: it is time consuming (symptom development requires up to 6 months post-inoculation); it requires dedicated containment facilities such as temperature-controlled insect-proof greenhouses; and it requires experienced staff who can accurately interpret disease symptoms that can be confused with symptoms of other graft-transmissible agents. In addition, CTV isolates that do not induce symptoms (latent isolates) are not detectable on indicator plants (e.g. the CTV "strain K" described by Albertini et al. (1988) and for which no molecular data is available). Consequently, biological indexing should always be used in combination with another test.

4 | IDENTIFICATION

4.1 | Identification of CTV

The tests described in Section 3.3 allow both detection and identification of CTV. However, in the case of findings in the EPPO region, it is important to be able to identify isolates containing genotypes which are not present or have a limited distribution in the EPPO region and are (potentially) able to cause severe symptoms (stem pitting) in citrus orchards or break resistance.

4.2 | Assignment of isolates to phylogenetic groups and/or strains[3]

The assignment of isolates (genotypes) to phylogenetic groups and strains is described in Sections 4.2.1 and 4.2.2, respectively. Table 2 provides an overview of CTV phylogenetic groups, their pathogenicity (assessed by biological indexing) and the recommended tests. Since molecular tests alone appear of limited value for the prediction of pathogenic properties of CTV isolates (Bar-Joseph et al., 2010; Harper et al., 2010), a combination of molecular and/or biological tests is needed for a conclusive characterization of the genetic and pathogenic characteristics of a CTV isolate.

4.2.1 | Molecular tests

For the assignment of CTV isolates to particular phylogenetic groups, the molecular tests included in Table 2 can be used. Validation data for these tests is currently not available. Their analytical specificity (inclusivity-exclusivity) needs to be evaluated using representative isolates of the different phylogenetic groups. The molecular tests include:

[3]The monoclonal antibody MCA13 (Permar et al., 1990) has been used to confirm severe CTV isolates, including those able to induce the decline of trees grafted on sour orange or lemon rootstocks. However, this antibody is no longer commercially available (Colomer PlantPrint, pers. comm., 2021). However, the hybridoma secreting MCA13 antibody is available upon request to the American Type Culture Collection (ATCC) for non-profit institutions and research purposes.

TABLE 2 Available tests to characterize CTV isolates

Phylogenetic group	T36[a]	T68[a]	RB[a,1]	T3[b]	VT[b]	T30[b]
Method/Test						
Biological indexing (Appendix 8) (assignment to strains)	SY, QD, SP	SP	Potentially all types of symptoms	SY, SP	All types of symptoms	Leaf yellowing, vein clearing, leaf cupping
HTS	✓	✓	✓	✓	✓	✓
Multiplex conventional RT-PCR (Roy et al., 2010)	✓[2]	✓	✓[2]	✓	✓	✓
Multiplex real-time RT-PCR (Yokomi et al., 2010)	✓	✓[3]	✓	✓[3]	✓[3]	✓[4]
Conventional RT-PCR (Roy et al., 2013)	–	–	✓	–	–	–
Conventional RT-PCR (Cook et al., 2016)[5]	✓	–	✓	–	–	–
Two-step conventional RT-PCR (Saponari et al., 2019)	–	–	✓	–	–	–
One-step real-time RT-PCR (Saponari et al., 2019)	–	–	✓	–	–	–

Abbreviations: QD, quick decline; SP, stem pitting; SY, seedling yellows;

✓, test (or combination of tests) considered suitable to assign isolates to phylogenetic groups;

–, test does not detect isolates from the phylogenetic group.

[1]Able to overcome *Citrus trifoliata* resistance.

[2]The test will detect T36 and RB isolates, but these cannot be distinguished.

[3]The test will detect T68, T3 and VT isolates, but these cannot be distinguished.

[4]Results obtained from the four different probes should be combined to assign an isolate to the T30 phylogenetic group.

[5]Two specific RT PCR tests are described in this publication.

Note: *a and b are additional information that relate to the presence of the phylogenetic groups in EU countries or not.*

[a]Not present in EU countries.

[b]Present in EU and also in non-EU countries.

- Conventional simplex and multiplex RT-PCR tests followed by sequencing and sequence analysis of the amplicon.
- Simplex and multiplex real-time RT-PCR tests using specific primers (and probes) for particular phylogenetic groups.
- High-Throughput Sequencing (HTS) and analysis of the sequences obtained.

Conventional RT-PCR tests followed by Sanger sequencing of amplicons or HTS analysis (Bester et al., 2021) can be used for assignment of an isolate to a phylogenetic group when the virus concentration allows (Ruiz-García et al., 2019). Obtaining a 'near' complete genome sequence is preferable. Sequence analysis should follow the guidelines described in Appendices 7 and 8 of the EPPO Standard PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2021b).

4.2.2 | Biological indexing

Biological indexing is recommended for the characterization of the pathogenic properties of CTV isolates. Although biological methods are time consuming and can be performed only for a limited number of samples, biological indexing is the only method to assess the pathogenic features of CTV isolates. Further information is given in Appendix 8.

5 | REFERENCE MATERIAL

CTV-infected and healthy citrus controls, and CTV-specific oligonucleotide primer sequences are available for non-profit institutions from Instituto Valenciano de Investigaciones Agrarias (IVIA), Centro Protección Vegetal y Biotecnología, Carretera de Moncada-Náquera km 5, 46113 Moncada, Valencia, Spain. A Olmos (aolmos@ivia.es) and DSMZ Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH Inhoffenstraβe 7 B 38124 Braunschweig (DE).

6 | REPORTING AND DOCUMENTATION

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7 | PERFORMANCE CHARACTERISTICS

When performance characteristics are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

Instituto Valenciano de Investigaciones Agrarias (IVIA), Centro Protección Vegetal y Biotecnología, Carretera de Moncada-Náquera km 5, 46113 Moncada (Valencia). Spain. E-mail: aolmos@ivia.es.

Council for Agronomic Research and the bioeconomy – Research Centre for Plant Protection and Certification, Via C. G. Bertero 22–00156 Rome. Italy. E-mail: luca.ferretti@crea.gov.it.

9 | FEEDBACK ON THIS DIAGNOSTIC STANDARD

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

10 | STANDARD REVISION

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

ACKNOWLEDGEMENTS

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APPENDIX 1 - SAMPLE PREPARATION

1. Preparation of tissue prints for serological and molecular tests

The freshly cut sections of young shoots, leaf petioles, fruit peduncles or flower ovaries are carefully pressed against a nitrocellulose or cellulose-ester membrane (0.45 mm) and prints are allowed to dry for 2–5 min. For routine serological and molecular testing, at least two prints should be made per selected shoot (one from each end of the shoot) or peduncle and one per leaf petiole or flower ovary. Printed membranes can be kept for several months in a dry and dark place.

2. Preparation of plant extracts for serological and molecular tests

For serological testing, 0.2–0.5 g fresh plant material (leaf midribs, petioles or phloem scrapings) is cut into small pieces with disposable razor blades or bleach-treated scissors and placed into a suitable tube or plastic bag. The sample is homogenized thoroughly in 2–10 mL (from 1:10 to 1:20 w/v) extraction buffer (PBS with DIECA see below) using an electrical tissue homogenizer, a manual roller, a hammer or a similar tool.

For molecular testing, fresh plant material, 0.2 g for samples from individual trees up to 2 g for pooled samples (pooled samples consisting of equal amounts of each tree), is cut into small pieces as described above, placed into individual plastic bags and homogenized thoroughly in 1–20 mL (from 1:10 to 1:5 w/v) extraction buffer (PBS with DIECA, see below). Using a 1:5 w/v ratio was evaluated in a small experiment at the Benaki Phytopathological Institute (GR) and showed to yield a higher amount of RNA compared to the ratio 1:10 w/v ratio used for serological testing (Varveri, pers. comm.).

Extraction buffer for leaf, bark tissues, tissue prints and squashes, Phosphate-buffered saline (PBS) with DIECA.

NaCl	8.0 g
KCl	0.2 g
$Na_2HPO_4.12H_2O$	2.9 g
KH_2PO_4	0.2 g
Distilled water to	1 L
Adjust pH to 7.2–7.4	

The extraction buffer is supplemented with sodium diethyl dithiocarbamate (DIECA) just before use to give a final concentration of 0.2% (2 g/L).

APPENDIX 2 - SEROLOGICAL TESTS

Instructions on how to perform an ELISA test are described in the EPPO Standard PM 7/125 *ELISA tests for viruses* (EPPO, 2015).

ELISA using validated monoclonal antibodies or polyclonal antibodies is recommended for screening large numbers of samples. Information on the use of serological tests for the detection and identification of CTV is presented below.

1. ELISA

Commercial kits are available from Agdia (combination of polyclonal and monoclonal), Bioreba (polyclonal), Loewe (polyclonal), PlantPrint (two reference universal monoclonal antibodies i.e., 3DF1 and 3CA5) and Sediag (monoclonal) and should be used according to the manufacturer's instructions. The tests have been validated in the framework of the VALITEST project.

Validation data from the VALITEST test performance study based on a panel of 24 samples including 14 samples composed of naturally contaminated tangelo diluted in orange (2–3 replicates, 5 concentrations), 1 naturally contaminated orange sample, 1 naturally contaminated Bearss lime sample, 3 commercial positive controls, and 5 healthy plant samples (tangor, Meyer lemon, Tahiti lime, orange and pomelo).

Validation was carried out in accordance with EPPO Standard PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* (EPPO, 2021c).

TPS data for Agdia and PlantPrint should be taken with care because results from fewer than seven laboratories (4 and 6 laboratories respectively) were included in the statistical analysis.

When data has been obtained during the TPS preliminary studies only this is specified.

- Analytical sensitivity data
  Probability of detection (POD) 95
  Agdia $10^{-1.95}$
  Bioreba $10^{-1.55}$
  Loewe $10^{-0.47}$
  Sediag $10^{-0.73}$
  PlantPrint $10^{-0.12}$

- Analytical specificity data
  Evaluated during preliminary studies of the TPS (VALITEST):
  Inclusivity tested on 10 CTV isolates[4] (5 from Reunion Island, 1 from Italy, 1 from Israel, 2 from USA, 1 from Spain): 100% for Agdia; 100% for Bioreba; 100% for Loewe; 100% for PlantPrint; 100% for Sediag.
  Exclusivity was evaluated for a limited number of non-targets, i.e. '*Candidatus* Liberibacter asiaticus', citrus variegation virus (CVV), citrus psorosis virus A (CPsVa), citrus psorosis virus B (CPsVb): 100% for Agdia; 100% for Bioreba; 100% for Loewe; 100% for PlantPrint; 100% for Sediag.

- Repeatability data
  Average accordance: 98% for Agdia; 99% for Bioreba; 97% for Loewe; 95% for PlantPrint; 98% for Sediag.

- Reproducibility data
  Average concordance: 98% for Agdia; 96% for Bioreba; 92% for Loewe; 81% for PlantPrint; 89% for Sediag.

- Diagnostic sensitivity data
  51% for Agdia; 40% for Bioreba; 36% for Loewe; 36% for PlantPrint; 43% for Sediag.

- Diagnostic specificity data
  100% for Agdia; 98% for Bioreba; 100% for Loewe; 97% for PlantPrint; 100% for Sediag.

2. Direct tissue print-ELISA

2.1. Generic CTV detection
A complete kit based on CTV-universal 3DF1 + 3CA5 reference monoclonal antibodies, including pre-printed membranes with positive and negative controls and all reagents, buffers and substrate, is available from Plant Print Diagnòstics S.L. The kit should be used according to the manufacturer's instructions.

Validation data from VALITEST test performance study is based on a panel of 22 samples (13 positive samples and 9 negative samples) consisting of membranes with imprints from citrus trees.

Validation was carried out in accordance with EPPO Standard PM 7/98 (EPPO, 2021c).

TPS data should be taken with care because results from fewer than seven laboratories (4 laboratories) were included in the statistical analysis.

- Analytical specificity data
  Inclusivity tested on 10 CTV isolates (5 from Reunion Island, 1 from Italy, 1 from Israel, 2 from USA, 1 from Spain): 100%

- Diagnostic sensitivity data
  100%

- Diagnostic specificity data
  92%
  Results from previous validation studies (DIAGPRO) showed that direct tissue print-ELISA using 3DF1 + 3CA5 monoclonal antibodies was found to be a reliable, simple and economical method for routine testing of plant material when compared with biological indexing on Mexican lime, ELISA, or molecular methods (Cambra et al., 2002; Vidal et al., 2012). For more information see FAO, ISPM 27, Annex 15.

3. Immunostrip Flashkit (Agdia)

An Immunostrip® Flashkit kit for CTV detection is available from Agdia Inc. and should be used according to the manufacturer's instructions. The test has been validated in the framework of the VALITEST project.

[4]Information on the phylogenetic groups of the isolates is not available.

Validation data from the VALITEST project on a panel of 24 samples including 14 samples composed of naturally contaminated tangelo diluted in orange (2–3 replicates, 5 concentrations), 1 naturally contaminated orange sample, 1 naturally contaminated Bearss lime sample, 3 commercial positive controls, and 5 healthy plant samples (Tangor, Meyer lemon, Tahiti lime, Orange and Pomelo).

Validation was carried out in accordance with EPPO Standard PM 7/98 (EPPO, 2021c).

- Analytical sensitivity data
  CTV could be detected in less than 95 % of the undiluted (positive) samples.

- Analytical specificity data
  Inclusivity tested on 10 CTV isolates[5] (5 from Reunion Island, 1 from Italy, 1 from Israel, 2 from USA, 1 from Spain): 100%

- Repeatability data
  Average accordance: 97%

- Reproducibility data
  Average concordance: 94%

- Diagnostic sensitivity data
  22%

- Diagnostic specificity data
  100%

APPENDIX 3 - RNA EXTRACTION

RNA extraction from plant tissue

This appendix describes RNA extraction methods for plant material. These initial steps are critical for the results of a test and are often more related to the matrix than the specific test. Therefore, they are described in this separate appendix.

A wide range of RNA extraction methods may be used, from commercial kits to methods published in scientific journals.

Care should be taken to prevent cross contamination when handling samples especially when high concentrations of virus are expected.

Extracted RNA should be stored refrigerated for short-term storage (<8 h), at −20°C (<1 month) or at −80°C for longer periods.

The RNeasy Plant Mini kit (Qiagen), the *PureLink RNA Mini Kit* (ThermoFisher Scientific) can be used on all tissue types. Briefly, 200 µL of the homogenized extract is added to 500 µL lysis buffer (without β-mercaptoethanol), the resulting mixture is directly loaded on the kit columns and the manufacturer's instructions are followed.

RNA extraction using CTAB (e.g. Saponari et al., 2019) or other extraction methods or kits may be used. It should be noted that the RNA extraction method should be validated in combination with the molecular test to be used.

RNA extraction from membranes

A piece of membrane of approximately 0.5 cm^2 with several partially overlapping imprints is placed in a 2 mL tube and 100 µL of extraction buffer Glycine, 0.1 M; NaCl, 0.05 M; EDTA, 1 mM is added. The tube is vortexed for several seconds. The piece of membrane should be well immersed in the buffer during all the extraction.

The tube is then placed in a thermostatically controlled water bath at 95°C for 10 min and quickly centrifuged.

The RNA released should be stored at a temperature of ≤ –18°C until its use as a template for the molecular tests.

APPENDIX 4 - CONVENTIONAL RT-PCR (OLMOS ET AL., 1999)

The test below differs from the one described in the original publication.

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The test can be used for the detection of CTV in plant material
1.2. The test is adapted from Olmos et al. (1999)
1.3. The target sequence is located in the 3′ UTR region at positions 19094–19225 based on GenBank accession number NC_001661
1.4. Oligonucleotides and average amplicon size:

Primers	Sequence	Amplicon size
Forward primer PIN1	5′- GGT TCA CGC ATA CGT TAA GCC TCA CTT - 3′	131 bp
Reverse primer PIN2	5′- TAT CAC TAG ACA ATA ACC GGA TGG GTA - 3′	

2. Methods

2.1. Nucleic Acid Extraction and Purification
2.1.1. RNA extraction is performed according to Appendix 3
2.2. One step RT-PCR

[5]Information on the phylogenetic groups of the isolates is not available.

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	11.375	N.A.
OneStep RT-PCR buffer (Qiagen)	5×	5	1×
dNTPs	10 mM	0.625	250 µM
Forward primer PIN1	10 µM	2.5	1 µM
Reverse primer PIN2	10 µM	2.5	1 µM
OneStep RT-PCR Enzyme Mix (Qiagen)		1	
Subtotal		23	
RNA extract		2	
Total		25	

2.2.2. Conventional RT-PCR conditions: 50°C for 30 min, 95°C for 15 min, followed by 40 cycles of (94°C for 30 s, 60°C for 30 s, 72°C for 1 min), 72°C for 10 min and keep at 4°C until use.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPCs) can be used to monitor each individual sample separately. IPC can include an endogenous nucleic acid of the matrix using conserved primers, preferably amplifying RNA targets, such as *nad5* (Menzel et al., 2002).

Laboratories should take additional care to prevent risks of cross contamination when using high concentration positive controls (e.g. cloned products, gBlocks, and whole genome amplicons) directly or when preparing dilutions of them.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results

In order to assign results from PCR-based tests the following criteria should be followed:

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC, PAC (and if relevant IC) a band of the expected size 131 bp is visualized.

When these conditions are met:

- A test will be considered positive if a band of the expected size 131 bp is visualized.
- A test will be considered negative, if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

It should be noted that in virology bands of different sizes may correspond to strains of the target organism and care should be taken when interpreting conventional PCR products.

4. Performance characteristics available

Validation data from the VALITEST test performance study based on a panel of 24 samples including 14 samples composed of naturally contaminated tangelo diluted in orange (2–3 replicates, 5 concentrations), 1 naturally contaminated orange sample, 1 naturally contaminated Bearss lime sample, 3 commercial positive controls, and 5 healthy plant samples (Tangor, Meyer lemon, Tahiti lime, Orange and Pomelo).

Validation was carried out in accordance with EPPO Standard PM 7/98 (EPPO, 2021c).

When data has been obtained during the TPS preliminary studies only this is specified.

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO

database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

4.1. Analytical sensitivity data
Probability of detection (POD) 95% obtained for a dilution of $10^{-4.6}$.

4.2. Analytical specificity data
Inclusivity tested on 10 CTV isolates[6] (5 from Reunion Island, 1 from Italy, 1 from Israel, 2 from USA, 1 from Spain): 100%
Exclusivity evaluated during the TPS preliminary study on a limited number of non-target organisms, i.e. '*Candidatus* Liberibacter asiaticus', citrus variegation virus (CVV), citrus psorosis virus (CPsV, isolates A and B): 100%

4.3. Repeatability data
Average accordance: 94%

4.4. Reproducibility data
Average concordance: 88%

4.5. Diagnostic sensitivity data: 98%
4.6. Diagnostic specificity data: 72%

APPENDIX 5 - REAL-TIME RT-PCR (SAPONARI ET AL., 2008)

The test below differs from the one described in the original publication.

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The test can be used for the detection of CTV in plant material and tissue prints
1.2. The test is based on Saponari et al. (2008)
1.3. The target sequence is located in the CP region at positions 16376–16477, position 16376–16399 and position 16457–16477 for the primers and position 16412–16436 for the probe based on GenBank accession number AF260651
1.4. Oligonucleotides:

Primers/probe	Sequence
Forward primer P25F	5′- AGC RGT TAA GAG TTC ATC ATT RC- 3′
Reverse primer P25R	5′- TCR GTC CAA AGT TTG TCA GA- 3′
Probe CTVp	5′ CY5 -CRC CAC GGG YAT AAC GTA CAC TCG G- BHQ3 3′

[6]Information on the phylogenetic groups of the isolates is not available.

Note that the probe is labelled with Cy5 fluorophore in the original publication, but another fluorophore can be used depending on the compatibility of the filter of the quantitative thermal cycler used. The quencher (BHQ3) can also be changed based on what the provider of the TaqMan probe proposes in its catalogue according to the compatibility between reporter and quencher.

1.5. Real-time PCR system: iQ5 Real-Time PCR Detection System (BIORAD)
1.6. Software and settings (automatic or manual) for data analysis: iQ5 Optical System software version 2.0

2. Methods

2.1. Nucleic Acid Extraction and Purification
2.1.1. RNA extraction is performed according to Appendix 3.
2.2. One step real-time RT-PCR
2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	6.6	N.A.
RT-PCR reaction mix for probes (iScript™ One-step RT-PCR kit for Probes, BIORAD)	2×	12.5	1×
iScript™ reverse transcriptase Supermix (BIORAD)[a]		0.5	
Forward Primer P25F	10 µM	1	400 nM
Reverse Primer P25R	10 µM	2	800 nM
Probe CTVp	5 µM	0.4	80 nM
Subtotal		23	
RNA extract		2	
Total		25	

[a]The mastermix is no longer commercially available but it was used to produce the validation data described in Section 4.

2.2.2. Real-time RT-PCR cycling conditions: reverse transcription at 55°C for 2 min, denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 59°C for 30 s.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product).

As an alternative (or in addition) to the PIC, internal positive controls (IPCs) can be used to monitor each individual sample separately. IPC can include an endogenous nucleic acid of the matrix using conserved primers, preferably amplifying RNA targets, such as *nad5* (Botermans et al., 2013; Saponari et al., 2008).

Laboratories should take additional care to prevent risks of cross contamination when using high concentration positive controls (e.g. cloned products, gBlocks, and whole genome amplicons) directly or when preparing dilutions of them.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results

In order to assign results from PCR-based tests the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation data from VALITEST test performance study based on a panel of 24 samples including 14 samples composed of naturally contaminated tangelo diluted in orange (2–3 replicates, 5 concentrations), 1 naturally contaminated orange sample, 1 naturally contaminated Bearss lime sample, 3 commercial positive controls, and 5 healthy plant samples (Tangor, Meyer lemon, Tahiti lime, Orange and Pomelo).

Validation was carried out in accordance with EPPO Standard PM 7/98 (EPPO, 2021c).

When data has been obtained during the TPS preliminary studies only this is specified.

It should be noted that during the TPS this real-time PCR had a lower performance than other molecular tests, although laboratories which had experience with this test obtained results equivalent to those obtained with the other molecular tests.

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

4.1. Analytical sensitivity data

Less than 95% of the undiluted samples could be detected.

4.2. Analytical specificity data

Inclusivity tested on 10 CTV isolates[7] (5 from Reunion Island, 1 from Italy, 1 from Israel, 2 from USA, 1 from Spain): 100%

Exclusivity evaluated during the TPS preliminary study on a limited number of non-target organisms, i.e. '*Candidatus* Liberibacter asiaticus', citrus variegation virus (CVV), citrus psorosis virus (CPsV, isolates A and B): 100%

4.3. Repeatability data

Average accordance: 93%

4.4. Reproducibility data

Average concordance: 63%

4.5. Diagnostic sensitivity data

41%

Data for the tissue print test: 89%, these data should be taken with care because results from only 4 laboratories were included in the statistical analysis.

4.6. Diagnostic specificity data

88%

[7]Information on the phylogenetic groups of the isolates is not available.

Data for the tissue print test: 86%, these data should be taken with care because results from only 4 laboratories were included in the statistical analysis.

APPENDIX 6 - REAL-TIME RT-PCR KIT FROM IPADLAB BASED ON BERTOLINI ET AL. (2008)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (*see PM 7/98*) *is carried out.*

1. General Information

1.1. The test can be used for the detection of CTV in plant material and tissue prints.
1.2. A molecular kit has been developed by Ipadlab based on Bertolini et al. (2008) and was evaluated in the framework of the EU funded VALITEST project
1.3. The target sequence is located on the 3′ UTR region at positions from 19152 to 19254 based on GenBank accession number NC_001661.1
1.4. Oligonucleotides:

Primers/probe	Sequence
Forward primer 3′UTR1	5′- CGT ATC CTC TCG TTG GTC TAA GC - 3′
Reverse primer 3′UTR2	5′- ACA ACA CAC ACT CTA AGG AGA ACT TCT T - 3′
Probe 181T	5′FAM- TGG TTC ACG CAT ACG TTA AGC CTC ACT TG -TAMRA 3′

1.5. Thermal cycler or real-time PCR system: ABI Prism 7000 Sequence Detection System software (Applied Biosystems).
1.6. Software for data analysis: ABI prism 7000 Sequence Detection System software (Applied Biosystems).

2. Methods

2.1. Nucleic Acid Extraction and Purification
2.1.1. RNA extraction is performed according to Appendix 3.
2.2. One step real-time RT-PCR
2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Direct Master mix (*Ipadlab*)		17.5	1×
RT-enzyme (*Ipadlab*)		0.5	
Subtotal		18	
RNA extract		2	
Total		20	

2.2.2. Real-time RT-PCR cycling conditions: reverse transcription at 55°C for 15 min; denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 1 min.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product).

As an alternative (or in addition) to the PIC, internal positive controls (IPCs) can be used to monitor each individual sample separately. IPC can include an endogenous nucleic acid of the matrix using conserved primers, preferably amplifying RNA targets, such as *nad5* (Botermans et al., 2013; Saponari et al., 2008).

Laboratories should take additional care to prevent risks of cross contamination when using high concentration positive controls (e.g. cloned products, gBlocks, and whole genome amplicons) directly or when preparing dilutions of them.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results

In order to assign results from PCR-based tests the following criteria should be followed:

Verification of the controls
- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:
- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation was carried out in accordance with EPPO Standard PM 7/98 (EPPO, 2021c) based on a panel of 24 samples including 14 samples composed of naturally contaminated tangelo diluted in orange (2-3 replicates, 5 concentrations), 1 naturally contaminated orange sample, 1 naturally contaminated Bearss lime sample, 3 commercial positive controls, and 5 healthy plant samples (Tangor, Meyer lemon, Tahiti lime, Orange and Pomelo).

When data has been obtained during the TPS preliminary studies only this is specified.

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

4.1. Analytical sensitivity data
Probability of detection (POD) 95% obtained for a dilution between 10^{-4} and 10^{-5}

4.2. Analytical specificity data
Inclusivity tested on 10 CTV isolates[8] (5 from Reunion Island, 1 from Italy, 1 from Israel, 2 from USA, 1 from Spain): 100%

Exclusivity evaluated during the preliminary study on a limited number of non-target organisms, i.e. '*Candidatus* Liberibacter asiaticus', citrus variegation virus (CVV), citrus psorosis virus (CPsV, isolates A and B): 100%

4.3. Repeatability data
Average accordance: 94%

4.4. Reproducibility data
Average concordance: 88%

4.5. Diagnostic sensitivity data
98%
Data for the tissue print test: 96% these data should be taken with care because results from only 4 laboratories were included in the statistical analysis.

4.6. Diagnostic specificity data
76%
Data for the tissue print test: 92% these data should be taken with care because results from only 4 laboratories were included in the statistical analysis.

[8]Information on the phylogenetic groups of the isolates is not available.

APPENDIX 7 - RT-LAMP (WANG ET AL., 2013)

The test below differs from the one described in the original publication.

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The test can be used for the detection of CTV in plant material and tissue prints.
1.2. The test is adapted from Wang et al. (2013).
1.3. The target sequence is located on the conserved region of CTV *p25* at positions from 16445 to 16621 based on GenBank accession number AF260651
1.4. Oligonucleotides:

Primers	Sequence
Primer p25F3	5′- CGA AGT GGA TTT GTC TGA CA- 3′
Primer p25B3	5′- GGA ATC CCT GCA TCT AGC G- 3′
Primer p25FIP	5′- ACT CGA AGG GCG TTA GTA CGG CTT TGG ACT GAC GTC GTG TT- 3′
Primer p25BIP	5′- CTG GGG TAG GAC TAA CGA TGC CGA CGT CCG CCA TAA CTC AA- 3′

1.5. LAMP reactions should be performed in an equipment dedicated to LAMP isothermal amplification (e.g. Genie® II (OptiGene) or in a real-time PCR equipment).

2. Methods

2.1. Nucleic Acid Extraction and Purification
2.1.1. RNA extraction is performed according to Appendix 3.

2.2. One step RT-LAMP

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	3.2	N.A.
Isothermal Master Mix ref ISO-DR004 (*Optigene*)		15	
Primer p25F3	10 µM	0.5	200 nM
Primer p25B3	10 µM	0.5	200 nM
Primer p25FIP	100 µM	0.4	1.6 µM
Primer p25BIP	100 µM	0.4	1.6 µM
Subtotal		20	
RNA extract		5	
Total		25	

2.2.2. RT-LAMP conditions: 65°C for 45 min, 98–80°C, ramping at 0.1°C per second

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPCs) can be used to monitor each individual sample separately. IPC can include an endogenous nucleic acid of the matrix using conserved primers, preferably amplifying RNA targets, such as *nad5* (Botermans et al., 2013; Saponari et al., 2008).

Laboratories should take additional care to prevent risks of cross contamination when using high concentration positive controls (e.g. cloned products, gBlocks, and whole genome amplicons) directly or when preparing dilutions of them.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results

In order to assign results from PCR-based tests the following criteria should be followed:

Verification of the controls

- NIC and NAC should produce no fluorescence
- PIC, PAC (and if relevant IC) should produce exponential amplification curves. The Tm (melting temperature) should be between 86.0 and 89.0°C when samples are analysed on real-time LightCycler 480 thermocycler (Roche Diagnostics). Similar Tm range is expected when analysed on any other device, but it needs to be verified

When these conditions are met:

- A test will be considered positive if it produces a positive reaction as for PIC and PAC (see above).
- A test will be considered negative, if it produces no turbidity/colour change or no fluorescence.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation data from VALITEST test performance study based on a panel of 24 samples including 14 samples composed of naturally contaminated tangelo diluted in orange (2–3 replicates, 5 concentrations), 1 naturally contaminated orange sample, 1 naturally contaminated Bearss lime sample, 3 commercial positive controls, and 5 healthy plant samples (Tangor, Meyer lemon, Tahiti lime, Orange and Pomelo).

Validation was carried out in accordance with EPPO Standard PM 7/98(EPPO, 2021c).

When data has been obtained during the TPS preliminary studies only this is specified.

TPS data for this test should be taken with care because results from fewer than seven laboratories (3 laboratories) were included in the statistical analysis.

The test was also evaluated as a tissue print test however results were obtained from a limited number of dataset.

The test may have been adapted further and validated or verified using other critical reagents, instruments and/

or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

4.1. Analytical sensitivity data
Probability of detection (POD) 95% obtained for a dilution between $10^{-2.3}$

4.2. Analytical specificity data
Inclusivity tested on 10 CTV isolates[9] (5 from Reunion Island, 1 from Italy, 1 from Israel, 2 from USA, 1 from Spain): 100%
Exclusivity evaluated during the preliminary study on a limited number of non-target organisms, i.e. '*Candidatus* Liberibacter asiaticus', citrus variegation virus (CVV), citrus psorosis virus (CPsV, isolates A and B): 100%

4.3. Repeatability data
Average accordance: 93%

4.4 Reproducibility data
Average concordance: 79%

4.5. Diagnostic sensitivity data
68%
Data for the tissue print test: 81% these data should be taken with care because results from only 3 laboratories were included in the statistical analysis.

4.6. Diagnostic specificity data
97%
Data for the tissue print test: 93% these data should be taken with care because results from only 3 laboratories were included in the statistical analysis.

APPENDIX 8 - BIOLOGICAL INDEXING

The objective of biological indexing is:

- To detect the presence of CTV in plant accessions or selections
- To estimate the severity of the CTV isolate

Detailed procedures for biological indexing (including scoring) for graft-transmissible diseases of citrus, including CTV, are described in Roistacher (1991), see link.

[9]Information on the phylogenetic groups of the isolates is not available.

1. Indicator plants

Indicator plants as recommended in Roistacher, 1991; Ballester-Olmos et al., 1993; Garnsey et al., 2005 are:

- *C. sinensis* (sweet orange) scion grafted on *C. aurantium* (sour orange) rootstock for the evaluation of stunting and leaf chlorosis (decline, tristeza "sensu stricto").
- *C. aurantifolia* (Mexican, key or Omani lime), *C. macrophylla*, *C. sinensis* or *C. paradisi* Macfadyen (Duncan grapefruit) for stem pitting evaluation.
- *C. aurantium, C. limon* or *C. paradisi* Macfadyen (Duncan grapefruit) seedlings for seedling yellows evaluation.

2. Preparation of indicator plants

Allow all shoots to develop for the first three growth flushes (approximately 8 weeks), then prune and train as a single shoot.

3. Growing conditions

Plants are maintained at approximately 24–27°C maximum during the day and 18–21°C minimum during the night.

4. Inoculation

Buds (including buds with eyes, blind buds, chip buds), leaf pieces, or leaf discs are used for graft inoculation. A detailed description of inoculation is given in Roistacher (1991).
The indicator is graft inoculated according to conventional methods and held under standard conditions (Roistacher, 1991), preferably in four to six replicates. Symptoms are compared to those observed on positive and negative control plants. Illustrations of symptoms caused by CTV on indicator plants can be found in Roistacher (1991) and Moreno et al. (2008).

5. Symptoms

First symptoms appear 3 to 5 weeks (end of first or second growth flush). More information on symptoms is available from Roistacher (1991).

6. Performance characteristics

Not available.