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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

PM 7/118 (1)

Diagnostics
Diagnostic

PM 7/118 (1) *Tomato chlorosis virus* and *Tomato infectious chlorosis virus*

Specific scope

This Standard describes a diagnostic protocol for *Tomato chlorosis virus* and *Tomato infectious chlorosis virus*[1].

Specific approval and amendment

Approved in 2013-09.

Introduction

Tomato chlorosis virus (ToCV) and *Tomato infectious chlorosis virus* (TICV) are two distinct whitefly-transmitted viruses from tomato (*Solanum lycopersicum*) originally identified in the USA in 1998 and 1993, respectively (Duffus *et al.*, 1996; Wisler *et al.*, 1998). Since then, the viruses have spread worldwide in all major tomato-production areas. *Tomato chlorosis virus* and TICV are not mechanically transmitted, but are vectored by whiteflies in a semi-persistent manner (maximum persistence: 3–5 days): TICV is transmitted exclusively by *Trialeurodes vaporariorum* (Westwood), whereas ToCV is vectored by *T. vaporariorum*, *Bemisia tabaci* (Gennadius) biotypes B and Q (predominant in Mediterranean area) and, less efficiently, by *T. abutilonea* (Halderman) and *B. tabaci* biotype A (Dalmon *et al.*, 2009). Tomato is the major natural host of both viruses. Natural hosts of ToCV include *Solanum nigrum*, *Datura stramonium*, *Physalis peruviana*, tomatillo (*Physalis ixocarpa*), *Physalis angulata* as well as some varieties of sweet pepper (*Capsicum annuum*) and potato (*Solanum tuberosum*) (Lozano *et al.*, 2004; EPPO, 2005; Trenado *et al.*, 2007; Freitas *et al.*, 2012; de Noronha Fonseca *et al.*, 2012). A further 20 species were shown to be experimental hosts of ToCV. In addition to tomato, TICV has been reported from lettuce (*Lactuca sativa*), escarole (*Cichorium endivia*), tomatillo, artichoke (*Cynara scolymus*), petunia (*Petunia hybrida*), china aster (*Callistephus chinensis*), ranunculus (*Ranunculus* spp.) and zinnia (*Zinnia elegans*) (Parrella, 2008; EPPO, 2009). Naturally infected weeds have been identified in California (*Picris echioides*, *Nicotiana glauca*, *Cynara cardunculus*), Spain and Jordan (*S. nigrum*, *Chenopodium album* and *C. murale*). Other species belonging to eight different botanical families were susceptible to TICV in whitefly-inoculation experiments (Duffus *et al.*, 1996).

Tomato chlorosis virus and TICV have a bipartite, single-stranded and positive-sense RNA genome. Additional information on the biology of ToCV and TICV can be found in EPPO datasheets (2005, 2009).

A flow diagram describing the diagnostic procedure for ToCV and TICV is presented in Fig. 1.

Identity

Name: *Tomato chlorosis virus*
Synonyms: Tomato chlorosis crinivirus
Acronym: ToCV
Taxonomic position: viruses: *Closteroviridae*: *Crinivirus*.
EPPO code: TOCV00
Phytosanitary categorization: EPPO A2 list no. 323

Name: *Tomato infectious chlorosis virus*
Synonyms: Tomato infectious chlorosis crinivirus
Acronym: TICV
Taxonomic position: viruses: *Closteroviridae*: *Crinivirus*.
EPPO code: TICV00
Phytosanitary categorization: EPPO A2 list no. 348

Detection

Tomato chlorosis virus and TICV cause infection in both open fields and protected crops; in the latter, virus spread is more rapid due to the environmental conditions that favour vector development and multiplication.

Symptoms

Tomato plants affected by the criniviruses show similar symptoms, which consist of interveinal yellowing beginning on lower leaves. Initially, delimited chlorotic patches evolve with a light yellow colour; then they coalesce and

[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.

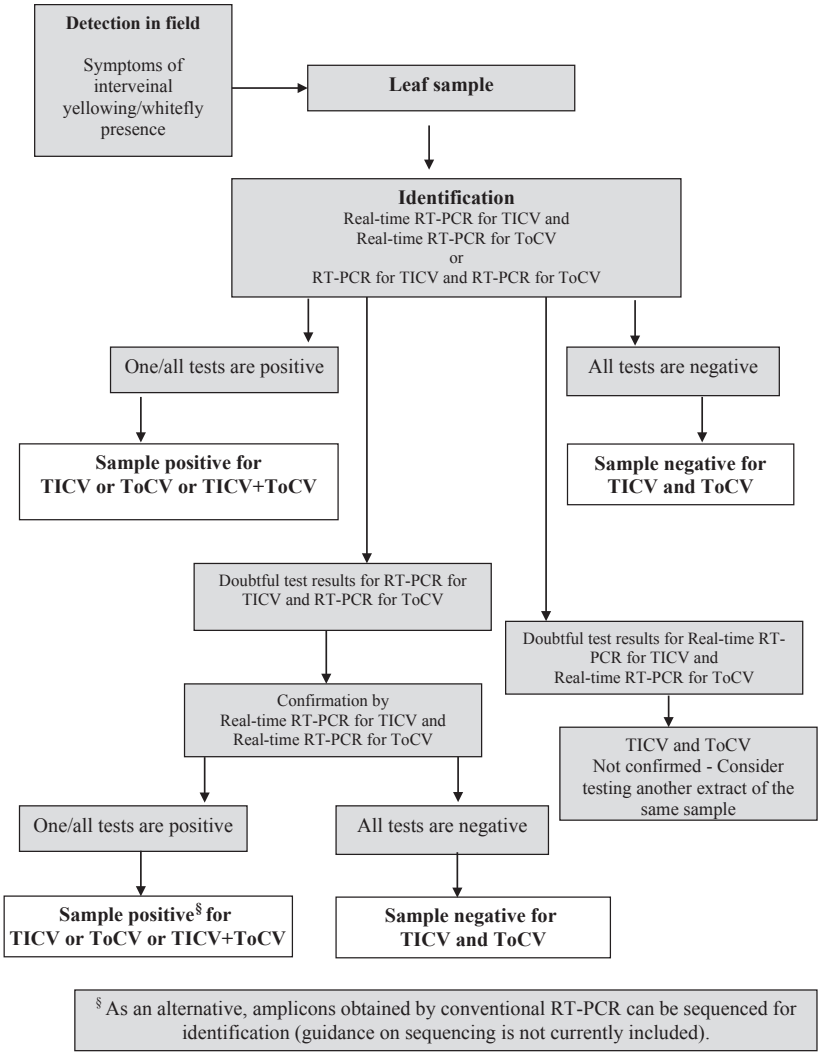

Fig. 1 *Tomato chlorosis virus*/*Tomato infectious chlorosis virus* diagnostic decision tree. The confirmatory test should be different from that used for primary identification.

the whole leaf appears bright yellow except for the veins, which remain green. These symptoms are similar to those produced by nutritional disorders, phytotoxicity or senescence. Symptomatic leaves are also thickened and brittle when hand crushed. Yellowing develops from the base to the top but is rarely visible on younger leaves. In addition, reddening, bronzing, necrosis and rolling of older leaves may be observed. Infected plants show early senescence and less vigour. There have been several reports that fruit ripening is delayed and fruit yields are decreased. Figs 2 and 3 show symptoms on leaves.

Sampling

Fully developed leaves, showing mild interveinal yellowing, should be sampled. Virus concentration is likely to be low or undetectable in old symptomatic leaves (bright yellowing, bronzing or necrosis) and in young asymptomatic leaves. *Tomato infectious chlorosis virus* can also be detected in fruits.

Bioassay using whitefly transmission

Efficient transmission of ToCV and/or TICV is obtained by allowing adult whiteflies (*T. vaporariorum*) a 48 h acquisition access period on diseased samples and a 48 h inoculation access period on test plants of tomato, *Nicotiana benthamiana* or *Physalis wrightii* (these indicator plants both work well). Interveinal chlorosis and mild yellowing generally appear 6–8 weeks after inoculation but criniviruses are detectable after 4 weeks by dsRNA analysis or reverse transcription-polymerase chain reaction (RT-PCR) testing (Duffus *et al.*, 1996; Hartono *et al.*, 2003; Wintermantel *et al.*, 2008; Dalmon *et al.*, 2009). The two criniviruses can be transmitted by *T. vaporariorum* with the same efficiency and, in the case of a

Fig. 2 Symptoms of ToCV or TICV on tomato leaves.

Fig. 3 Symptoms of ToCV or TICV on tomato leaves.

doubly-infected source plant, with the same probability. Subsequently, the resulting infections of indicator plants need to be assigned to the responsible virus using suitable identification tests.

Molecular methods

Double-stranded RNA analysis

Since the symptoms of criniviruses (and some other viruses) can be mistaken for nutritional deficiencies, dsRNA analysis (Morris & Dodds 1979) has been used to establish the nature of leaf yellowing. However, this method is not commonly used in routine diagnostics and is consequently not described in this protocol.

Conventional RT-PCR and real-time RT-PCR

Conventional RT-PCR and real-time RT-PCR can be used for both detection and identification, and are described in the following section (Identification) and in Appendices 1 and 2, respectively.

Identification

Real-time RT-PCR and conventional RT-PCR are recommended for virus identification. In addition, sequence analysis of amplicons can be used for identification (guidance on sequencing is not currently included). Antisera to TICV and/or ToCV have been produced mainly for research purposes. A commercial kit is available for ToCV detection only and is consequently not described in full in this protocol.

Nucleic acid hybridization

The use of digoxigenin-labelled complementary RNA synthesized by an *in vitro* transcription reaction and of dsDNA probes for dot blot analysis (Hadidi & Yang, 1990; Vaira *et al.*, 2002; Anfoka & Abhary, 2007; Velasco *et al.*, 2008) has proved to be reliable and sensitive in particular for mass screening of samples (Testing Service AGDIA, Ekhart, IN, USA). This is not commonly used, and for routine diagnosis the method can be replaced by RT-PCR tests.

Reverse transcription-polymerase chain reaction

Conventional RT-PCR is widely used for the diagnosis of tomato criniviruses. Several specific primers for TICV and ToCV have been derived from the heat-shock protein (HSP70) homologue gene or the coat protein (CP) gene.

The efficiencies of several published primer sets for detecting the two criniviruses have been compared (Tomassoli, unpublished data). In this comparison, the TICV primers from Vaira *et al.* (2002) and Hartono *et al.* (2003), derived from the HSP70 and CP region, respectively, showed the highest relative analytical sensitivity (10^{-5}), the best analytical specificity, and good repeatability in detecting TICV in replicated tests. TICV primers from Li *et al.* (1998) showed a one-fold lower sensitivity.

Tomato chlorosis virus primers from Louro *et al.* (2000) and Trenado *et al.* (2007) detected the virus only up to a dilution of 10^{-2} (confirmed in three different tests) but showed a good specificity and repeatability.

A duplex RT-PCR test was performed using TICV primers from Li *et al.* (1998) and ToCV primers from Trenado *et al.* (2007) as they have similar melting temperature (T_m) and a different amplicon size that allows a good separation of PCR products. The test was able to detect the viruses present in a sample with the same relative analytical sensitivity of 10^{-4} for TICV, but of only 10^{-1} for ToCV. Consequently, performing a duplex PCR is not recommended.

Appendix 1 provides details of a one-step singleplex RT-PCR test that is recommended for TICV or ToCV identification. The test was evaluated in a test performance study in the framework of an Italian project (ARNADIA) involving nine laboratories.

Real-time RT-PCR

Several real-time RT-PCR tests have been developed to test for TICV and ToCV. Wintermantel *et al.* (2008) described a real-time RT-PCR test for both TICV and ToCV based on SYBR-Green chemistry. Real-time RT-PCR tests based on TaqMan have been developed for detection of ToCV (Morris *et al.*, 2006); for TICV (A. Fox, FERA, York, GB, pers. comm.) during the whitefly-transmitted virus project in the framework of EUPHRESCO (NCM1[2]); and for both ToCV and TICV (Tiberini *et al.*, 2011). A real-time RT-PCR test has also been developed for simultaneous identification of ToCV and TICV (Papayiannis *et al.*, 2011). However, in a test performance study (ARNADIA Italian project) comparing the aforementioned real-time RT-PCR tests based on TaqMan, some variability was observed in the quality of the amplification curves and ΔCt value between target and non-target samples with the primers and probes of Papayiannis *et al.* (2011). As consequence, two protocols based on the best ToCV primers and ToCV probes by Morris *et al.* (2006), and the TICV primers and TICV probe from Tiberini *et al.* (2011), respectively, were validated in a test performance study involving five laboratories.

Appendix 2 provides details of the real-time RT-PCR that is recommended for TICV or ToCV identification.

Reference material

TICV and ToCV infected tomato controls are available for non-profit institutions from:
Plant Pathology Research Centre (CRA-PAV), C.G. Bertero 22, 00156 Rome, Italy.
Food Environment Research Agency (Fera), Sand Hutton, York, Y041 1LZ, United Kingdom.

Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM-7/77 (1) *Documentation and reporting on a diagnosis.*

Performance criteria

When performance criteria 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).

[2]http://www.euphresco.org/downloadFile.cfm?id=674

Further information

Further information on these viruses can be obtained from:

L. Tomassoli, Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Centro di Ricerca per la Patologia Vegetale, CRA, Via G.C. Bertero 22, 00156 Rome, Italy.

Feedback on this Diagnostic Protocol

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 send it to diagnostics@eppo.int.

Protocol 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.

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Appendix 1 – One-step RT-PCR for detection of ToCV or TICV

1. General information

1.1 The following one-step RT-PCR protocol is performed using primer sets from Louro *et al.* (2000) and Vaira *et al.* (2002) for detection of ToCV and TICV, respectively. The test has been validated only for tomato leaf material.

1.2 Fresh, frozen or dried tissue from symptomatic leaves.

1.3 Primers have been derived from the heat shock protein 70 homologue (HSP70) gene located on RNA2 of ToCV (GenBank acc. no. AY903448) and TICV (GenBank acc. no. FJ542305 or -6) (see Table 1).

1.4 RT-PCR is performed using SuperScript III One Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, CA, USA).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 50–100 mg of tomato symptomatic leaf tissue for fresh or frozen material, or 10–30 mg for dried material, is ground in liquid nitrogen using an iced mortar and pestle and dissolved with the buffer provided with the RNA extraction kit. For dried material, the alternative grinding procedure is to use Homex 6 and homogenization bags (Bioreba, cat. no. 411 000) and to grind 1 g leaf material in 5 mL 0.1 M PBS (3.63 g Na_2HPO_4 12 H_2O, 0.24 g KH_2PO_4, 8.0 g NaCl and 0.2 g KCl)[3]. 100 µL of PBS leaf extract is added to 350 µL of the buffer provided in the kit.

Table 1 Derivation of primers

	Primer name	RNA2 location of amplicon	Oligonucleotide sequences	Amplicon size (bp)
ToCV	ToCV-172 (F)	904-1342 (GenBank acc. No. AY903448)	5′-GCTTCCGAAACTCCGTCTTG-3′	439
	ToCV-610 (R)		5′-TGTCGAAAGTACCGCCACC-3′	
TICV	TICV-32 (F)	578-1078 (GenBank acc. no. FJ542305)	5′-TCAGTGCGTACGTTAATGGG-3′	501
	TICV 532 (R)		5′-CACAGTATACAGCAGCGGCA-3′	

[3]Preparation of additional PBS is needed as there is not enough buffer provided with the kit. This is based on the experience of several laboratories.

Table 2 One-step singleplex RT- PCR using SuperScript III One Step RT-PCR System with Platinum Taq DNA Polymerase

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water	N.A.	9.5	N.A.
Reaction Mix	2×	12.5	1×
Primer (F)	10 μM	0.5	0.2 μM
Primer (R)	10 μM	0.5	0.2 μM
Reverse transcriptase (RT)/Taq Mix	N.A.	1.0	N.A.
Subtotal		24	
RNA	–	1	–
Total		25	

2.1.2 Total RNA should be extracted using the commercial kit RNeasy Plant Mini Kit (Qiagen, Germany), which proved to be efficient. Other nucleic acid extraction kits (e.g. RealTotal RNA from Tissue and Cell, Valencia, Spain) also have been shown to yield a good quantity and quality of total RNA.

2.1.3 Total RNA extract is eluted in 50–100 μL of elution buffer.

2.1.4 Total RNA extract can be kept under short-term storage (<1 month) at −20°C or longer at −80°C.

2.2 One-step singleplex RT-PCR using SuperScript III One Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, CA, USA) (see Table 2).

2.3 RT-PCR cycling parameters: reverse transcriptase at 50°C for 30 min; denaturation step at 94°C for 5 min; 35 cycles consisting of denaturation at 94°C for 15 s, annealing at 57°C for 30 s, elongation at 72°C for 30 s; a terminal elongation step at 72°C for 5 min.

2.4 Visualization of amplicons on 1.2% agarose gels in horizontal gel electrophoresis apparatus.

3. Essential procedural information

3.1 Controls:

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation 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 nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- *Negative amplification control (NAC)* to rule out false positives due to contamination during preparation of the reaction mix: amplification of 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 nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

3.2 Interpretation of results: in order to assess results from PCR-based tests, the following criteria should be followed.

Verification of the controls:

- NIC and NAC should produce no amplicons.
- PIC, PAC should produce amplicons of 439 or 501 bp (for ToCV and TICV, respectively).

When these conditions are met:

- a test will be considered positive for ToCV if amplicons of 439 bp are produced
- a test will be considered positive for TICV if amplicons of 501 bp are produced
- a test will be considered negative if it produces no band or a band of a different size
- tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Validation data were generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*. Validation was performed by the following working group within an Italian Project (ARNADIA) funded by the Agricultural Ministry: Centro di Ricerca per la Patologia Vegetale, CRA-PAV, Roma, (L. Tomassoli); Instituto di Virologia Vegetale, IVV – CNR, Torino, Italy (M. Turina); Università degli Studi di Bari (D. Gallitelli); Università degli Studi di Palermo (S. Davino).

4.1 Analytical sensitivity (relative)

From ARNADIA test performance: tenfold dilution series were obtained by diluting infected RNA extracts in RNA extracts from healthy plants; three series have been prepared and tested in three experiments. Results: TICV: 10^{-5}ToCV: 10^{-2}.

4.2 Analytical specificity

From ARNADIA test performance study: each test has been validated against 12 target isolates from different varieties and origin (10 Italy, one Spain, one France). The tests have also been validated against non-target viruses belonging to the genus *Crinivirus* [(*Beet pseudoyellows virus* (BPYV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Lettuce infectious yellows*

virus (LYIV)], and *Tomato yellow leaf curl virus* (TYLCV), *Tomato spotted wilt virus* (TSWV), *Pepino mosaic virus* (PepMV) and TICV or ToCV according to the simplex test under validation.

4.3 Repeatability
From ARNADIA test performance study: two samples for each virus at medium and low detection levels (from the analytical sensitivity test) were replicated three times in duplicate.
TICV: 100%
ToCV: 100%.

4.4 Reproducibility
From ARNADIA test performance study: nine laboratories participated in the test performance study. In total, 18 samples were analysed for each virus/test using healthy and naturally infected tomato leaf tissue (12 TICV/ToCV infected samples; three non-target infected samples; three healthy samples).
TICV 99%
ToCV 99%.

4.5 Other performance criteria available
For both viruses:
Diagnostic sensitivity: 100%
Diagnostic specificity: 100%
Relative accuracy: 100%
Comparison with samples of known status.

Appendix 2 – Real-time RT-PCR for detection of ToCV or TICV

1. General information

1.1 The following real time RT-PCR test was prepared and evaluated in the framework of the ARNADIA project. It is performed for the detection and identification of ToCV and TICV using specific primers and probes from Morris *et al.* (2006) and Tiberini *et al.* (2011), respectively (see Tables 3 and 4).

1.2 Fresh, frozen or dried tissue from symptomatic tomato leaves.

1.3 Primers and probes have been derived from the CP gene located on RNA2 of ToCV and TICV after multiple sequence alignment of published GenBank sequences for both viruses (ToCV: AY048854; TICV: FJ815441, EU625351, FJ542305, FJ542306).

1.4 An internal control is included with primers and probe designed in a highly conserved region of the plant mtCOX1 gene (X83206) (Weller *et al.*, 2000).

1.5 TaqMan probes: different combinations of dyes can be used to suit specific real-time systems.

1.6 The method has been successfully performed using reagents from TaqMan One-Step RT-PCR Master Mix Kit (Applied Biosystem Cod. N. 4309169).

1.7 The test has been successfully performed on a range of different real-time PCR systems including ABI (7900, 7500 fast, StepOne), Bio-Rad (MJ Chromo4, MiniOpticon CFX96).

1.8 All samples should be run in duplicate.

2. Methods

2.1 Nucleic acid extraction

2.1.1 Total RNA should be extracted using the commercial kit RNeasy Plant Mini Kit (Qiagen, Germany) that has been shown to be efficient.

2.1.2 50–100 mg of leaf tissue is ground in liquid nitrogen using an iced mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled 2 mL

Table 3 Primers

	Primer name	Location of amplicon	Reference	Oligonucleotide sequences	Amplicon size (bp)
ToCV	ToCV – 258F	258–280	GenBank acc. AY048854	5′-GTCTGTTCCGGCTGATTACAAGT-3′	74
	ToCV 331R	331–308		5′-AATTGAAACCCAAAGAGGAACAAA-3′	
TICV	TICV 463F	4750–4772	GenBank acc. FJ815441	5′-GCGGGACATTTTTTATCATATGC-3′	115
	TICV 577R	4864–4841		5′-TCAGCCCAACATCTTGTAGTTGTT-3′	
COX	COX F	1488–1508	GenBank acc. X83206	5′-CGTCGCATTCCAGATTATCCA-3′	78
	COX R	1565–1538		5′- CAACTACGGATATATAAGRRCCRRAACTG -3′	

Table 4 Probes

	Probe name	Location of amplicon	Reference	Oligonucleotide sequences
ToCV	ToCV probe	282–306	GenBank acc. AY048854	5′(FAM)-TGGGCAGAGACTTTTCATGCAGGCA-(MGB)3′
TICV	TICV-497 probe	4784–4809	GenBank acc. FJ815441	5′(FAM)-CGTCAGGTCACCCAAACGCTCTAAGG-(MGB)3′
COX	COX SOL 1511	1534–1511	GenBank acc. X83206	5′(VIC)- AGG GCA TTC CAT CCA GCG TAA GCA -(MGB)3′

Table 5 Singleplex one-step real-time RT-PCR for ToCV or TICV or internal control (COX)

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	8.85	N.A.
Master mix	2 x	12.5	1 x
RNase inhibitor (included in the kit)	40 x	0.65	1 x
Primer F (TICV or ToCV or COX)	10 µM	0.75	0.3 µM
Primer R (TICV or ToCV or COX)	10 µM	0.75	0.3 µM
Probe (TICV or ToCV or COX)	5 µM	0.5	0.1 µM
Subtotal		24	
Total RNA		1	
Total		25	

microcentrifuge tube. Add 450 µL buffer from the kit and proceed with RNA extraction.

2.1.3 Alternative grinding procedure: use Homex 6 and homogenization bags (Bioreba, cat. no. 411 000) and grind 1 g leaf material in 5 mL 1× PBS (dissolve the following in 800 mL of distilled water: 3.63 g Na_2HPO_4 12 H_2O, 0.24 g KH_2PO_4, 8.0 g NaCl and 0.2 g KCl; adjust pH to 7.4, adjust volume to 1 L and sterilize by autoclaving). Take an aliquot of 100 µL, add 450 µL buffer from the kit and proceed with RNA extraction.

2.1.4 Total RNA is eluted in 50–100 µL elution buffer.

2.1.5 Total RNA can be kept under short-term storage (<1 month) at −20°C or longer at −80°C.

2.2 Singleplex one-step real-time RT-PCR for ToCV or TICV or internal control (COX) (see Table 5).

2.3 Real-time RT-PCR cycling parameters: reverse transcriptase at 50°C for 30 min; denaturation step at 94°C for 3 min; 40 cycles consisting of 10 s at 94°C and 30 s at 60°C.

Duplex one-step real-time RT-PCR can be performed using primers and probes for ToCV and COX, or TICV and COX in the same test. However, the performance criteria provided for this test are for a singleplex use. The quantities for primers and probes are as indicated for the simplex reaction, adjusting molecular-grade water volume. Multiplex with the three targets (ToCV, TICV and COX) showed a decrease in analytical sensitivity.

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation 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 nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism). As an alternative (or in addition) to the external positive controls (PIC), internal positive controls can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or mitochondrial *nad5* gene), amplification of samples spiked with exogenous nucleic acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

– *Negative amplification control (NAC)* to rule out false positives due to contamination during preparation of the reaction mix: amplification of 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 nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

3.2 Interpretation of results:

The cycle cut-off value for TICV and ToCV is set at 38, and was obtained using the equipment/materials and chemistry used as described in this appendix. The COX Ct value is usually between 18 and 30.

The cycle cut-off values needs to be verified in each laboratory when implementing the test for the first time.

- The PIC, PAC and COX amplification curves should be exponential.
- NIC and NAC should be negative (Ct > cut-off).
- PIC, PAC should have a Ct value below the cut-off value.
- For each sample the COX value should be as expected.

When these conditions are met:

- a sample will be considered positive if it produces an exponential amplification curve and a Ct value below the cut-off value.
- a sample will be considered negative if it produces no exponential amplification curve and a Ct value equal or above the cut-off value.

• tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Validation data were generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*. Validation was performed by the following working group within an Italian Project (ARNADIA) funded by the Agricultural Ministry: Centro di Ricerca per la Patologia Vegetale, CRA-PAV, Roma, (L. Tomassoli); Instituto di Virologia Vegetale, IVV – CNR, Torino, Italy (M. Turina); Università degli Studi di Bari (D. Gallitelli); Università degli Studi di Palermo (S. Davino).

4.1 Analytical sensitivity (relative)

Tenfold dilution series were obtained by diluting infected RNA extracts in RNA extracts from healthy plants; five series have been prepared and tested in three experiments. Results: TICV: 10^{-6}, ToCV: 10^{-4}.

4.2 Analytical specificity

From ARNADIA test performance study: each test has been validated against 12 target isolates from different varieties and origin (10 Italy, one Spain, one France). The tests have also been validated against non-target viruses belonging to the genus *Crinivirus* [(*Beet pseudoyellows virus* (BPYV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Lettuce infectious yellows virus* (LYIV)], and *Tomato yellow leaf curl virus* (TYLCV), *Tomato spotted wilt virus* (TSWV), *Pepino mosaic virus* (PepMV), and TICV or ToCV according to the simplex test under validation.

4.3 Repeatability

Two samples for each virus at medium and low detection levels (from the analytical sensitivity test) were replicated three times in duplicate:

TICV: 100%

ToCV: 100%.

4.4 Reproducibility

In total, 18 samples (12 TICV/ToCV infected samples; three non-target infected samples; three healthy samples) were analysed for each virus/test using healthy and naturally infected tomato leaf tissue by five laboratories:

TICV: 100%

ToCV: 100%.

4.5 Other performance criteria available

Diagnostic sensitivity: 100%

Diagnostic specificity: 100%

Relative accuracy: 100%

Comparison with samples of known status.