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EPPO STANDARD ON DIAGNOSTICS

# PM 7/152 (1) Begomoviruses

**Specific scope:** This Standard describes a diagnostic protocol for detection and identification of begomoviruses[1] on cucurbits, eggplant, pepper and tomato.

This protocol replaces the EPPO Standard PM 7/50 Tomato yellow leaf curl and Tomato mottle begomoviruses.

This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

**Specific approval and amendment:** Approved in 2022–07.

Authors and contributors are given in the Acknowledgements section.

## 1 | INTRODUCTION

The genus *Begomovirus* (Family Geminiviridae) is the largest genus of plant viruses with more than 400 species (https://talk.ictvonline.org/taxonomy). Virions of viruses belonging to Geminiviridae are typically twinned ('geminate'). Begomoviruses have monopartite or bipartite genomes. Twinned virions of monopartite begomoviruses contain a single copy of circular single stranded DNA (ssDNA). The genomes of bipartite begomoviruses consist of two components, referred to as DNA-A and DNA-B. The DNA-A component of the bipartite begomoviruses can replicate autonomously and produce virions but requires the DNA-B component for systemic infection (Zerbini et al., 2017). The most important means of begomovirus transmission is by the whitefly *Bemisia tabaci*, a cryptic species complex, which can infest many crops and is globally widespread (Fiallo-Olivé et al., 2020; Stansly et al., 2010). Begomoviruses infect a wide range of economically important dicotyledonous plants and represent an emerging problem worldwide. The main hosts are in the families Cucurbitaceae, Euphorbiaceae, Malvaceae and Solanaceae.

In the EPPO region, the oldest reports of begomovirus outbreaks date from the 1980s. Sporadically, outbreaks with variable levels of damage continue to be reported by different countries (see EPPO Global Database). In the EPPO region, only a few members of this genus have been detected on tomato, and a few begomoviruses have been found on other crops of economic importance (e.g. cucurbits), mainly in the Mediterranean basin. Begomoviruses found on tomato and cucurbits already reported in the EPPO region are tomato yellow leaf curl Axarquia virus (TYLCAxV), tomato yellow leaf curl virus (TYLCV), tomato yellow leaf curl Malaga virus (TYLCMaV), tomato leaf curl New Delhi virus (ToLCNDV) and tomato yellow leaf curl Sardinia virus (TYLCSV).

The flow diagram describing the diagnostic procedures for begomoviruses is presented in Figure 1.

## 2 | IDENTITY

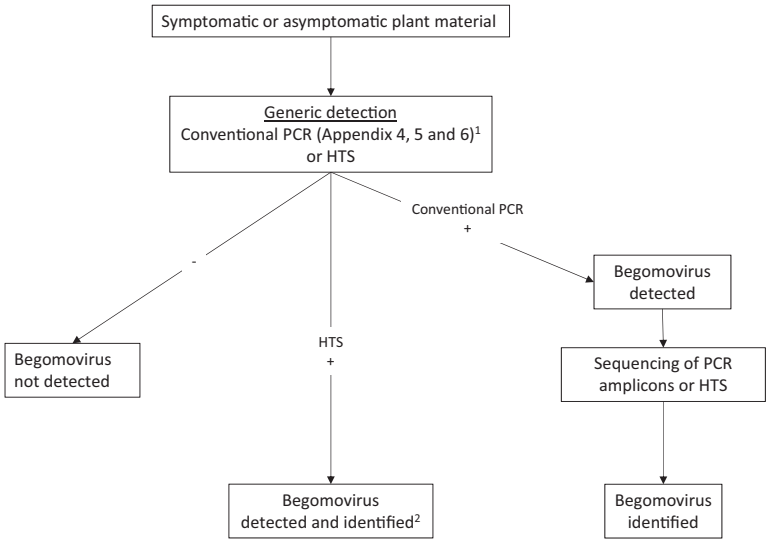
**Name:** Begomoviruses
**Taxonomic position:** Viruses, Geminiviridae
**EPPO Code:** 1BEGOG
**Phytosanitary categorization:** In the European Union (EU), all begomoviruses not known to occur in the Union territory are quarantine pests with the exception of tomato yellow leaf curl virus (TYLCV), tomato yellow leaf curl Sardinia virus (TYLCSV), tomato yellow leaf curl Axarquia virus (TYLCAxV), tomato yellow leaf curl Malaga virus (TYLCMaV), abutilon mosaic virus (AbMV), papaya leaf crumple virus (PaLCrV) and sweet potato leaf curl virus (SPLCV). The begomoviruses that are recommended for regulation by EPPO are listed below.

**Name:** Bean golden mosaic virus
**Acronym:** BGMV
**Taxonomic position:** Viruses, Geminiviridae, Begomovirus
**EPPO Code:** BGMV00
**Phytosanitary categorization:** EPPO A1 List, EU Annex II A

**Name:** Bean golden yellow mosaic virus
**Acronym:** BGYMV
**Taxonomic position:** Viruses, Geminiviridae, Begomovirus
**EPPO Code:** BGYMV00
**Phytosanitary categorization:** EPPO A1 List, EU Annex II A

**Name:** Tomato mottle virus
**Acronym:** ToMoV

[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.




[1] For symptomatic tomato and plants belonging to the family *Cucurbitaceae*, one of the PCRs that detect the core region of the capsid protein gene (Appendix 4 or 6) and the PCR that detects the sequence located on the open reading frame AC2 and AC1 (Appendix 5) should be used. However, the use of all three PCR tests described in Appendices 4-6 is recommended for testing of asymptomatic or other hosts.
[2] Confirmation with another test is recommended after HTS

**FIGURE 1** Flow diagram describing the diagnostic procedure for begomoviruses in plant samples. Appendix 1 gives an overview of other tests that can be used for detection, confirmation of detection and/or identification of begomoviruses. Other tests for the semi-specific and specific detection of begomoviruses are described in Appendices 7–9.

**Taxonomic position:** Viruses, Geminiviridae, Begomovirus
**EPPO Code:** TOMOV0
**Phytosanitary categorization:** EPPO A1 List, EU Annex II A

**Name:** Squash leaf curl virus
**Acronym:** SLCV
**Taxonomic position:** Viruses, Geminiviridae, Begomovirus
**EPPO Code:** SLCV00
**Phytosanitary categorization:** EPPO A2 List

**Name:** Tomato yellow leaf curl Sardinia virus
**Acronym:** TYLCSV
**Taxonomic position:** Viruses, Geminiviridae, Begomovirus
**EPPO Code:** TYLCSV
**Phytosanitary categorization:** EPPO A2 List

**Name:** Tomato yellow leaf curl virus
**Acronym:** TYLCV
**Taxonomic position:** Viruses, Geminiviridae, Begomovirus
**EPPO Code:** TYLCV0
**Phytosanitary categorization:** EPPO A2 List, EU Annex IV

**Name:** Tomato leaf curl New Delhi virus
**Acronym:** ToLCNDV
**Taxonomic position:** Viruses, Geminiviridae, Begomovirus
**EPPO Code:** TOLCND
**Phytosanitary categorization:** EPPO A2 List, EU Annex II B

# 3 | DETECTION

## 3.1 | Symptoms

A description of the symptoms adapted from the EFSA opinion (EFSA, 2013) is presented below. Symptoms of begomovirus infections in plants usually consist of leaf curling, vein yellowing and/or yellow mosaic. However, symptoms vary depending on the growth stage at the time of initial infection, weather conditions, host species/cultivar and begomovirus species. Green to bright yellow mosaic symptoms, leaf deformation and chlorosis are often a sign of severe disease. Early infections result in severe growth reduction and stunting of the plant. In the case of early infection, flowering is reduced, and fruit development is aborted, and thus early infections can lead to the entire loss of the crop while infections at later stages of development are often mild, and losses are tolerable.

### 3.1.1 | Cucurbitaceae

Typical symptoms on begomovirus infected Cucurbitaceae are yellowing and leaf curl. The main symptoms associated with ToLCNDV infection are leaf distortion, yellow mosaic, vein clearing and leaf curling. These symptoms can also include short internodes, vein swelling, roughness of the fruit skin, smaller and fewer fruits and fruit bursting (Figures 2–5).

**FIGURE 2** Early stage of infection of ToLCNDV, with leaf curling of young leaves, on *Cucurbita pepo* in open field cultivation. Courtesy of CREA (IT).

**FIGURE 3** ToLCNDV infection with severe leaf curling and yellow mosaic, on *Cucurbita pepo* in open field cultivation. Courtesy of CREA (IT).

### 3.1.2 | *Solanum melongena*

Begomovirus-infected eggplants can exhibit yellow leaf mosaic and mottling symptoms, leaf distortion and leaf curling. Eggplants infected with ToLCNDV may show severe yellow mosaic and mottling of leaves at later stages of infection.

### 3.1.3 | *Capsicum annuum*

Typical symptoms of begomovirus-infected pepper plants are yellowing and leaf curling. Chilli plants infected with ToLCNDV show leaf curling, shortening of internodes and petioles, bunching of leaves and stunting of the whole plant (Figure 6). Fruit symptoms are either not observed or, in severe cases, no fruits are formed, and complete crop loss can occur.

**FIGURE 4** ToLCNDV infection with leaf curling and swelling of veins, on *Cucurbita pepo* in protected cultivation. Courtesy of CREA (IT).

**FIGURE 5** ToLCNDV infection with symptoms on plant apex, on *Cucurbita pepo* in protected cultivation. Courtesy of CREA (IT).

### 3.1.4 | *Solanum lycopersicum*

All known tomato cultivars infected by begomoviruses show symptoms such as, but not limited to, leaf curling and yellow leaf curling (Figure 7). Symptoms of the tomato yellow leaf curl disease can be caused by several begomovirus species and consist of a more or less prominent upward curling of leaflet margins, reduction in leaflet area and yellowing of young leaves, together with stunting and

**FIGURE 6** ToLCNDV infection with symptoms of yellowing and leaf curling, on *Capsicum annuum* in protected cultivation. Courtesy of CREA (IT).

**FIGURE 7** Yellow leaf curling caused by TYLCSV infection on *Solanum lycopersicum*. Courtesy of CREA (IT).

flower abortion. Infection results in a general decrease in plant growth and reduced yields, and production is almost entirely lost if plants are infected at an early stage. Typical symptoms of tomatoes infected with ToLCNDV are yellowing, severe leaf curling and stunting.

## 3.2 | Test sample requirements

### 3.2.1 | Plants

Virus concentration is dependant on the begomovirus strain or species, the host plant species, the developmental stage of the plant and weather conditions. Furthermore, the virus concentration in different plant parts can vary significantly; therefore, whenever possible, testing of symptomatic plants is recommended for the diagnosis of begomoviruses.

For the majority of begomoviruses, the virus is present at higher concentrations in freshly expanded young leaves in the uppermost parts of the plant rather than in older plant parts.

#### *3.2.1.1 | Symptomatic plants*

For symptomatic plants, samples for laboratory testing should consist of at least 3 symptomatic leaflets collected from the young shoots of the plants.

#### *3.2.1.2 | Asymptomatic plants*

Asymptomatic infections can occur in some host plants, and if there is a suspicion of a begomovirus infection it is important to sample different parts of the plant.

Experimental data to recommend a sample size in the case of testing asymptomatic plants is lacking.

No data is available on the testing of young plants grown in nurseries. It is not known at which growth stage of the plants begomoviruses can be detected reliably.

### 3.2.2 | Vectors

*Bemisia tabaci* specimens can also be tested for the presence of begomoviruses (Bertin et al., 2018; Lefeuvre et al., 2007; Papayiannis et al., 2010; van Brunschot et al., 2013). Samples should be made of single and/or pooled (up to 5) specimens of *B. tabaci*. After collection, it is recommended to preserve the whiteflies at −20°C for short-term storage, or in absolute or 70% ethanol for long-term storage (ideally absolute ethanol as the use of alcohol solutions mixed with water may decrease the yield of DNA).

## 3.3 | Screening tests

For the detection of begomoviruses, molecular tests, including high-throughput sequencing, are recommended.

### 3.3.1 | Molecular methods

The tests recommended in this diagnostic protocol have been evaluated during a Test Performance Study (TPS) organized in the framework of the Euphresco project BegomoVal (2016-A-212) and in the framework of the activities of the European Union Reference Laboratory

for plant pests for viruses, viroids and phytoplasmas (EURL Virology thereafter). Based on the Euphresco BegomoVal TPS (Gentit et al., 2022) and EURL Virology evaluation, the following conventional PCR tests using degenerate primers are recommended for the generic detection of begomoviruses:

- conventional PCR adapted from Wyatt and Brown (1996), as described in Appendix 4.
- conventional PCR adapted from Li et al. (2004), as described in Appendix 5.
- conventional PCR according to Saison and Gentit (2015), as described in Appendix 6.

None of the currently known generic PCR tests can detect all begomovirus isolates. In particular, the conventional PCR adapted from Li et al. (2004) has shown poor performance in the Euphresco BegomoVal TPS as many isolates were not detected with this test. Nevertheless, the test is recommended because it can detect some species (e.g., Squash leaf curl China virus – SLCCNV and luffa yellow mosaic virus – LYMV) that are not detected by the conventional PCR tests of Saison and Gentit (2015) and Wyatt and Brown (1996).

For symptomatic tomato plants and plants belonging to the family Cucurbitaceae, one of the PCR tests that detects the core region of the capsid protein gene (Appendix 4 or 6) and the PCR that detects the sequence located in the open reading frame AC2 and AC1 (Appendix 5) should be used.

For asymptomatic plants and plants other than tomato and Cucurbitaceae, the three PCR tests should be performed in parallel.

*In-silico* analysis showed that the PCR tests that target the core region of the capsid protein gene (Appendices 4 and 6) and the open reading frame AC2 and AC1 (Appendix 5) can detect most of the begomoviruses that can infect tomato and/or plants belonging to the family Cucurbitaceae (altogether at least 213 species [Natasa Mehle, EURL Virology, NIB, pers. comm.]).

In addition, there are other conventional PCR tests based on degenerate primers that can be used for generic detection of begomoviruses, such as, the test based on Accotto et al. (2000) which was also included in the Euphresco BegomoVal TPS. This PCR test targets the core region of the capsid protein gene. Compared to other tests included in the Euphresco BegomoVal TPS that target the same region i.e. Wyatt and Brown (1996) and Saison and Gentit (2015), the test from Accotto et al. (2000) showed lower performance characteristics (lower inclusivity, lower diagnostic sensitivity 79.9% compared to 93.5% and 87% for the Wyatt & Brown and Saison & Gentit PCR tests).

Appendix 1 lists some other tests that can be used to detect a number of begomoviruses. Other molecular tests, including conventional PCR, real-time PCR and LAMP tests can be used for the detection (and identification) of a number of begomovirus species. A test for the semi-specific detection of ToLCNDV is described in Appendix 7. For detection and identification of ToLCNDV, Appendices 8 and 9 describe the recommended tests. In addition, for ToLCNDV species-specific primers as published by Mizutani et al. (2011) or Gawande et al. (2007) can also be used. TYLCV, TYLCSV and tomato mottle virus (ToMoV) can be detected using the PCR test according to Accotto et al. (2000), Belabess et al. (2015) and Deng et al. (1994). It is important to note that when (semi-) specific tests are used for detection, negative results do not exclude the presence of a begomovirus.

### 3.3.2 | High-throughput sequencing

High-throughput sequencing (HTS) analysis can be used as a screening test for begomoviruses, these being identified at the same time.

Input for HTS can be total DNA, total RNA, small RNAs and Rolling Circle Amplification (RCA) products.

### 3.3.3 | Other tests

#### *3.3.3.1 | Serological tests*

Double-antibody sandwich-enzyme-linked immunosorbent assay (DAS)-ELISA or triple-antibody sandwich (TAS)-ELISA can be used for the detection of a number of begomoviruses (see Appendix 1), such as ToLCNDV, TYLCV and TYLCSV in symptomatic plants (EURL Virology, personal communication 2021). Instructions to perform an ELISA test are described in the EPPO Standard PM 7/125 *ELISA tests for viruses* (EPPO, 2015).

An on-site serological test is available as a preliminary screening test for ToLCNDV (ImmunoStrip, Agdia) (see Appendix 1). It allows detection of ToLCNDV in symptomatic plant material and provides results within a few minutes. Tests should be performed according to the manufacturer's instructions. A positive result should be confirmed by an additional serological and/or molecular test.

#### *3.3.3.2 | Electron microscopy*

Electron microscopy (EM) can be used for detection of begomoviruses since they have a typical morphology, i.e. twinned ('geminate') virions. Instructions to perform EM are described in EPPO Standard PM 7/126 *Electron microscopy in diagnosis of plant viruses* (EPPO, 2015).

## 4 | IDENTIFICATION

For the identification of begomoviruses the following tests are recommended: sequence analysis of PCR

products, HTS analysis or species-specific real-time PCR or LAMP tests. Specific conventional PCR tests can be used in certain cases, provided that the test is validated.

### 4.1 | Sequencing

Sanger sequencing of amplicons obtained from generic PCR tests (3.3.1) or HTS analysis can be used for the identification of begomoviruses. Sequence analysis should follow the guidelines described in Appendices 7 and 8 of the EPPO Standard PM 7/129 (2) *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2021).

### 4.2 | Conventional PCR, real-time PCR and LAMP tests

For the identification of ToLCNDV, the real-time PCR or LAMP tests described in Appendices 8 and 9 are recommended. Other specific tests that can be used for the identification of begomoviruses are listed in Appendix 1.

## 5 | REFERENCE MATERIAL

Begomovirus isolates for reference are available from:

DSMZ Leibniz-Institut DSMZ-Deutsche Sammlung von Mikro- organismen und Zellkulturen GmbH Inhoffenstraße 7 B 38124 Braunschweig Germany.

## 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:

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## 9 | FEEDBACK ON THIS DIAGNOSTIC STANDARD

If you have any feedback concerning this Diagnostic Standard, 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 Standards. Standards 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 - OVERVIEW OF OTHER TESTS THAT CAN BE USED FOR DETECTION AND/OR IDENTIFICATION OF BEGOMOVIRUSES

When available, validation data can be downloaded from the EPPO Database on diagnostic expertise https://dc.eppo.int/validation_data/validationlist

| Method | Reference/Company | Target virus | Comment |
|---|---|---|---|
| ELISA | Adgen | TYLCV, TYLCSV, some other begomoviruses | Validation data has been generated by ILVO (BE) |
| | Agdia | ToLCNDV (some other begomoviruses may be detected) | Cross reactions reported by the producer of the antiserum: ACMV, SLCV, TYLCV, BGMV<br>Validation data has been generated by CREA-DC (IT) |
| | Bioreba | TYLCV and some other begomoviruses | Validation data not available |
| | DSMZ | ToLCNDV | Validation data has been generated by CREA-DC (IT) |
| | DSMZ | TYLCV, TYLCSV | Validation data not available |
| | Loewe | TYLCV, TYLCSV, some other begomoviruses | Validation data not available |
| ImmunoStrip | Agdia | ToLCNDV (some other begomoviruses may be detected) | Cross reactions reported by the producer of the antiserum: BGMV, SLCV, TGMV |
| LAMP | Jeevalatha et al. (2018) | ToLCNDV | Test recommended for detection and identification of ToLCNDV in plant material and *B. tabaci* (see Appendix 9). |
| PCR | Accotto et al. (2000) | TYLCV, TYLCSV and some other begomoviruses | Validation data has been generated by ANSES (FR) |
| | Ali-Shtayeh, Jamous, Hussein, et al. (2014) | SLCV | Validation data not available |
| | Ali-Shtayeh, Jamous, Mallah, et al. (2014) | WmCSV | Validation data not available |
| | Belabess et al. (2015) | TYLCV, TYLCSV | RFLP or sequence analysis of PCR products can be used for identification of begomoviruses |
| | Briddon et al. (2002) | Monopartite begomoviruses | Limited validation data: only analytical specificity (inclusivity) has been generated by ANSES (FR) |
| | Chigurupati et al. (2011) | ToLCNDV | Validation data not available |
| | Davino et al. (2008) | TYLCV, TYLCSV, TYLCMalV | Validation data not available |
| | Deng et al. (1994) | TYLCV, TYLCSV, ToMoV and some other begomoviruses | RFLP or sequence analysis of PCR products can be used for identification of begomoviruses<br>Limited validation data: only analytical specificity (inclusivity) has been generated by ANSES (FR) |
| | Gawande et al. (2007) | ToLCNDV | Validation data not available |
| | Mizutani et al. (2011) | ToLCNDV | Validation data (available for B2 primer pair only) has been generated by CREA-DC (IT) |
| | Rojas et al. (1993) | bipartite begomoviruses | Limited validation data: only analytical specificity (inclusivity) has been generated by ANSES (FR) |
| | Souza et al. (2020) | SiMMV, TGVV, ToALCV, ToCMoV, ToMoLCV, ToRMV, ToSRV | Validation data not available |
| | Tsai et al. (2011) | ToLCNDV, TYLCV, TYLCSV, SLCV and some other begomoviruses | Sequence analysis of PCR products can be used for identification of begomoviruses<br>Validation data has been generated by DSMZ (DE) |
| Real-time PCR | Luigi et al. (2020) | ToLCNDV | Test recommended for detection and identification of ToLCNDV in plant material and *B. tabaci* (see Appendix 8) |
| | Powell et al. (2012) | TYLCV, TYLCSV | Validation data has been generated by ILVO (BE) and Fera (GB) |
| | Simon et al. (2018) | ToLCNDV (some other begomoviruses may be detected) | Test recommended for detection of ToLCNDV in plant material and *B. tabaci* (see Appendix 7) |

## APPENDIX 2 - NUCLEIC ACID EXTRACTION

### 1 | DNA EXTRACTION FROM PLANT MATERIAL

Different procedures for DNA extraction from plant material are available. The extraction procedures suitable for the tests described in Appendices 4–9 are listed below. Other extraction methods may be used but should be validated in combination with the molecular test to be used. Extracted total DNA can be stored at −20°C.

#### 1.1 | DNA extraction methods with a magnetic bead processor

These DNA extraction methods can be used with a large number of plant samples. They combine a simple and quick homogenization step of crude extracts with DNA extraction based on the binding of DNA to magnetic beads. These extraction methods were found to be suitable for all tests described in Appendices 4–9.

##### 1.1.1 | DNA extraction using a QuickPick™ SML Plant DNA kit (Bio-Nobile)

200 mg of tissue is homogenized in 1 mL of lysis buffer (from a QuickPick™ SML Plant DNA kit, Bio-Nobile) using a tissue homogenizer (e.g. FastPrep®-24, MP Biochemicals). An alternative grinding procedure is homogenization in extraction bags using a Homex 6 homogenizer (BIOREBA). Total DNA can be extracted using a QuickPick™ SML Plant DNA kit (Bio-Nobile) and a magnetic particle processor (e.g. KingFisher® mL, Thermo Scientific) as described by Mehle et al. (2013). Total DNA extract is eluted in 200 µL of elution buffer (from a QuickPick™ SML Plant DNA kit, Bio-Nobile). Undiluted DNA is suitable for testing.

##### 1.1.2 | DNA extraction using the Nucleomag Plant Kit (Macherey-Nagel)

The Nucleomag Plant Kit can be used in combination with a King-Fisher KF96 system for high throughput extraction or with a KingFisher mL or Biosprint (Qiagen) for 15 samples.

1 g of fresh or 20–50 mg of dried plant material is ground in 4.5 mL PBS-extraction buffer (see Appendix 3) by using homogenization bags and Homex 6 (Bioreba). An aliquot of 500 µL is taken and 500 µL of MC1 buffer (Macherey-Nagel, included in the kit)[2] added; DNA extraction is performed following the manufacturer's instructions. Total DNA is eluted in 100-µL MC6 Elution buffer (included in the kit).

[2]If samples contain large amount of RNA, the addition of 10 µL of RNase A solution (stock solution 12 mg/mL, included in the kit) to the MC1 lysis mixture is recommended.

#### 1.2 | CTAB

200 mg of tissue is ground using liquid nitrogen. The powder is rapidly transferred in an extraction bag and 2–3 mL of CTAB extraction buffer (see below) is added. Homogenize the sample using a wooden/plastic wallpaper roller or similar. Transfer the ground sap into a 1.5 mL microfuge tube. Incubate for 10–15 min at 65°C. Centrifuge for 5 min at 13 000 rpm. Transfer 700 µL of the supernatant into a new microcentrifuge tube. Extract with an equal volume (700 µL) of chloroform: IAA (24:1). Vortex at low speed for 2 s. Centrifuge at 13 000 rpm at ambient temperature for 10 min. Transfer the upper aqueous layer to a new microcentrifuge tube. The centrifugation step can be repeated to retrieve any residual aqueous phase. Precipitate with 0.5 volume of 5 M sodium chloride and an equal volume of ice-cold isopropanol. Incubate at −20°C overnight. Pellet the DNA by centrifugation at 13 000 rpm for 10 min (optionally, this centrifugation step can be performed at 4°C). Wash by addition of 500 µL of 70% ethanol and centrifuge for 5 min at 13 000 rpm. Carefully discard ethanol. Desiccate for 15 min to dry the pellet. Resuspend pellet in 100 µL of 1 × TE (see below).

#### 1.3 | DNeasy mericon Food Kit or DNeasy Plant Mini kit (Qiagen)

DNeasy mericon Food Kit and DNeasy Plant Mini kit (Qiagen) according to the manufacturer's instructions were shown to be suitable for the tests described in the appendices.

### 2 | DNA EXTRACTION FROM *BEMISIA TABACI* SPECIMENS

DNA can be extracted from single or pooled (up to 5) specimens of *B. tabaci*. If whiteflies have been stored in absolute ethanol after collection, it is recommended to transfer the specimens onto a Whatman paper to evaporate the ethanol before the DNA extraction. At least two DNA extraction methods were found to be suitable for the tests described in Appendices 7–9:

- The Xpert directXtract Lysis Buffer (GriSP). Grind each whitefly pool/specimen in a 1.5 mL vial with a micro-pestle in 50 µL of reaction mixture (35 µL PCR-grade dd$H_2O$, 10 µL Xpert directXtract buffer A, and 5 µL Xpert directXtract buffer B) and follow the manufacturer's instructions.
- QuickPick™ SML Plant DNA (Bio-Nobile) in combination with King Fisher (as described for plant material)

Other extraction methods may be used but should be validated in combination with the molecular test to be used. Extracted total DNA can be stored at -20°C.

## 3 | ROLLING CIRCLE AMPLIFICATION WITH XPERT DIRECTXTRACT LYSIS BUFFER

A DNA enrichment by means of Rolling Circle Amplification (RCA) can be performed for both plant material or insects before performing the tests described in Appendices 7–9. It has not been evaluated so far for tests described in Appendices 4–6. This pre-amplification step can be done using the TempliPhi kit (GE Healthcare) according to the manufacturer's instructions. A volume of 2 µL of total DNA is briefly mixed with 5 µL of Sample Buffer and then denatured at 95°C for 3 min. The samples are incubated for 2 h at 30°C followed by 10 min at 65°C. A volume of 2 µL of the RCA product is used for the molecular tests.

In addition, the RCA product can be used as template for sequencing based on primer walking strategy (Sanger sequencing) and/or for HTS. In the latter case it is recommended to clean-up the reaction mix to remove primers, dNTPs, enzymes and buffer components.

## APPENDIX 3 - BUFFERS

*PBS-extraction buffer*

| | |
|---|---|
| NaCl | 8.0 g |
| $Na_2HPO_4$ $12H_2O$ | 2.7 g |
| $NaH_2PO_4$ | 0.4 g |

Dissolve in 800 mL of distilled water, adjust volume to 1 L.

Adjust pH to 7.2.

*CTAB buffer*

| | |
|---|---|
| CTAB | 2% |
| Tris-HCl pH 8.0 | 100 mM |
| EDTA | 20 mM |
| NaCl | 1.4 M |
| $Na_2SO_3$ | 1% |
| PVP-40 | 2.0% |

Mix the first 4 reagents. Make up to 1 L with distilled water.

Store solution at ambient temperature. Add PVP and sodium sulphite fresh to aliquot of stock buffer (this will keep for about 2 weeks).

*TE buffer*

| | |
|---|---|
| Tris–HCl | 10 mM |
| EDTA | 1 mM |

Molecular grade water up to 1 L.

Adjust to pH 8.0.

## APPENDIX 4 - CONVENTIONAL PCR WYATT AND BROWN (1996)

*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 from the Euphresco TPS 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. This PCR is suitable for the detection of several begomoviruses in plant material.

1.2. The test is based on primers published by Wyatt and Brown (1996).

1.3. The target sequence is located on the core region of the capsid protein (CP) gene (reference isolates that were used to design the primers are listed in Wyatt & Brown, 1996).

1.4. Oligonucleotides:

| Primers | Sequence | Amplicon size |
|---|---|---|
| AV494 | 5′- GCC YAT RTA YAG RAA GCC MAG -3′ | 580 bp |
| AC1048 | 5′- GGR TTD GAR GCA TGH GTA CAT G -3′ | |

### 2. Methods

2.1. Nucleic Acid Extraction

2.1.1. DNA extraction methods that are described in Appendix 2 can be used.

2.2. Conventional PCR

2.2.1. Master Mix

| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
|---|---|---|---|
| Molecular grade water | N.A. | 14.8 | N.A. |
| Taq polymerase® buffer (*Invitrogen*) | 10× | 2.5 | 1× |
| $MgCl_2$ (*Invitrogen*) | 50 mM | 1.0 | 2 mM |
| dNTPs (*Invitrogen*) | 5 mM each | 0.5 | 0.1 mM |
| Forward primer AV494 | 10 µM | 2.0 | 0.8 µM |
| Reverse primer AC1048 | 10 µM | 2.0 | 0.8 µM |
| Platinum Taq® DNA polymerase (*Invitrogen*) | 10 U/µL | 0.2 | 2 U |
| Subtotal | | 23.0 | |
| Genomic DNA | | 2.0 | |
| Total | | 25.0 | |

Abbreviation: N.A., not applicable.

2.2.2. PCR conditions: 2 min at 94°C followed by 10 cycles (15 s at 94°C, 20 s at ramping 65°C −1°C to 56°C (annealing T down 1°C/cycle) and 30 s at

72°C), 30 cycles (15 s at 94°C, 20 s at 55°C, 30 s at 72°C) and 10 min at 72°C.

## 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 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, whole genome amplified DNA 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 external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- specific amplification or co-amplification of nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

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:

*Verification of the controls*

- NIC and NAC: no band is visualized,
- PIC, PAC (and if relevant IC): a band of 580 bp is visualized,
- IPC (if relevant) a band of the expected size is visualized.

*When these conditions are met*:

- A test will be considered positive if a band of 580 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

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).

### A) Validation data available from the Euphresco test performance study (project 2016-A-212 BegomoVal)

Validation was carried out in accordance with PM 7/98

#### A 4.1 Analytical specificity data

Inclusivity: all isolates of begomoviruses included in the study were detected by at least 5 of 7 participating laboratories.

Bipartite begomoviruses (isolates) included in the study: abutilon mosaic virus (AbMV, origin: the Netherlands, collection: ANSES), African cassava mosaic virus (ACMV, collection: DSMZ PV-0421), bean golden mosaic virus (BGMV, collection: DSMZ PV-0094), pepper golden mosaic virus (PepGMV, origin: Mexico, collection: ANSES), potato yellow mosaic virus (PYMV, origin: Martinique, collection: ANSES), tomato leaf curl New Delhi virus ($10^{-2}$ dilution of ToLCNDV, origin: Spain, collection: ANSES), tomato mottle virus (ToMoV, origin: Florida USA, collection: ANSES), tomato severe rugose virus (ToSRV, origin: Brazil, collection: ANSES), Sri Lankan cassava mosaic

virus (SLCMV, collection: DSMZ PV-0424), watermelon chlorotic stunt virus (WmCSV, collection: DSMZ PV-0830).

Monopartite begomoviruses (isolates: chilli leaf curl virus [ChiLCV, origin: India, collection: ANSES]), tomato yellow leaf curl virus (TYLCV, origin: Reunion Island and, Mauritius, collection: ANSES), $10^{-2}$ and $10^{-3}$ dilution of TYLCV (origin: New Caledonia, collection: ANSES); tomato leaf curl Comoros virus (ToLCYTV, origin: Mayotte, collection: ANSES), tomato yellow leaf curl Sardinia virus (TYLCSV, origin: Spain, collection: ANSES), tomato leaf curl Mali virus (ToLCMLV, origin: Senegal, collection: ANSES).

Exclusivity: 100%

Non-target viruses (isolates) included in the study: banana bunchy top virus (BBTV, origin: Reunion Island, collection: ANSES), maize streak virus (MSV, origin: Reunion Island, collection: ANSES), pea necrotic yellow dwarf virus (PNYDV, origin: Austria, collection: ANSES), pepino mosaic virus (CH2 PepMV, collection: ANSES), tomato chlorosis virus (ToCV, collection: ANSES).

**A 4.2 Data on Repeatability**
97.1%

**A 4.3 Data on Reproducibility**
85.8%

**A 4.4 Diagnostic sensitivity data**
93.5%

**A 4.5 Diagnostic specificity data**
100%

**B) Validation data from EURL Virology (NIB, SI)**

Validation was carried out in accordance with PM 7/98

**B 4.1 Analytical specificity data**
Inclusivity: test did not detect isolates of TYLCSV PV-0561 and ToLCNDV PV-1285, and gBlocks of SLCCNV and LYMV. All other isolates and gBlocks of begomoviruses included in the study were detected.

Begomoviruses (isolates) included in the study: Isolate of squash leaf curl virus (SLCuV) obtained from Israel (Volcani center, ARO) and nine begomovirus isolates from DSMZ collection (chayote yellow mosaic virus, ChaYMV: PV-0843; tomato leaf curl New Delhi virus, ToLCNDV: PV-1109, PV-1111 and PV-1285; tomato yellow leaf curl Sardinia virus, TYLCSV: PV-0561; tomato yellow leaf curl Thailand virus, TYLCTHV: PV-0952; tomato yellow leaf curl virus, TYLCV: PV-0844 and PV-0560; watermelon chlorotic stunt virus, WmCSV: PV-0830). In addition, ten gBlocks representing fragments of viral sequences of the core region of the capsid protein (CP) gene and parts located on the open reading frame AC2 and AC1 were included in the study (in brackets accession number of NCBI sequence used for designing the gBlock): chayote enation yellow mosaic virus (KX259339); squash leaf curl China virus, SLCCNV (MN437657); luffa yellow mosaic virus, LYMV (NC_004824); croton yellow vein mosaic virus, CroYVMV (LN878119); squash leaf curl Yunnan virus, SLCuYV (MN563794); cucumber chlorotic leaf virus (MN013786); tomato golden leaf spot virus, ToGLSV (NC_021579); tomato bright yellow mosaic virus, ToBYMV (NC_038467); tomato leaf curl Sulawesi virus, ToLCSuV (NC_013413); coccinia mosaic Tamil Nadu virus, CMTNV (NC_024810). These gBlocks have been tested in concentrations about 1 million copies per reaction.

**B 4.2 Diagnostic sensitivity data**
80%

## APPENDIX 5 - CONVENTIONAL PCR LI ET AL. (2004)

*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 from the Euphresco TPS 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 several species of begomoviruses in plant material.

1.2. The test is adapted from Li et al. (2004), originally developed for detection of begomoviruses in sweet potato.

1.3. The target sequence is located on the open reading frame AC2 and AC1 (locations of primers on the sequence of AF104036: 1490-1508 and 2412-2391; other reference sequences that were used to design primers are listed in Li et al., 2004).

1.4. Oligonucleotides:

| Primers | Sequence | Amplicon size |
|---|---|---|
| SPG1 | 5′- CCC CKG TGC GWR AAT CCA T -3′ | 912 bp |
| SPG2 | 5′- ATC CVA AYW TYC AGG GAG CTA A -3′ | |

### 2. Methods

2.1. Nucleic Acid Extraction

2.1.1. DNA extraction methods that are described in Appendix 2 may be used.

2.2. Conventional PCR

2.2.1. Master Mix

| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
|---|---|---|---|
| Molecular grade water | N.A. | 22.76 | N.A. |
| Taq polymerase® buffer (*Invitrogen*) | 10× | 3.00 | 1× |
| $MgCl_2$ (*Invitrogen*) | 50 mM | 1.20 | 2 mM |
| dNTPs (*Invitrogen*) | 5 mM each | 0.60 | 0.1 mM |
| Forward primer SPG1 | 10 µM | 0.60 | 0.2 µM |
| Reverse primer SPG2 | 10 µM | 0.60 | 0.2 µM |
| Platinum Taq® DNA polymerase (*Invitrogen*) | 10 U/µL | 0.24 | 2.4 U |
| Subtotal | | 29.00 | |
| Genomic DNA | | 1.00 | |
| Total | | 30.00 | |

Abbreviation: N.A., not applicable.

2.2.2. PCR conditions: 2 min at 94°C followed by 11 cycles (40 s at 94°C, 40 s at ramping 61°C +1°C to 71°C (annealing T up 1°C/cycle) and 90 s at 72°C), 24 cycles (40 s at 94°C, 40 s at 60°C, 90 s at 72°C) and 10 min at 72°C.

## 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 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, whole genome amplified DNA 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 external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- specific amplification or co-amplification of nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

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:

*Verification of the controls*

- NIC and NAC: no band is visualized,
- PIC, PAC (and if relevant IC): a band of 912 bp is visualized,
- IPC (if relevant) a band of the expected size is visualized.

*When these conditions are met*:

- A test will be considered positive if a band of 912 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**

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).

**A) Validation data available from the Euphresco test performance study (project 2016-A-212 BegomoVal)**

Validation was carried out in accordance with PM 7/98

**A 4.1 Analytical specificity data**

Inclusivity: all isolates of begomoviruses included in the study (see below), except ToLCMLV and TYLCV-IL-NC (Israel strain, isolate from New Caledonia) were detected by participating laboratories. ACMV, TYLCV (Israel strain, isolate from Reunion Island), SLCMV and WmCSV were detected by less than 5 of 7 participating laboratories; while all other isolates of begomoviruses included in the study were detected by at least 5 of 7 participating laboratories.

Bipartite begomoviruses (isolates) included in the study: abutilon mosaic virus (AbMV, origin: the Netherlands, collection: ANSES), african cassava mosaic virus (ACMV, collection: DSMZ PV-0421), bean golden mosaic virus (BGMV, collection: DSMZ PV-0094), pepper golden mosaic virus (PepGMV, origin: Mexico, collection: ANSES), potato yellow mosaic virus (PYMV, origin: Martinique, collection: ANSES), tomato leaf curl New Delhi virus ($10^{-2}$ dilution of ToLCNDV, origin: Spain, collection: ANSES), tomato mottle virus (ToMoV, origin: Florida USA, collection: ANSES), tomato severe rugose virus (ToSRV, origin: Brazil, collection: ANSES), Sri Lankan cassava mosaic virus (SLCMV, collection: DSMZ PV-0424), watermelon chlorotic stunt virus (WmCSV, collection: DSMZ PV-0830).

Monopartite begomoviruses (isolates) included in the study: chilli leaf curl virus (ChiLCV, origin: India, collection: ANSES), tomato yellow leaf curl virus (TYLCV, origin: Reunion Island and Mauritius, collection: ANSES), $10^{-2}$ and $10^{-3}$ dilution of TYLCV (origin: New Caledonia, collection: ANSES), tomato leaf curl Comoros virus (ToLCYTV, origin: Mayotte, collection: ANSES), tomato yellow leaf curl Sardinia virus (TYLCSV, origin: Spain, collection: ANSES), tomato leaf curl Mali virus (ToLCMLV, origin: Senegal, collection: ANSES).

Exclusivity: 100%

Non-target viruses (isolates) included in the study: banana bunchy top virus (BBTV, origin: Reunion Island, collection: ANSES), maize streak virus (MSV, origin: Reunion Island, collection: ANSES), pea necrotic yellow dwarf virus (PNYDV, origin: Austria, collection: ANSES), pepino mosaic virus (CH2 PepMV, collection: ANSES), tomato chlorosis virus (ToCV, collection: ANSES).

**A 4.2 Data on Repeatability**

100%

**A 4.3 Data on Reproducibility**

82.2%

**A 4.4 Diagnostic sensitivity data**

53.9%

**A 4.5 Diagnostic specificity data**

100%

**B) Validation data available from EURL Virology (NIB, SI)**

Validation was carried out in accordance with PM 7/98

**B 4.1 Analytical specificity data**

Inclusivity: test did not detect isolates of SLCuV and WmCSV PV-0830, and gBlocks of CCLV, ToGLSV and ToLCSuV. All other isolates and gBlocks of begomoviruses included in the study were detected.

Begomoviruses (isolates) included in the study: Isolate of squash leaf curl virus (SLCuV) obtained from Israel (Volcani center, ARO) and nine begomovirus isolates from DSMZ collection (chayote yellow mosaic virus, ChaYMV: PV-0843; tomato leaf curl New Delhi virus, ToLCNDV: PV-1109, PV-1111 and PV-1285; tomato yellow leaf curl Sardinia virus, TYLCSV: PV-0561; tomato yellow leaf curl Thailand virus, TYLCTHV: PV-0952; tomato yellow leaf curl virus, TYLCV: PV-0844 and PV-0560; watermelon chlorotic stunt virus, WmCSV: PV-0830) were used in the study. DNA from those isolates were extracted using QuickPick™ SML Plant DNA kit (Bio-Nobile) and a magnetic particle processor. In addition, ten gBlocks fragments of viral sequences of the core region of the capsid protein (CP) gene and parts located on the open reading frame AC2 and AC1 were included in the study (in brackets accession number of NCBI sequence used for designing the gBlock is given): chayote enation yellow mosaic virus, CEYMV (KX259339);

squash leaf curl China virus, SLCCNV (MN437657); luffa yellow mosaic virus, LYMV (NC_004824); croton yellow vein mosaic virus, CroYVMV (LN878119); squash leaf curl Yunnan virus, SLCuYV (MN563794); cucumber chlorotic leaf virus, CCLV (MN013786); tomato golden leaf spot virus, ToGLSV (NC_021579); tomato bright yellow mosaic virus, ToBYMV (NC_038467); tomato leaf curl Sulawesi virus, ToLCSuV (NC_013413); coccinia mosaic Tamil Nadu virus, CMTNV (NC_024810). These gBlocks have been tested in two concentrations: about 1 million copies per reaction and about 1000 copies per reaction.

**B 4.2 Diagnostic sensitivity data**

75% when including data of gBlocks tested at a concentration of about 1 million copies per reaction; 65% when including data of gBlocks tested at a concentration of about 1000 copies per reaction.

## APPENDIX 6 - CONVENTIONAL PCR SAISON AND GENTIT (2015)

*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 from the Euphresco TPS 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. This PCR is suitable for detection of several species of begomoviruses in plant material.

1.2. The test is based on Saison and Gentit (2015).

1.3. The target sequence is located on the core region of the capsid protein (CP) gene and the amplicon size can differ depending on the begomovirus species. For WmCSV (DNA-B), the primers are located at position 905-922 and 1764-1745 of sequence KC462553 (860 bp). For other begomoviruses (DNA-A), the primers are located at position 459-476 and 1040-1021 of sequence HM448447 (580 bp).

1.4. Oligonucleotides:

| Primers | Sequence | Amplicon size |
|---|---|---|
| Beg-CP-F | 5′-GCC CAT GTA YMG RAA RCC-3′ | 580 bp or 860 bp |
| Beg-580-R | 5′-GGR TTA GAR GCA TGM GTA CA-3′ | |

### 2. Methods

2.1. Nucleic Acid Extraction.

2.1.1. DNA extraction methods that are described in Appendix 2 may be used.

2.2. Conventional PCR.

2.2.1. Master Mix.

| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
|---|---|---|---|
| Molecular grade water | N.A. | 17.50 | N.A. |
| Taq polymerase® buffer (*Invitrogen*) | 10× | 2.50 | 1× |
| $MgCl_2$ (*Invitrogen*) | 50 mM | 0.80 | 1.6 mM |
| dNTPs (*Invitrogen*) | 5 mM each | 0.50 | 0.1 mM |
| Forward primer Beg-CP-F | 10 µM | 0.75 | 0.3 µM |
| Reverse primer Beg-580-R | 10 µM | 0.75 | 0.3 µM |
| Platinum Taq® DNA polymerase (*Invitrogen*) | 10 U/µL | 0.20 | 2 U |
| Subtotal | | 23.00 | |
| Genomic DNA | | 2.00 | |
| Total | | 25.00 | |

Abbreviation: N.A., not applicable.

2.2.2. PCR conditions: 3 min at 94°C followed by 35 cycles (30 s at 94°C, 35 s at 58°C, 30 s at 72°C) and 7 min at 72°C.

### 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 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, whole genome amplified DNA 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 external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- specific amplification or co-amplification of nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

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:

*Verification of the controls*

- NIC and NAC: no band is visualized,
- PIC, PAC (and if relevant IC): a band of 580 bp or 860 bp is visualized,
- IPC (if relevant) a band of the expected size is visualized.

*When these conditions are met*:

- A test will be considered positive if a band of 580 bp or 860 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**

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).

**A) Validation data available from the Euphresco test performance study (project 2016-A-212 Begomoval)**

Validation was carried out in accordance with PM 7/98

**A 4.1 Analytical specificity data**

Inclusivity: all isolates of begomoviruses included in the study were detected by at least 5 of 7 participating laboratories.

Bipartite begomoviruses (isolates) included in the study: abutilon mosaic virus (AbMV, origin: the Netherlands, collection: ANSES), african cassava mosaic virus (ACMV, collection: DSMZ PV-0421), bean golden mosaic virus (BGMV, collection: DSMZ PV-0094), pepper golden mosaic virus (PepGMV, origin: Mexico, collection: ANSES), potato yellow mosaic virus (PYMV, origin: Martinique, collection: ANSES), tomato leaf curl New Delhi virus ($10^{-2}$ dilution of ToLCNDV, origin: Spain, collection: ANSES), tomato mottle virus (ToMoV, origin: Florida USA, collection: ANSES), tomato severe rugose virus (ToSRV, origin: Brazil, collection: ANSES), Sri Lankan cassava mosaic virus (SLCMV, collection: DSMZ PV-0424), watermelon chlorotic stunt virus (WmCSV, collection: DSMZ PV-0830).

Monopartite begomoviruses (isolates) included in the study: chilli leaf curl virus (ChiLCV, origin: India; collection: ANSES), tomato yellow leaf curl virus (TYLCV, origin: Reunion Island and Mauritius, collection: ANSES), $10^{-2}$ and $10^{-3}$ dilution of TYLCV (origin: New Caledonia, collection: ANSES), tomato leaf curl Comoros virus (ToLCYTV, origin: Mayotte, collection: ANSES), tomato yellow leaf curl Sardinia virus (TYLCSV, origin: Spain, collection: ANSES), tomato leaf curl Mali virus (ToLCMLV, origin: Senegal, collection: ANSES).

Exclusivity: 100%

Non-target viruses (isolates) included in the study: banana bunchy top virus (BBTV, origin: Reunion Island, collection: ANSES), maize streak virus (MSV, origin: Reunion Island, collection: ANSES), pea necrotic yellow dwarf virus (PNYDV, origin: Austria, collection: ANSES), pepino mosaic virus (CH2 PepMV, collection: ANSES), tomato chlorosis virus (ToCV, collection: ANSES).

**A 4.2 Data on Repeatability**

97.4%

**A 4.3 Data on Reproducibility**

87.6%

**A 4.4 Diagnostic sensitivity data**

87.0%

**A 4.5 Diagnostic specificity data**

100%

**B) Validation data available from EURL Virology (NIB, SI)**

Validation was carried out in accordance with PM 7/98

**B 4.1 Analytical specificity data**

Inclusivity: test did not detect gBlocks of SLCCNV and LYMV, and the probability of detection of the isolate ToLCNDV PV1285 was 50% (half replicates positive and half negative). All other isolates and gBlocks of begomoviruses included in the study were detected.

Begomoviruses (isolates) included in the study: Isolate of squash leaf curl virus (SLCuV) obtained from Israel (Volcani center, ARO) and nine begomovirus isolates from DSMZ collection (chayote yellow mosaic virus, ChaYMV: PV-0843; tomato leaf curl New Delhi virus, ToLCNDV: PV-1109, PV-1111 and PV-1285; tomato yellow leaf curl Sardinia virus, TYLCSV: PV-0561; tomato yellow leaf curl Thailand virus, TYLCTHV: PV-0952; tomato yellow leaf curl virus, TYLCV: PV-0844 and PV-0560; watermelon chlorotic stunt virus, WmCSV: PV-0830) were used in the study. DNA from those isolates were extracted using QuickPick™ SML Plant DNA kit (Bio-Nobile) and a magnetic particle processor. In addition, ten gBlocks fragments of viral sequences of the core region of the capsid protein (CP) gene and parts located on the open reading frame AC2 and AC1 were included in the study (in brackets accession number of NCBI sequence used for designing the gBlock is given): chayote enation yellow mosaic virus, CEYMV (KX259339); squash leaf curl China virus, SLCCNV (MN437657); luffa yellow mosaic virus, LYMV (NC_004824); croton yellow vein mosaic virus, CroYVMV (LN878119); squash leaf curl Yunnan virus, SLCuYV (MN563794); cucumber chlorotic leaf virus, CCLV (MN013786); tomato golden leaf spot virus, ToGLSV (NC_021579); tomato bright yellow mosaic virus, ToBYMV (NC_038467); tomato leaf curl Sulawesi virus, ToLCSuV (NC_013413); coccinia mosaic Tamil Nadu virus, CMTNV (NC_024810). These gBlocks have been tested in two concentrations: about 1 million copies per reaction and about 1000 copies per reaction.

**B 4.2 Diagnostic sensitivity data**

85% when including data of gBlocks tested at a concentration of about 1 million copies per reaction; 60% when including data of gBlocks tested at a concentration of about 1000 copies per reaction

## APPENDIX 7 - REAL-TIME PCR SIMON ET AL. (2018)

*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 tomato leaf curl New Delhi virus in plants and in *B. tabaci*.

1.2. The test is based on primers and probe published by Simon et al. (2018).

1.3. The target sequence is located on AV2 gene in the DNA-A (reference sequences: KF749223, KF749224, KF749225, KF891468, KM9777733), position 129-238.

1.4. Oligonucleotides:

| Primers/probe | Sequence |
|---|---|
| ToLA-up | 5′-CAT TAT TGC ACG AAT TTC CG-3′ |
| ToLA-low | 5′-ATC GTA GCC GAC TGT GTC TG-3′ |
| ToLA-probe | CY5[a]-CAT GCA CCT TAG ACC ATG GAC GCT-BHQ2 |

[a] Can be replaced by the combination FAM/BHQ1.

**2. Methods**

2.1. Nucleic acid extraction.

2.1.1. DNA extraction methods that are described in Appendix 2 may be used.

2.2. Real-time PCR.

2.2.1. Master mix

| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
|---|---|---|---|
| Molecular grade water | N.A. | 7.7 | N.A. |
| TaqMan™ Universal PCR Master Mix (Life technologies) | 2× | 10 | 1× |
| Forward primer ToLA-up | 10 µM | 0.1 | 0.05 µM |
| Reverse primer ToLA-low | 10 µM | 0.1 | 0.05 µM |
| ToLA-probe | 10 µM | 0.1 | 0.05 µM |
| Subtotal | | 18.00 | |
| Genomic DNA | | 2.00 | |
| Total | | 20.00 | |

Abbreviation: N.A., not applicable.

2.2.2. Real-time PCR conditions: initial denaturation at 95°C for 10 min; 40 cycles consisting of 15 s at 95°C and 1 min at 60°C.

## 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 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 external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- specific amplification or co-amplification of nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

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:

*Verification of the controls*

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

*When these conditions are met*:

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

## 4. Performance Characteristics Available

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).

### A) Validation data available EURL Virology (CREA-DC, IT)

Validation was carried out in accordance with PM 7/98

#### A 4.1 Analytical specificity data

Inclusivity: 100%

The isolates of tomato leaf curl New Delhi virus included in the study were: ToLCNDV DSMZ isolate (PV1109), ToLCNDV DSMZ isolate (PV1111), ToLCNDV Italian isolate on *Cucurbita pepo* (CREA 102), ToLCNDV Italian isolate on *Capsicum annuum* (CREA 126), Italian isolate of ToLCNDV (CREA 102) in *Bemisia tabaci* sample.

Exclusivity: 100%

Non-target viruses (isolates) included in the study: tomato yellow leaf curl virus (TYLCV) (M; IL) (CREA collection), tomato yellow leaf curl Sardinia virus (TYLCSV) (CREA collection), tomato yellow leaf curl Thailand virus (TYLCTHV) (DSMZ collection PV0952); squash leaf curl virus (SLCuV) (DSMZ collection PC1271); watermelon chlorotic stunt virus (WmCSV) (DSMZ collection PV0830), chayote yellow mosaic virus (ChaYMV) (DSMZ collection PV0843). In the framework of in-house validation CREA-DC (IT) and the European Union Reference Laboratory PT no cross-reaction was observed. In the framework of the EURL TPS, cross reaction was observed with high-titre

TYLCV samples (see diagnostic specificity for plant samples)

**B) Validation data from EURL Virology test performance study (plant samples). Three laboratories participated in the TPS**

Validation was carried out in accordance with PM 7/98

**B 4.1 Diagnostic sensitivity data**
100%

**B 4.2 Diagnostic specificity data**
60%

**B 4.3 Data on Repeatability**
100%

**B 4.4 Data on Reproducibility**
100%

**C) Validation data from EURL Virology test performance study (*B. tabaci* samples*). Three laboratories participated in the TPS**

Validation was carried out in accordance with PM 7/98

**C 4.1 Diagnostic sensitivity data**
100%

**C 4.2 Diagnostic specificity data**
100%

**C 4.3 Data on Repeatability**
100%

**C 4.4 Data on Reproducibility**
100%

## APPENDIX 8 - REAL-TIME PCR FROM LUIGI ET AL. (2020)

*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 specific detection and identification of tomato leaf curl New Delhi virus in plants and in *B. tabaci*.

1.2. The test is based on primers and probe published by Luigi et al. (2020).

1.3. The target sequence is located on the BC1 gene in the DNA-B (reference sequence: NC_004612.1), position 1800–1890.

1.4. Oligonucleotides:

| Primers/probe | Sequence |
|---|---|
| B-Rev | 5′-CAA-GCA-GAA-TTC-ACA-ATT-CCA-ATC-3′ |
| B-Fow | 5′-TCC-AAG-GAT-TCT-TAT-CCT-TKA-GAG-AG-3′ |
| B-Probe | HEX[a]- TGA GGA AGA GTA GTA GTG GAG GTT ACA GT -BHQ1 |

[a] Can be replaced by the combination FAM/BHQ1.

### 2. Methods

2.1. Nucleic acid extraction

2.1.1. DNA extraction methods that are described in Appendix 2 may be used.

2.2. Real-time PCR

2.2.1. Master mix

| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
|---|---|---|---|
| Molecular grade water | N.A. | 7.7 | N.A. |
| TaqMan™ Universal PCR Master Mix (Life technologies) | 2× | 10 | 1× |
| Forward primer B-Rev | 10 µM | 0.1 | 0.05 µM |
| Reverse primer B-Fow | 10 µM | 0.1 | 0.05 µM |
| B-Probe | 10 µM | 0.1 | 0.05 µM |
| Subtotal | | 18.00 | |
| Genomic DNA | | 2.00 | |
| Total | | 20.00 | |

Abbreviation: N.A., not applicable.

2.2.2. Real-time PCR conditions: initial denaturation at 95°C for 10 min; 40 cycles consisting of 15 s at 95°C and 1 min at 60°C.

### 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 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.

*with or without preliminary RCA enrichment step.

- 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 external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- specific amplification or co-amplification of nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

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:

*Verification of the controls*

- The PIC and PAC (as well as IC and IPC) 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**

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).

**A) Validation data available from EURL Virology (CREA-DC, IT)**

Validation was carried out in accordance with PM 7/98

**A 4.1 Analytical specificity data**

Inclusivity: 100%

The isolates of tomato leaf curl New Delhi virus included in the study were: ToLCNDV DSMZ isolate (PV1109), ToLCNDV DSMZ isolate (PV1111), ToLCNDV Italian isolate on *Cucurbita pepo* (CREA 102), ToLCNDV Italian isolate on *Capsicum annuum* (CREA 126), Italian isolate of ToLCNDV (CREA 102) in *Bemisia tabaci* sample.

Furthermore, *in silico* analysis confirms that all ToLCNDV isolates from the Mediterranean region should be detected by this test.

Exclusivity: 100%

Non-target viruses (isolates) included in the study: tomato yellow leaf curl virus (TYLCV) (M; IL) (CREA collection), tomato yellow leaf curl Sardinia virus (TYLCSV) (CREA collection), tomato yellow leaf curl Thailand virus (TYLCTHV) (DSMZ collection PV0952); squash leaf curl virus (SLCuV) (DSMZ collection PC1271); watermelon chlorotic stunt virus (WmCSV) (DSMZ collection PV0830), chayote yellow mosaic virus (ChaYMV) (DSMZ collection PV0843). Furthermore, *in silico* analysis was also performed. As the primers used for this test are specific for the DNA B, that is not present in the majority of the other begomoviruses, the data provided is sufficient to exclude cross-reactions with other begomoviruses spp.

**B) Validation data from EURL Virology test performance study (plant samples). Three laboratories participated in the TPS**

Validation was carried out in accordance with PM 7/98

**B 4.1 Diagnostic sensitivity data**

82%

**B 4.2 Diagnostic specificity data**

100%

**B 4.3 Data on Repeatability**
96%

**C) Validation data from EURL Virology test performance study (*B. tabaci* samples*). Three laboratories participated in the TPS**

Validation was carried out in accordance with PM 7/98

**C 4.1 Diagnostic sensitivity data**
83%

**C 4.2 Diagnostic specificity data**
100%

**C 4.3 Data on Repeatability**
100%

**C 4.4 Data on Reproducibility**
89%

## APPENDIX 9 - LAMP JEEVALATHA ET AL. (2018)

*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 following LAMP protocol is performed for the specific detection and identification of tomato leaf curl New Delhi virus in plants and in *B. tabaci*.

1.2. The test is based on primers published by Jeevalatha et al. (2018). The primers were designed based on the complete coat protein gene sequences available in GenBank (KC205219, KC205286, KC205239, KC205217, KC205284, KC205223, KC205285 and KC205257).

1.3. Oligonucleotides:

| Primers/probe | Sequence |
|---|---|
| LCV-F3 | 5′-GAA AAC ATC AAG ACA AAA AAC CA-3′ |
| LCV-B3 | 5′-CTT AGA TGC ATA TGT TCC TCC-3′ |
| LCV-FIP | 5′-TGT CAA ACA TGT TAA AAA CTT CCC CAA CAG TGT CAT GTT TTT TTT GGT-3′ |
| LCV-LF | 5′-GAG ATC CTG TAG GAC GAC GG-3′ |
| LCV-LB | 5′-CGT GAT CGT TAT CAA GTC TTA CGG-3′ |
| LCV-BIP | 5′-GAG CAC AGC AAC GGT GAA GAG TCA CAG TAG CAT GCC AC-3′ |

1.4. LAMP reactions must be performed in equipment dedicated to LAMP isothermal amplification (e.g. Genie® II - OptiGene) or in real-time PCR equipment. If real-time PCR equipment is used, it is necessary to activate the detection of fluorescent emissions corresponding to the FAM or SYBR Green channel to measure the fluorescence every 30 s during the 45 min step and to plot the annealing curves.

*with or without preliminary RCA enrichment step.

**2. Methods**

2.1. Nucleic acid extraction

2.1.1. DNA extraction methods that are described in Appendix 2 may be used.

2.2. Real-time PCR

2.2.1. Master mix

| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
|---|---|---|---|
| Molecular grade water | N.A. | 1 | N.A. |
| Isothermal Master Mix (OptiGene, ISO-001) | N.A. | 15 | N.A. |
| LCV-F3 | 10 µM | 0.5 | 0.2 µM |
| LCV-B3 | 10 µM | 0.5 | 0.2 µM |
| LCV-FIP | 100 µM | 1 | 4 µM |
| LCV-BIP | 100 µM | 1 | 4 µM |
| LCV-LF | 100 µM | 0.5 | 2 µM |
| LCV-LB | 100 µM | 0.5 | 2 µM |
| Subtotal | | 20.00 | |
| Genomic DNA | | 5.00 | |
| Total | | 25.00 | |

Abbreviation: N.A., not applicable.

2.2.2. LAMP amplification conditions: 65°C for 45 min; melting curve analysis: 98°C–80°C, 0.1°C per second.

**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 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.

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:

*Verification of the controls*
- The PIC and PAC (as well as IC and IPC) amplification curves should be exponential. The Tm (melting temperature) should be between 84.0 and 87.0°C when samples are analysed on real-time CFX96 Bio-rad. Similar Tm range is expected when analysed on any other device, but it needs to be verified.
- 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 and specific Tm.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential or Tm is not specific.
- Tests should be repeated if any contradictory or unclear results are obtained.

**4. Performance Characteristics Available**

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).

**A) Validation data available from EURL Virology CREA-DC (IT)**

Validation was carried out in accordance with PM 7/98

**A 4.1 Analytical specificity data**

Inclusivity: 100%

The isolates of tomato leaf curl New Delhi virus included in the study were: ToLCNDV DSMZ isolate (PV1109), ToLCNDV DSMZ isolate (PV1111), ToLCNDV Italian isolate on *Cucurbita pepo* (CREA 102), ToLCNDV Italian isolate on *Capsicum annuum* (CREA 126), Italian isolate of ToLCNDV (CREA 102) in *Bemisia tabaci* sample.

Exclusivity: 100%

Non-target viruses (isolates) included in the study: tomato yellow leaf curl virus (TYLCV) (M; IL) (CREA collection), tomato yellow leaf curl Sardinia virus (TYLCSV) (CREA collection), tomato yellow leaf curl Thailand virus (TYLCTHV) (DSMZ collection PV0952); squash leaf curl virus (SLCuV) (DSMZ collection PC1271); watermelon chlorotic stunt virus (WmCSV) (DSMZ collection PV0830), chayote yellow mosaic virus (ChaYMV) (DSMZ collection PV0843).

**B) Validation data from EURL Virology test performance study (plant samples). Three laboratories participated in the TPS**

Validation was carried out in accordance with PM 7/98

**B 4.1 Diagnostic sensitivity data**

82%

**B 4.2 Diagnostic specificity data**

93%

**B 4.3 Data on Repeatability**

81%

**B 4.4 Data on Reproducibility**

74%

**C) Validation data from EURL Virology test performance study (*B. tabaci* samples*). Three laboratories participated in the TPS**

Validation was carried out in accordance with PM 7/98

**C 4.1 Diagnostic sensitivity data**

67%

**C 4.2 Diagnostic specificity data**

100%

**C 4.3 Data on Repeatability**

100%

**C 4.4 Data on Reproducibility**

78%

**with or without preliminary RCA enrichment step.*