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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/132 (1)

Diagnostics
Diagnostic

PM 7/132 (1) *Andean potato latent virus* and *Andean potato mild mosaic virus*

Specific scope

This Standard describes a diagnostic protocol for *Andean potato latent virus* and *Andean potato mild mosaic virus*[1]

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

Specific approval and amendment

Approved in 2018-09.

1. Introduction

Andean potato latent virus (APLV) and *Andean potato mild mosaic virus* (APMMV) belong to the genus *Tymovirus* (Gibbs *et al.*, 1966). Both viruses have a single-stranded RNA genome of 6.0–6.7 kb in size and consist of isometric particles of about 30 nm in diameter. In the past, host range and serological characteristics were the main criteria for species demarcation within the genus *Tymovirus* (Gibbs *et al.*, 1966; Koenig *et al.*, 1979). Koenig *et al.* (2005) showed that these criteria have their limitations and stated that the molecular criteria appeared to be the most reliable and comprehensive for species demarcation. These criteria have been described in the ninth ICTV report (Dreher *et al.*, 2012). For APLV three major serological strain groups were recognized, CCC, Col-Cay and Hu (Fribourg *et al.*, 1977; Koenig *et al.*, 1979). Recently, however, Kreuze *et al.* (2013) have suggested that APLV should be subdivided into two species, APLV and APMMV based on comparison of the complete genomic RNA sequences of Hu and Col isolates. The name *Andean potato mild mosaic virus* is now accepted for the former isolates of the Hu group.

The natural host range of APLV and APMMV is narrow, including potato (*Solanum tuberosum*) and ulluco (*Ullucus tuberosus)* (Lizarraga *et al.*, 1996). In addition, APMMV has been found once in plants of *Solanum acaule* grown from a seed accession from a gene bank (Roenhorst & Verhoeven, 1998). The main means of transmission is transmission by plant to plant contact and via machinery. The virus is also transmitted by insect vectors of the genus *Epitrix* (flea beetles) and through true potato seed (Jones & Fribourg, 1977). APLV and APMMV are restricted to the Andean region of South America, although they have occasionally been reported in gene collections outside this region.

This protocol focuses on the detection and identification of APLV and APMMV in leaf material. It has not been validated for testing true seed or tubers.

A flow diagram describing the procedure for detection and identification of APLV and APMMV is presented in Fig. 1.

2. Identity

Name: *Andean potato latent virus*
Acronym: APLV
Taxonomic position: Viruses: *Tymovirales*: *Tymoviridae*: *Tymovirus*
EPPO Code: APLV00
Phytosanitary categorization: EU IA1

Name: *Andean potato mild mosaic virus*
Formerly Andean *potato latent virus* Hu
Acronym: APMMV
Taxonomic position: Viruses: *Tymovirales*: *Tymoviridae*: *Tymovirus*
EPPO Code: APMMV0
Phytosanitary categorization: EU IA1

[1]The use of brand names of chemicals or equipment in this EPPO Standard implies no approval of them to the exclusion of others that may also be suitable.

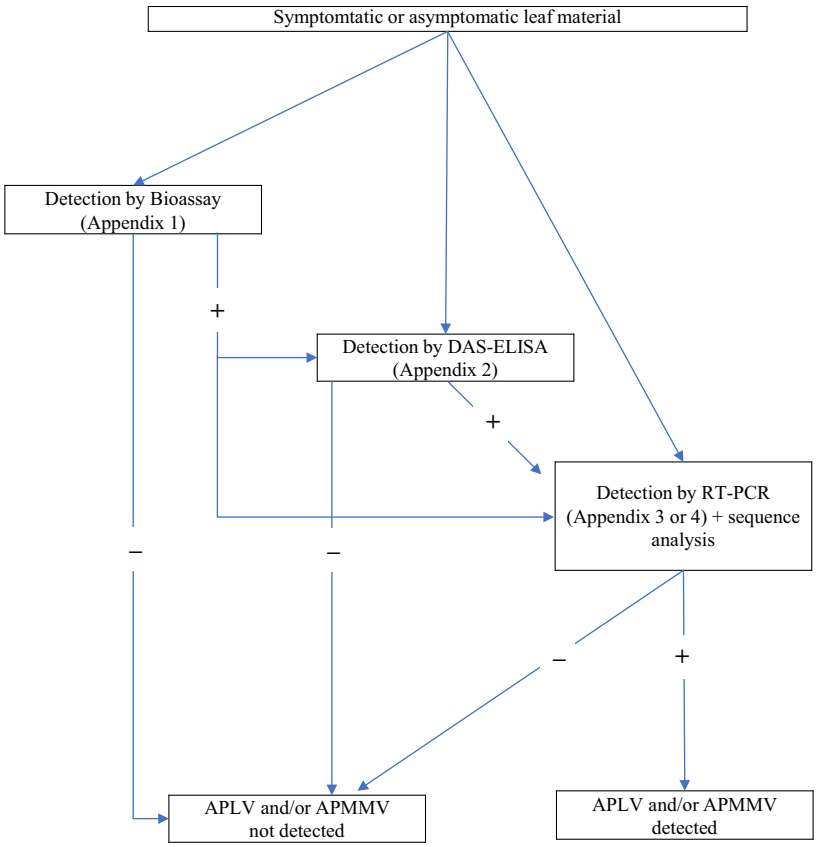

Fig. 1 Flow diagram for the detection and identification of APLV and APMMV in leaf material (bioassay, DAS-ELISA, RT-PCR). Note that validation data is limited; therefore, additional validation might be required in relation to the use of individual tests (Roenhorst *et al.*, 2018). [Colour figure can be viewed at wileyonlinelibrary.com]

3. Detection

Given that the geographical distribution of APLV and APMMV is restricted to the Andean region of South America, detection of these viruses will most likely take place during post-entry quarantine testing of material from this region or during screening of gene bank material originally collected from this region. Reports of these viruses outside this region are limited to *Solanum* spp. from gene banks.

3.1. Symptoms

The natural host range of APLV and APMMV is narrow, including potato (*Solanum tuberosum*), *Solanum acaule* and ulluco (*Ullucus tuberosus*) (Lizarraga *et al.*, 1996; Roenhorst & Verhoeven, 1998). Disease symptoms can be used as an indication of the presence of APLV, APMMV and related viruses (Jones & Fribourg, 1978). Symptoms depend on the virus species, strain, potato cultivar and climatic conditions. Usually, APLV is latent in *Solanum* spp., but occasionally it causes chlorotic netting of minor veins or mild or even severe mosaic symptoms (Fribourg *et al.*, 1977). APMMV does not usually cause symptoms, but in rare cases, it induces mild mosaic symptoms (Kreuze *et al.*, 2013).

3.2. Sampling

Fully expanded leaflets should be sampled from the top and the midway position of each stem of a plant of approximately 25 cm in height. Leaf material from 5 plants can be bulked together since both viruses usually reach relatively high concentrations at this stage. In the case of true seed, seeds should be germinated and grown up for testing. For microplants whole stems of at least 4–6-week-old plants should be sampled (Jeffries, 1998).

3.3. Screening tests

The following tests are recommended for detection of APLV and APMMV.

3.3.1. Bioassay

Mechanical inoculation into herbaceous test plants is a simple method for virus detection. The strength of a biological assay is that it will potentially detect a broader range of *Tymovirus* species and strains, thus providing an excellent

screening tool. The combination of *Nicotiana benthamiana*, *Nicotiana hesperis* 67A and *Nicotiana occidentalis* P1 has been shown to allow the detection of all isolates of APLV and APMMV tested so far (Verhoeven & Roenhorst, 2000). Symptoms on test plants, however, do not enable identification of the species. The bioassay is described in Appendix 1.

3.3.2. Double antibody sandwich (DAS)-ELISA

DAS-ELISA is the preferred method for detection of APLV and APMMV. Antisera are available from different suppliers, i.e. DSMZ and Prime Diagnostics. The polyclonal antiserum from Prime Diagnostics has been validated for the screening of leaf material of *N. occidentalis* P1 and *S. tuberosum*. This antiserum was found to react not only with all available isolates of APLV and APMMV but also with other related tymoviruses. Since more specific antisera might not detect all strains, it is important to address specificity in the validation of serological tests. Further details on DAS-ELISA can be found in EPPO Standard PM 7/125 *ELISA tests for viruses* (EPPO, 2015a) and Appendix 2.

3.3.3. Molecular tests

Two conventional RT-PCR tests have been described that can be used for generic detection of tymoviruses, including APLV and APMMV (Kreuze *et al.*, 2013). Primers EM13 and EM14, amplifying part of the replicase and putative movement protein genes, are derived from the type strain of *Eggplant mosaic virus* (EMV; GenBank accession no. NC_001480). The sequences of these primers are highly conserved in the respective regions of tymoviruses. Therefore they are likely to detect not only EMV, APLV and APMMV isolates but also most, if not all, other potato-infecting tymoviruses such as *Belladonna mottle virus* (Salazar, 1996). Primers EM2 and EM3, derived from conserved regions upstream and downstream of the EMV coat protein gene, will also allow generic detection of all known strains of APLV, APMMV, EMV and possibly other related tymoviruses. Both RT-PCR tests are described in Appendices 3 and 4, respectively. Note that neither of these tests has been validated for detection of APLV and APMMV thus far.

3.3.4. Other tests

Immunoelectron microscopy (IEM) can give an indication of the presence of APLV and APMMV in plant sap and is described in EPPO Standard PM 7/126 *Electron microscopy in diagnosis of plant viruses* (EPPO, 2015b). The source of antibodies is critical because of differences in specificity. Antisera are described in Appendix 2.

4. Identification

Molecular tests are recommended for identification of APLV and APMMV.

4.1. Conventional RT-PCR and sequencing

Identification should be performed by sequence analysis of the amplicons obtained by the generic conventional RT-PCR tests described in Appendices 3 and 4. Guidance for sequence analysis is given in appendices 7 and 8 of EPPO Standard PM 7/129 (1) *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2016). Analysis of the amplicon produced by primers EM2 and EM3 (Appendix 4) is preferable since one of the species demarcation criteria within the genus *Tymovirus* is based on the amino acid sequence of the coat protein (Dreher *et al.*, 2012).

4.2. 'Specific' RT-PCR tests

Specific detection of APLV (GenBank accession no. NC_20470) and APMMV (GenBank accession no. NC_20471) is obtained by combining the EM3 primer with the modified A1-a-mod3C and AM-a-mod4C primers, respectively (Koenig & Ziebell, 2014). It should be noted that these tests have only been used for identification of purified virus and have not been validated for identification of APLV and APMMV in plant material; consequently, they are not described in this protocol.

4.3. Other tests

Serological tests, i.e. DAS-ELISA and IEM, can be used for identification depending on the specificity of the antiserum. It should be noted, however, that antisera that react with all strains of APLV/APMMV are likely to cross-react with other tymoviruses, while strain-specific antisera might fail to react with others. Therefore, unless specificity of the antiserum is validated, molecular tests are required for correct identification of the virus isolate.

The Panel on Diagnostics in Virology and Phytoplamsology noted that high-throughput sequencing (HTS) is a technology that may be used for obtaining (almost) complete genome sequences, analysis of which can be used for identification of a virus isolate. An EPPO Standard on the use of HTS (also called Next Generation Sequencing, NGS) for plant pest diagnostics is in preparation.

5. Reference material

Reference isolates and/or controls* of APLV, APMMV and related tymoviruses are available from:

Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7B, 38124, Braunschweig, Germany; National Plant Protection Organization of the Netherlands (NPPO-NL), PO Box 9102, 6700 HC Wageningen, the Netherlands; and Wageningen Plant Research International (WPR), Postbus 16, 6700 AA Wageningen, the Netherlands.

*Specifications and characteristics of available isolates can be found via Q-bank (http://www.q-bank.eu).

6. Reporting and documentation

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

7. Performance criteria

When performance criteria are available they are provided with the description of the test. Validation data may be available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int) and consultation of it this database is recommended 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 these viruses can be obtained from:

Julius Kühn-Institut, Messeweg 11–12, 38104 Braunschweig, Germany (bioassay, DAS-ELISA and RT-PCR) and National Plant Protection Organization, PO Box 9102, 6700 HC Wageningen, the Netherlands (bioassay, DAS-ELISA and RT-PCR).

9. 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.

10. 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.

Acknowledgements

This protocol was originally drafted by J. W. Roenhorst and A. W. Werkman, National Plant Protection Organization, PO Box 9102, 6700 HC Wageningen, the Netherlands.

It was reviewed by the Panel on Diagnostics in Virology and Phytoplasmology.

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Appendix 1 – Bioassay

Glasshouse-grown plants should be tested when they are about 25 cm tall, at or near flowering. Samples should be taken from at least two positions on each plant, including a young, fully expanded terminal leaflet at the top of a stem

and an older terminal leaflet from a midway position. Leaves from up to 5 plants belonging to the same line may be 'bulked' together to prepare the inoculum.

Extraction buffer and test-plant inoculation

The following buffer can be used for mechanical inoculation of test plants: 0.02 M Na/K phosphate buffer, pH 7.0, containing 2% (w/v) polyvinylpyrrolidone (PVP) (MW 10 000 to 40 000). Leaf material should be ground in extraction buffer at a ratio of 1:10. Celite or carborundum is then added to the inoculum as an abrasive or used for dusting the leaves prior to inoculation. This should be washed off after inoculation to avoid damage to the inoculated leaves, which will mask reactions. At least two plants from each indicator plant species should be used. Young (3–6 leaf stage), soft, actively growing test plants are inoculated by rubbing plant sap on their leaves. Inoculated plants should be kept at 18–22°C in a glasshouse or growth chamber with at least a 14 h photoperiod. Further details on quality control of mechanical inoculation of test plants are described by Roenhorst *et al.* (2013).

Recommended test plants and their symptoms

APLV and APMMV may cause the following symptoms:

Nicotiana benthamiana: chlorotic local lesions for some isolates; systemically infected leaves may show mosaic symptoms and rugosity.

Nicotiana hesperis 67A and *N. occidentalis* P1: chlorotic and necrotic local lesions; systemically infected leaves may show chlorosis, vein clearing and sometimes necrotic lesions.

Symptoms caused by other related tymoviruses, such as *Eggplant mosaic virus* (EMV) and *Physalis mottle virus* (PhyMV) might be similar. Therefore, it is not possible to identify APLV and APMMV on the basis of test plant reactions.

Performance criteria available

Detection of APLV and APMMV by mechanical inoculation of a combination of test plants

Data provided by the National Reference Centre of the NPPO, the Netherlands.

1.1 Analytical sensitivity data

An isolate of APMMV was mechanically inoculated on *N. benthamiana*, *N. hesperis* 67A and *N. occidentalis* P1 (two plants of each species). The latter two test plants reacted with symptoms upon inoculation with infected leaf material of *S. tuberosum* up to a relative infection rate of 0.1% and in *N. benthamiana* up to 1%. In addition, *N. benthamiana* often recovered, which makes it less suitable for screening. For *N. hesperis* 67A and *N. occidentalis* P1, the presence or absence of symptoms always agreed with results of DAS-ELISA. In *N. benthamiana*, symptomless infections occurred.

1.2 Analytical specificity

The symptoms caused by different tymoviruses are not specific; therefore, this test cannot be used for identification of the species. For potato leaf material no matrix effects have been observed.

1.3 Data on repeatability

Not available.

1.4 Data on reproducibility

Not available.

Appendix 2 – Data on antisera for DAS-ELISA and IEM

Instructions for performing DAS-ELISA and IEM are provided in EPPO Standards PM 7/125 *ELISA tests for viruses* and PM 7/126 *Electron microscopy in diagnosis of plant viruses*, respectively.

The source of antibodies is critical as they may differ in reactivity. APLV antisera are available from DSMZ and Prime Diagnostics.

1. Performance criteria available for DSMZ Antisera AS-0002 and AS-0003

Data provided by W. Menzel, DSMZ.

Antisera AS-0002 and AS-0003 are available from DSMZ on request (W. Menzel, e-mail: plantvirus@dsmz.de).

1.1 Analytical sensitivity data

Not available.

1.2 Analytical specificity

AS-0002 detects APLV isolates Col (DSMZ, PV0060) and Col2 (DSMZ, PV0062) with relatively high OD values (>2) and APMMV (formerly APLV Hu) with low ODs (<0.5). In contrast, AS-0003 detects APLV isolates with relatively low ODs (<0.5) and APMMV with high ODs (>3.5). Both antisera were found to cross-react with *Eggplant mosaic virus* (EMV). No reactions were observed with *Tymovirus* isolates of *Belladonna mottle virus* (BeMV), *Nemesia ring necrosis virus*, *Okra mosaic virus*, *Scrophularia mottle virus* (ScrMV) and *Turnip yellow mosaic virus* (TYMV), or with isolates of other potato-infecting viruses *Alfalfa mosaic virus* (AMV), *Andean potato mottle virus* (APMoV), *Potato aucuba mosaic virus* (PAMV), *Potato black ringspot virus* (PBRSV), *Potato latent virus* (PotLV), *Potato leafroll virus* (PLRV), *Potato mop top virus* (PMTV), *Potato yellow dwarf virus*, *Potato yellowing virus*, *Potato virus A*, *M*, *S*, *T*, *V*, *X*, *Y*, *Tobacco rattle virus* (TRV), *Tomato spotted wilt virus* (TSWV), *Tomato black ring virus*, *Tomato chlorosis virus* and *Tomato leaf curl New Delhi virus.* No background reactions were observed for leaf material of *Solanum lycopersicum* (tomato), *S. tuberosum* (potato) and test plants *Nicotiana benthamiana*, *Nicotiana glutinosa*, *Nicotiana rustica*, *Nicotiana tabacum* (different varieties) and *Physalis floridana*.

1.3 Data on repeatability
Not available.
1.4 Data on reproducibility
Not available.

2. Performance criteria available for DSMZ antiserum AS-0978 (combination AS-0002 and AS-0003)

2.1 Data provided by the French quarantine unit of the Plant Health Laboratory of Anses.
2.2 Analytical sensitivity
The antiserum of DSMZ (AS-0978, batch no. 4986) has been validated for testing leaf material of *N. occidentalis* P1, *S. tuberosum* (potato) and related *Solanum* species. Following the instructions of the supplier, the test could detect the former APLV Hu isolate (PV-0061, DSMZ). Dilutions in healthy plant material showed 100% detection up to 1/5000 dilution level.
2.3 Analytical specificity (exclusivity)
The antiserum does not react with the potato-infecting viruses PLRV and PVM, S, X. No background reactions were observed for leaf material of *N. occidentalis* P1, *S. tuberosum* and the following *Solanum* species: *S. albicans*, *S. bulbocastanum*, *S. chacoense*, *S. fendleri*, *S. oplocense*, *S. polydanium*, *S. polytrichon*, *S. stoloniferum* and *S. trifidum*.
2.4 Data on repeatability
Not available.
2.5 Data on reproducibility
Not available.

3. Performance criteria available for Prime Diagnostics antiserum APLV

Data provided by the National Reference Centre of the NPPO, the Netherlands.
3.1 Analytical sensitivity data
The Prime Diagnostics antiserum has been validated for testing leaf material of *N. occidentalis* P1 and *S. tuberosum* by following the supplier's instructions; the test could detect all included APLV and APMMV isolates (see Analytical specificity) up to a relative infection rate of 0.001% for *S. tuberosum* and 0.0001% for *N. occidentalis* P1 (10^5 and 10^7 serial dilution in healthy plant material).
3.2 Analytical specificity data
The tests gave clear positive reactions with all included APLV and APMMV isolates, i.e. APLV isolates Col (DSMZ, PV0060) and Col2 (DSMZ, PV0062), and two APMMV isolates, the former APLV Hu isolates (DSMZ, PV0061) and an isolate from potato plants originating from Colombia (PD Q930460). Cross-reactions were observed for EMV and *Physalis mottle virus* (PhyMV). The antiserum did not react with the less related tymoviruses ScrMV and TYMV, nor with the potato-infecting viruses AMV, APMoV, PAMV, PBRSV, PotLV, PLRV, PMTV, *Potato virus A*, *M*, *P* (*Potato rough dwarf virus*), *S*, *T*, *V*, *X*, *Y*, TRV and TSWV. No background reactions were observed for leaf material of *N. occidentalis* P1 and *S. tuberosum* varieties Allure, Crisper, Michelle, Nicola, Santé and Seresta.
3.3 Data on repeatability
Repeatability scored 100%.
3.4 Data on reproducibility
Reproducibility scored 100%.
3.5 Other information
Note that the Science and Advice for Scottish Agriculture (SASA, GB) obtained comparable results for analytical sensitivity and analytical specificity.

Appendix 3 – One-step reverse transcription PCR (Kreuze *et al.*, 2013)

1. General information

1.1 This test can be used for generic detection of tymoviruses, using primers designed by Kreuze *et al.* (2013). The original protocol was adapted to a one-step format and further optimized for routine use by the NPPO, Wageningen, the Netherlands.
1.2 This test has been successfully used for testing fresh, frozen or dried leaf material from potato and different herbaceous test plants.
1.3 Generic primers are derived from the EMV type strain (GenBank accession no. NC_001480). EM13 and EM14 are located in the overlapping replicase and putative movement protein and produce amplicons of approximately 800 bp depending on the virus species and strain. Primers have been shown to detect isolates of APLV, APMMV, EMV, PhyMV and ScrMV, and might detect additional tymoviruses.
1.4 Oligonucleotides

Primer	Sequence
EM13 (forward)	5′-CCTTCAACTGTGATGTTCATG-3′
EM14 (reverse)	5′-TGCAGATTGTCCCACGC-3′

1.5 The test has been successfully performed on a Bio-Rad C1000/S1000 thermal cycler using deep-well blocks.

2. Methods

2.1 Nucleic acid extraction and purification
2.1.1 Approximately 250 mg of (symptomatic) plant material is used for total RNA extraction by using the RNeasy Mini Kit (Qiagen). Alternative procedures might also work.
2.1.2 Extracted RNA should be stored refrigerated for short-term storage (<8 h), at −20°C (<1 month) or −80°C for longer periods.
2.2 One-step reverse transcription PCR

2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water*	NA	9.0	NA
RT-PCR buffer (Invitrogen)	2×	12.5	1×
PCR forward primer: EM13	10 μM	0.5	0.2 μM
PCR reverse primer: EM14	10 μM	0.5	0.2 μM
Enzyme mix	NA	0.5	NA
Subtotal		23.0	
Genomic RNA extract		2.0	
Total		25.0	

*Molecular-grade water should preferably be used or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 μm filtered) and nuclease-free water.

2.2.2 One-step RT-PCR using the SuperScript™ One-Step RT-PCR system with Platinum™ Taq DNA polymerase (Invitrogen).

2.2.3 RT-PCR cycling parameters: reverse transcription at 50°C for 30 min; denaturation at 94°C for 2 min; 40 cycles consisting of denaturation at 95°C for 15 s, annealing at 48°C for 30 s, elongation at 72°C for 60 s; terminal elongation at 72°C for 10 min.

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 that 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 the 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[2]). The PAC should preferably be near to the limit of detection.

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

3.2 Interpretation of results

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC, PAC should produce amplicons of approximately 800 bp (depending on species used as positive control).

When these conditions are met:

- A test will be considered positive if amplicons of approximately 800 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.

It should be noted that for viruses and viroids bands of different sizes may correspond to strains of the target organism and care should be taken when interpreting the results of conventional PCR, in particular the sizes of bands.

4. Performance criteria available

Validation data was generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity.* A limited validation was performed at the NPPO of the Netherlands.

4.1 Analytical sensitivity data

Not determined.

4.2 Analytical specificity data

The RT-PCR test produced amplicons with isolates of APLV (DSMZ isolates Col, PV-0060, and Col2, PV-0062), APMMV (DSMZ isolate APLV Hu, PV-0061), BeMV (DSMZ isolate PV-0042), EMV (NPPO-NL isolate PD422517), PhyMV (NPPO-NL isolate PD1968347) and ScrMV (DSMZ isolate PV-0870). The identity of the isolates could be confirmed by sequence analysis, except for BeMV as no sequence data of the target region was available in NCBI GenBank. No amplicons were obtained with isolates of *Poinsettia mosaic virus* (NPPO-NL isolate PD23006511) or TYMV (DSMZ isolate PV-0299).

4.3 Data on repeatability

Not available.

4.4 Data on reproducibility

Not available.

[2]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)

Appendix 4 – Two-step reverse transcription PCR (Kreuze *et al.*, 2013)

1. General information

1.1 This test can be used for detection of APLV, APMMV, EMV and possibly other related tymoviruses, using primers EM2 and EM3 designed by Kreuze *et al.* (2013).

1.2 This test has been successfully used for testing leaf material from potato and *N. benthamiana* plants.

1.3 Generic primers are derived from the EMV type strain (GenBank accession No. NC_001480). EM2 and EM3 are located in conserved regions downstream and upstream of the coat protein gene and produce amplicons of approximately 700 bp. Primers have been shown to detect isolates of APLV, APMMV and EMV, and might detect additional tymoviruses.

1.4 Oligonucleotides

Primer	Sequence
EM2 (forward)	5′-TCTCAACTGGAGTCTGAATTGCTTC-3′
EM3 (reverse)	5′-CACCCAAATGGACCTCTGTGTGCTA-3′

1.5 The test has been successfully performed on a Flexcycler2 thermocycler (Analytik Jena).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Approximately 100 mg of (symptomatic) plant material is used for total RNA extraction using the innuPREP Plant RNA Extraction Kit (Analytik Jena). Alternative procedures might also work.

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

2.2 Reverse transcription (to produce cDNA from RNA)

2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water*	N.A.	9.0	N.A.
RT buffer (Protoscript II Buffer; New England BioLabs)	5×	4.0	1×
dNTPs (Carl Roth)	10 mM	1.0	0.5 mM
Dithiothreitol (DTT; Invitrogen)	0.1 M	2.0	10 mM
RNasin (Promega)	40 U μL^{-1}	1.0	40 U
Primer EM3	10 μM	1.0	0.5 μM
Reverse transcriptase (RT)	200 U μL^{-1}	1.0	200 U
Subtotal		19.0	
RNA		1.0	
Total		20.0	

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 μm filtered) and nuclease-free water.

2.2.2 Reverse transcription step using ProtoScript II Reverse Transcriptase (New England BioLabs).

2.2.3 Reverse transcription conditions: mix water, dNTPs and primer EM3 and denature at 65°C for 5 min; chill immediately on ice; add remaining ingredients (RT buffer, DTT, RNasin and RT) and incubate at 50°C for 30 min followed by a denaturation step at 65°C for 20 min.

2.3 Conventional PCR

2.3.1 Master mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water*	N.A.	11.72	N.A.
PCR buffer (Axon Labortechnik)	10×	2.0	1×
$MgCl_2$	25 mM	2.0	2.5 mM
dNTPs	10 mM	2.0	1 mM
Forward primer (EM2)	10 μM	0.6	0.3 μM
Reverse primer (EM3)	10 μM	0.6	0.3 μM
Polymerase	5 U μL^{-1}	0.08	0.4 U
Subtotal		19.0	
cDNA derived from RT-step		1.0	
Total		20.0	

*Molecular-grade water should preferably be used, or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 μm filtered) and nuclease-free water.

2.3.2 PCR using Taq DNA polymerase (Axon Labortechnik, cat. no. 2246) according to the manufacturer's protocol.

2.3.3 PCR cycling parameters: denaturation at 95°C for 5 min; 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 40 s, elongation at 72°C for 60 s; terminal elongation at 72°C for 7 min.

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 that 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 the 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[3]). The PAC should preferably be near to the limit of detection.

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

3.2 Interpretation of results

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC, PAC should produce amplicons of approximately 700 bp.

When these conditions are met:

- A test will be considered positive if amplicons of approximately 700 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.

It should be noted that for viruses and viroids bands of different sizes may correspond to strains of the target organism and care should be taken when interpreting the results of conventional PCR, in particular the sizes of bands.

4. Performance characteristics available

Validation data was generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity.* A limited validation was performed at the Julius Kühn-Institut, Braunschweig, Germany.

4.1 Analytical sensitivity data

APLV and APMMV could be detected in RNA-extracts of potato up to a 1:1000 dilution in water.

4.2 Analytical specificity data

Generic RT-PCR produced amplicons with different isolates of APLV (BO-14, BO-15,Col, Col-2, Col-3, Col-4, Ec-1) and APMMV (Ay, Hu).

4.3 Other information

Although the evaluation of the performance characteristics for repeatability and reproducibility has not been evaluated according to PM 7/98, the test has been repeated and reproduced on several occasions in JKI with consistent results.

[3]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)