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EPPO STANDARD ON DIAGNOSTICS

PM 7/162 (1) *Emaravirus rosae* (rose rosette virus)

Specific scope: This Standard describes a diagnostic protocol for detection and identification of rose rosette virus.[1]
This Standard should be used in conjunction with PM 7/76 *Use of EPPO Diagnostic Standards*.
Specific approval and amendment: Approved in 2026–06.
Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Rose rosette virus (RRV; genus *Emaravirus*) is the cause of rose rosette disease, a disease affecting roses in the United States and Canada since the 1940s (Conners, 1941; Thomas, 1953). Rose rosette disease was used as a prophylactic method to control *Rosa multiflora*, a rose variety considered a weed in 1980s (Hindal et al., 1988). During that period, roses grew in popularity, and thousands of RRV-susceptible plants were planted in private gardens and landscapes (Amrine, 1996). Since then, RRV has spread through the United States (US) and is now found in the majority of states. In 2017, RRV was detected for the first time outside North America, in India (Chakraborty et al., 2017).

Rosa species are the only confirmed natural hosts of RRV, and include different rose types such as climbers, hybrid tea roses, floribundas, shrubs, antique roses, and miniature roses (Stevens et al., 2022). Rose rosette virus has caused losses up to 25% in gross revenue in US rose production businesses (Stevens et al., 2022). RRV is transmitted by the eriophyid mites *Phyllocoptes fructiphilus* (Allington et al., 1968) and *Phyllocoptes arcani*, which is a recently discovered vector (Druciarek et al., 2023). Mite transmission is the primary mechanism for spread in the field, where mites are dispersed by the wind, or transported by pollinators (Byrne et al., 2015). It has also been demonstrated that RRV can be spread or transmitted through seeds and grafting (Shires, 2020).

For updated geographical distribution consult EPPO Global Database.

A mini datasheet providing more information on the biology is also available in EPPO Global Database.

A flow diagram describing the diagnostic procedure for the detection and identification of RRV in symptomatic plant material is presented in Figure 1.

2 | IDENTITY

Name: rose rosette virus.
Other scientific names: rose rosette virus.
Acronym: RRV.
Taxonomic position: Viruses, Riboviria, Fimoviridae, Emaravirus; *Emaravirus rosae*.
EPPO Code: RRV000.
Phytosanitary categorization: EPPO A1 List no. 415, Union Quarantine pest (Annex IIA[2]).

Note: Virus nomenclature in Diagnostic Protocols is based on the latest release of the official classification by the International Committee on Taxonomy of Viruses (ICTV, Release 2023, https://talk.ictvonline.org/taxonomy/). All accepted viral species have a binomial species name which is used in a taxonomic context, whereas their common English virus names and acronyms are used throughout the EPPO Diagnostic Protocols. Names of viruses not included in the official ICTV classification are based on first reports.

3 | DETECTION

3.1 | Symptoms

Roses infected with RRV can show few or no symptoms during early stages of infection (Dobhal et al., 2016), and can remain asymptomatic up to 6 months after infection (Babu et al., 2018). Plants infected with RRV show reduced growth and vigour (Epstein & Hill, 1999), and eventually infected plants die within a period of 3–5 years (Di Bello et al., 2017). Symptoms may vary between rose cultivars, stage of the disease and environmental factors, and are often confused with herbicide damage, symptoms from other pests, or nutrient

[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.

[2]Commission Implementing Regulation (EU) 2019/2072.

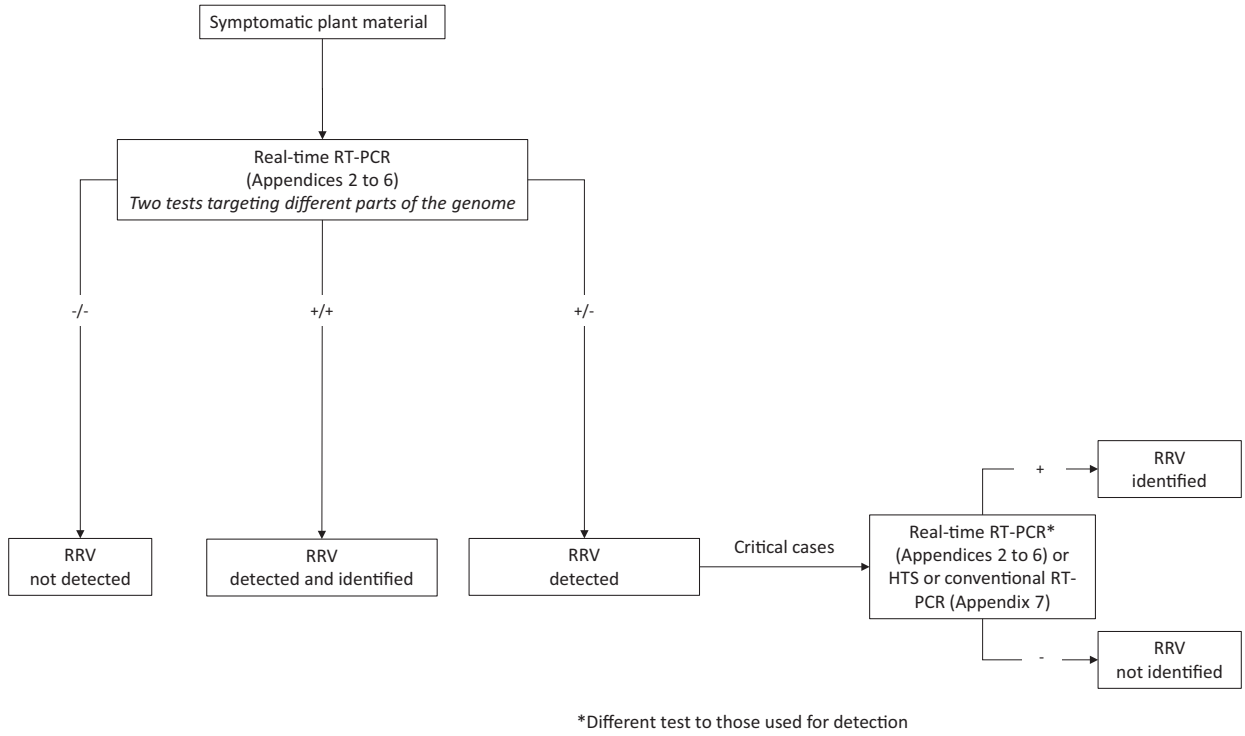

FIGURE 1 Flow diagram describing the diagnostic procedure for rose rosette virus in symptomatic plant material. The diagnostic procedure in asymptomatic plant material is not described as there is no experience in the EPPO region. This flow diagram is intended to provide an overview of the diagnostic process and may not cover all possible scenarios. In the flow diagram, '−/−' represents two negative tests, '+/+', two positive tests and '+/−' one test out of two is positive.

deficiencies. Mixed viral infections are commonly found in roses and the influence of this in symptoms expression has not been determined (Vazquez-Iglesias et al., 2020a).

The following virus symptoms may be observed on roses infected with RRV (Claros et al., 2022; Dobhal et al., 2016; Hong et al., 2012; Laney et al., 2011; Vazquez-Iglesias et al., 2020a):

- Leaves: extreme reddening (Figure 2a,d), deformation, mosaic, mottling, rough texture.
- Stems: witches' broom or rosetting (short internodal distances) (Figure 2b,d), thickening and excess of thorns (Figure 2c,d), lateral shoot growth, extreme reddening (Figure 2a), darkening of canes.
- Flowers: malformation, distortion, failure to open fully (Figure 3).

3.2 | Test sample requirements

Experimental data on the best sampling material and specific sample size is lacking. For symptomatic plants, it is recommended to collect leaves from shoots preferably with open flower buds (Ochoa-Corona, pers. comm.). The diagnostic procedure in asymptomatic plant material is not described as there is no experience in the EPPO region. Therefore, only preliminary recommendation can be made on test sample requirements, i.e., to collect young leaves from different parts of the plants.

3.3 | Detection tests

3.3.1 | Recommended molecular tests

For the detection of RRV, molecular tests are recommended. Several real-time RT-PCR tests have been described for the detection of RRV in leaves.

Given the fact that limited sequence data are available and considerable sequence variation exists between isolates, the use of two real-time RT PCR tests, that target different parts of the genome, is recommended. Furthermore, since matrix effects have been observed for undiluted RNA extracts of leaves of *Rosa* spp., the amount of buffer used for homogenisation of the leaf material or the use of additives can be critical. An alternative consists of capturing the RRV virions directly into the PCR tubes to cleanse putative rose inhibitors of PCR (Babu et al., 2017) and to avoid diluting the sap sample. In the case a positive and negative result are obtained, (due to sequence differences with the primers/probe of one of the tests), additional testing may be

FIGURE 2 Common symptoms of rose rosette virus (RRV): (a) reddening in the leaves and stems; (b) witches' broom or rosetting; and (c) excess of thorn production and thickened stems; (d) healthy-looking stem and leaves (left) compared with an RRV-infected stem and leaves (right). Courtesy: Vazquez-Iglesias et al. (2020a).

required for critical cases. When results are doubtful or inconsistent, retesting using the third real-time PCR test or conventional RT-PCR of Bratsch et al. (2017) or high-throughput sequencing (HTS) is recommended.

The following molecular tests are recommended for the detection of RRV in leaves:

- Real-time RT-PCR using the primers and probe from Babu et al. (2016) targeting the RNA 2 (hereafter referred to as real-time RT PCR RNA 2-2)—Appendix 2.
- Real-time RT-PCR using the primers and probe from Babu et al. (2016) targeting the RNA 3 (hereafter referred to as real-time RT PCR RNA 3-2)—Appendix 3.
- Real-time RT-PCR using the primers and probe from Babu et al. (2016) targeting the RNA 3 (hereafter referred to as real-time RT PCR RNA 3-5)—Appendix 4.
- Real-time RT-PCR using the primers and probe from Dobhal et al. (2016) targeting the RNA 3 (hereafter referred to as real-time RT PCR RNA 3)—Appendix 5.
- Real-time RT-PCR using the primers and probe from Vazquez-Iglesias et al. (2020b) targeting the RNA 1 (hereafter referred to as real-time RT-PCR RNA 1)—Appendix 6.

3.3.2 | Other molecular tests

Loop-mediated isothermal amplification (LAMP) (Salazar et al., 2021), recombinase polymerase amplification (RPA), and other real-time RT-PCR and conventional RT-PCR tests (Claros et al., 2022; Olmedo-Velarde et al., 2023) have been developed but are not described in detail in this Diagnostic Standard.

High-Throughput Sequencing (HTS) can be used for the detection of RRV in symptomatic plant material (see the test described in the Annex 1 of the supporting

FIGURE 3 Reduced flower development and malformation caused by rose rosette virus (RRV). Courtesy: Fera Science Ltd (GB).

information S2 of PM 7/151 *Considerations for the use of high throughput sequencing in plant health diagnostics*; EPPO, 2022), and the associated validation data available in the EPPO Database on Diagnostic Expertise (https://dc.eppo.int/validation_data/dwvalidation?id=512). The use of an HTS test should follow the guidelines provided in the EPPO Standard PM 7/151 (EPPO, 2022).

4 | IDENTIFICATION

The molecular tests described in Section 3.3.1 are recommended for identification of RRV in leaves. In addition, conventional RT-PCR from Bratsch et al., 2017 that targets RNA 4 is also recommended for identification of RRV in leaves (Appendix 7) when results are doubtful or inconsistent. Sequencing of amplicons is recommended in critical cases, following guidelines in the Appendices 5 and 6 of the EPPO Standard PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2021; under revision).

High-throughput sequencing (HTS) can be used as an identification test for RRV following positive detection and/or when results are doubtful or inconsistent. The application of HTS test should follow the guidelines in the EPPO Standard PM 7/151 *Considerations for the use of high throughput sequencing in plant health diagnostics* (EPPO, 2022). Validation data is available from the EPPO Database on Diagnostic Expertise.

5 | REFERENCE MATERIAL

RRV isolates for reference material are not available commercially.

Reference material is available from:

- Francisco Ochoa Corona, Oklahoma State University, 127 NRC, Stillwater, Oklahoma, 74078, United States (ochoaco@okstate.edu).
- Ioannis Tzanetakis, University of Arkansas, Dept. of Entomology & Plant Pathology, 495 *N. campus* Walk, PTSC 217, Fayetteville, AR 72701, United States (itzaneta@uark.edu).
- Denise Altenbach, Agroscope, Roggenweg 3, Wuerenlos CH-5436, Switzerland (denise.altenbach@agroscope.admin.ch)
- Marcel Westenberg, Netherlands Institute for Vectors, Invasive plants and Plant health (NIVIP-NVWA), Geertjesweg 15, 6706 EA Wageningen, the Netherlands, (m.westenberg@nvwa.nl). GBlocks can be used as positive amplification controls (PAC). Both gBlocks can be detected with four of the recommended tests (Appendices 2–5). The test described in Appendix 6 will only detect RRV-A. The sequences of the gBlocks are available in Appendix 8.

Additionally, an artificial positive amplification (PAC) control consisting of inserts of sense and antisense primer sequences in a circular plasmid has been developed to support diagnostics for RRV (Ruschel et al., 2023). It was designed considering most available molecular tests including those developed by Babu et al. (2016) and Dobhal et al. (2016). There is currently no experience with this artificial positive control in the EPPO region.

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:

Hejlova M (UKZUZ, CZ), Oplaat AG (NIVIP-NVWA, NL), Orsagova H (UKZUZ, CZ), Roenhorst JW (NIVIP-NVWA, NL: EURL for Pests of Plants on Viruses, Viroids and Phytoplasmas), van der Sman P (METK, EE), Vazquez Iglesias I (Fera Science Ltd, GB), and Westenberg M (NIVIP-NVWA, NL).

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 Standard that you wish to share please contact diagnostics@eppo.int.

10 | STANDARD REVISION

A regular 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

This Standard was originally drafted by an Expert Working Group composed of Vazquez-Iglesias I (Fera, GB lead author), Hejlova M (UKZUZ, CZ), Oplaat AG (NIVIP-NVWA, NL), Orsagova H (UKZUZ, CZ), Roenhorst JW (NIVIP-NVWA, NL; EURL for Pests of Plants on Viruses, Viroids and Phytoplasmas) and van der Sman P (METK, EE). It was reviewed by the EPPO Panel on Diagnostics in Virology and Phytoplasmology.

REFERENCES

Allington WB, Staples R, Viehmeyer G (1968). Transmission of rose rosette virus by eriophyid mite Phyllocoptes fructiphilus. *Journal of Economic Entomology* 61: 1137–1140

Amrine Jr J (1996). *4.1. 2 Phyllocoptes fructiphilus and biological control of multiflora rose, World Crop Pests*. Elsevier, pp. 741-749

Babu B, Jeyaprakash A, Jones D, Schubert TS, Baker C, Washburn BK, Miller SH, Poduch K, Knox GW, Ochoa-Corona FM, Paret ML (2016). Development of a rapid, sensitive TaqMan real-time RT-PCR assay for the detection of rose rosette virus using multiple gene targets. *Journal of Virological Methods* 235: 41-50. doi: 10.1016/j.jviromet.2016.05.010

Babu B, Knox G, Paret ML, Ochoa-Corona FM (2018). Rose rosette disease: recent advances on molecular diagnostic tools. *HortScience* 53: 596. doi: 10.21273/hortsci12551-17

Babu B, Washburn BK, Ertek TS, Miller SH, Riddle CB, Knox GW, *et al*. (2017). A field based detection method for Rose rosette virus using isothermal probe-based Reverse transcription-recombinase polymerase amplification assay. *Journal of Virological Methods* 247:81–90. doi: 10.1016/j.jviromet.2017.05.019

Bratsch S, Zlesak D, Mollov D, Lockhart B (2017). First Report of Rose rosette virus Associated with Rose Rosette Disease in Rosa hybrida in Minnesota. *Plant Health Progress* 18: 102-103. doi: 10.1094/php-01-17-0008-br

Byrne DH, Roundey E, Klein P, Yan M (2015). *Combating Rose rosette disease: are there resistant roses?, American Rose*. American Rose Society, USA, pp. 78-83

Chakraborty P, Das S, Saha B, Karmakar A, Saha D, Saha A (2017). *Rose rosette virus*: an emerging pathogen of garden roses in India. *Australasian Plant Pathology* 46: 223-226. doi: 10.1007/s13313-017-0479-y

Claros NA, Shires M, Mollov D, Hammond J, Jordan R, Ochoa-Corona F, Olson J, Ong K, Salamanca LR (2022). Rose Rosette Disease: A Diagnostic Guide. *Plant Health Progress* 23: 482-491. doi: 10.1094/PHP-05-22-0047-DG

Conners L (1941). *Twentieth Annual Report of the Canadian Plant Report Survey, 1940*. In: Domain of Canada Department of Agriculture Science Service, D.o.B.a.P.P., Ottawa. (Ed.)

Di Bello PL, Thekke-Veetil T, Druciarek T, Tzanetakis IE (2017). Transmission attributes and resistance to rose rosette virus. *Plant Pathology* 67: 499-504. doi: 10.1111/ppa.12738

Dobhal S, Olson JD, Arif M, Garcia Suarez JA, Ochoa-Corona FM (2016). A simplified strategy for sensitive detection of rose rosette virus compatible with three RT-PCR chemistries. *Journal of Virological Methods* 232: 47-56. doi: 10.1016/j.jviromet.2016.01.013

Druciarek T, Lewandowski M, Tzanetakis IE (2023). Identification of a second vector for rose rosette virus. *Plant Disease*

EPPO (2021) PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests. *EPPO Bulletin* 51: 100-143. doi: 10.1111/epp.12724

EPPO (2022). PM 7/151 (1) Considerations for the use of high throughput sequencing in plant health diagnostics. *EPPO Bulletin* 52: 619-642. doi: 10.1111/epp.12884

Epstein A, Hill J (1999). Status of Rose Rosette Disease as a biological control for Multiflora rose. *Plant Disease* 83: 92-101. doi: 10.1094/pdis.1999.83.2.92

Gambino G, Perrone I and Gribaudo I (2008). A Rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. *Phytochemical Analysis* 19: 520-525. 10.1002/pca.1078

Hindal DF, Amrine JW, Williams RL, Stasny TA (1988). Rose rosette disease on Multiflora rose (*Rosa multiflora*) in Indiana and Kentucky. *Weed Technology* 2: 442-444. doi: 10.1017/S0890037X00032243

Hong C, Hansen MA, Day E (2012). *Rose rosette disease*. Virginia State University Cooperative Extension, 450-620

Laney AG, Keller KE, Martin RR, Tzanetakis IE (2011). A discovery 70 years in the making: characterization of the rose rosette virus. *Journal of General Virology* 92: 1727-1732. doi: 10.1099/vir.0.031146-0

Olmedo-Velarde A, Ochoa-Corona FM, Larrea-Sarmiento AE, Elbeaino T, Flores F (2023). In-silico prediction of RT-qPCR-high resolution melting for broad detection of emaraviruses. *PLoS One* 8;18(5):e0272980. doi: 10.1371/journal.pone.0272980

Ruschel RG, Taylor M, Ochoa-Corona FM, Amirudeen AKJ, Druciarek T, Paret M (2023). An artificial positive control for routine detection of rose rosette virus and *Phyllocoptes fructiphilus* that fit most primers for PCR, LAMP and RPA based assays. *Annals of Applied Biology* n/a. doi: 10.1111/aab.12834

Salazar A, Ochoa-Corona FM, Olson JD, Babu B, Paret M (2021). Probing Loop-Mediated Isothermal Amplification (LAMP) targeting two gene-fragments of rose rosette virus. *bioRxiv*, 2021.2008.2017.456656. doi: 10.1101/2021.08.17.456656

Saponari M, Giampetruzzi A, Selvaraj V, Maheshwari Y, Yokomi R (2019). Identification and Characterization of Resistance-Breaking (RB) Isolates of *Citrus tristeza virus*. In: Catara, A.,

Bar-Joseph, M., Licciardello, G. (eds) *Citrus Tristeza Virus. Methods in Molecular Biology*, vol 2015. Humana, New York, NY. 10.1007/978-1-4939-9558-5_8

Shires M (2020). Study of resistance to rose rosette disease utilizing field research, molecular tools, and transmission methods. https://oaktrust.library.tamu.edu/bitstream/handle/1969.1/192819/SHIRES-DISSERTATION-2020.pdf?sequence=1

Stevens L, Ostoja-Starzewski J, Vazquez-Iglesias I (2022). *Rose rosette disease caused by rose rosette virus and its vector the eriophyoid mite* Phyllocoptes fructiphilus. Department for Environment Food and Rural Affairs

Thomas H (1953). Rosette of rose. *Phytopathology* 43: 218-219

Vazquez-Iglesias I, Boonham N, Robinson R, Clover GRG, Fox A (2023). A survey of rose viruses in England. *Crop Protection* 168: 106231. doi: 10.1016/j.cropro.2023.106231

Vazquez-Iglesias I, Ochoa-Corona FM, Tang J, Robinson R, Clover GRG, Fox A, Boonham N (2020a). Facing *Rose rosette virus*: A risk to European rose cultivation. *Plant Pathology* 69: 1603-1617. doi: 10.1111/ppa.13255

Vazquez-Iglesias I, Scrace J, McGreig S, Pufal H, Robinson R, Clover GRG, Adams I, Boonham N, Fox A (2020b). First report of Rose spring dwarf-associated virus in *Rosa* spp. in United Kingdom. *New Disease Reports* 42. doi: 10.5197/j.2044-0588.2020.042.013

APPENDIX 1 - SAMPLE PREPARATION AND RNA EXTRACTION

RNA extraction using CTAB (e.g. Gambino et al., 2008; Saponari et al., 2019) or other extraction methods or kits may be used but they should be validated in combination with the molecular test to be used.

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

To test individual plants and/or small samples the RNeasy Plant Mini Kit (Qiagen) can be used according to the manufacturer's instructions with some minor modifications:

A. Leaves are put in an extraction bag and homogenized in GH+ buffer (range 1:3.5–1:5 (w/v)) (see Table A1). One mL of homogenate is incubated for 10 min at 65°C. After centrifugation at 12 000*g* for 2 min, 500 µL of supernatant is loaded on the QIAshredder spin column and centrifuged. Thereafter, the manufacturer's instructions in the RNeasy Plant Mini kit (Qiagen) should be followed.
B. Leaves are put in an extraction bag and homogenized in extraction buffer (1:10 (w/v)) (see Table A2). Fifty µL of homogenate is added to 450 µL of RLT buffer (ß-mercaptoethanol included), loaded on the QIAshredder spin column, further followed by the manufacturer's instructions in the RNeasy Plant Mini kit (Qiagen).

TABLE A1 GH+ buffer.

	Amount	Final concentration
Guanidine hydrochloride	573.18 g	6 M
Sodium acetate (4 M, pH 5.2)	50 mL	0.2 M
EDTA $Na_2{\cdot}2H_2O$	9.3 g	25 mM
PVP-10	25.0 g	2.5% w/v
Distilled water to	1.0 L	

TABLE A2 Extraction buffer.

	Amount	Final concentration
Tween 20	0.5 mL	0.05%
PVP-10-40	20.0 g	2% w/v
PBS (pH 7.4) to	1.0 L	

APPENDIX 2 - REAL-TIME RT-PCR RNA 2-2 (ADAPTED FROM BABU ET AL., 2016)

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 and identification of RRV in leaves.
1.2 The test is adapted from Babu et al. (2016).
1.3 The target sequence is located on the glycoprotein gene on RNA 2.
1.4 Oligonucleotides:

Primer/probe	Sequence 5′–3′
RRV_2-2For	CCA TTG CAG GTT GTT GCA TT
RRV_2-2Rev	TTG GCT CTA CCC TTT CTT TCC
RRV_2-2Probe	FAM-TGA ACA AGG GTG GAC CAT TCC ACA-BHQ1

1.5 The test has been successfully performed using the TaqMan® RNA-to-Ct™ (Thermo Fisher Scientific) on the Bio-Rad CFX96™ Real-Time PCR system (Bio-Rad).
1.6 Data was analysed using the Bio-Rad CFX Maestro software using an automatic baseline and threshold.

2. Methods

2.1 Nucleic Acid Extraction and Purification
2.1.1 Tissue source: leaves.
2.1.2 RNA extraction procedure is described in Appendix 1A.
2.1.3 Extracted RNA should be stored refrigerated for short-term storage (<8 h), at −20°C (<1 month) or at −80°C for longer periods.
2.2 One-step real-time RT-PCR
2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	6.1	N.A.
TaqMan™ RT-PCR mix (RNA-to-Ct™ 1-Step kit, *Thermo Fisher Scientific*)	2×	10	1×
Forward primer (RRV_2-2For)	10 µM	0.6	0.3 µM
Reverse primer (RRV_2-2Rev)	10 µM	0.6	0.3 µM
Probe (RRV_2-2Probe)	10 µM	0.2	0.1 µM
TaqMan™ RT Enzyme mix (RNA-to-Ct™ 1-Step RT kit, *Thermo Fisher Scientific*)	40×	0.5	1×
Subtotal		18	
RNA extract		2	
Total		20	

2.2.2 Real-time RT-PCR cycling conditions: reverse transcription at 48°C for 15 min; denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 60 s.

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 the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- 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, gBlock). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene, NADH dehydrogenase subunit 5 or eukaryotic 18S rDNA).
- Specific amplification or co-amplification of nucleic acid from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

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. Duplicate of the plant sample material spiked with nucleic acid from the target organism.

3.2 Interpretation of results

Verification of the controls:

- NIC and NAC should give no amplication.
- The PIC and PAC (and if relevant IC and IPC) amplification curves should be exponential.

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 was validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* by NIVIP-NVWA (NL) during preparation of the proficiency test organised by the EURL.

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

4.1 Analytical sensitivity data

RRV was detected up to a 10^{-4} dilution in 10-fold serial dilutions of two isolates (MD and 'Knock out'), and the deviating OK-1 isolate was detected up to a 10^{-3} dilution.

4.2 Analytical specificity data

Inclusivity: all three tested RRV isolates were detected.
Exclusivity: no cross reaction observed with prunus necrotic ringspot virus (PNRSV, genus *Ilarvirus*); based on sequence differences at primer and probe positions no cross reactions are expected for other rose-infecting viruses, i.e., apple mosaic virus (ApMV; genus *Ilarvirus*), arabis mosaic virus (ArMV; genus *Nepovirus*), cucumber mosaic virus (CMV; genus *Cucumovirus*), strawberry latent ringspot virus (SLRSV; genus *Stralarivirus*), tomato spotted wilt virus (TSWV; genus *Orthotospovirus*), rose cryptic virus-1 (RoCV1; genus *Alphapartitivirus*), and rose spring dwarf-associated virus (RSDaV; genus *Luteovirus*) (Vazquez-Iglesias et al., 2023).

4.3 Selectivity

Leaf material of *Rosa* spp. has been found to cause inhibition, thus a matrix effect can be expected.

4.4 Other information

In the EURL proficiency test 20 out of 25 laboratories used this test and all had 100% conforming results, although Ct values differed considerably. Nevertheless, these results show the robustness of the test, as laboratories performed variations on the test by using different extraction methods. Most laboratories used the RNeasy Plant Mini kit (Qiagen). Other kits used were innuPREP plant RNA Kit (Analytik Jena); MagMax total RNA Isolation Kit (Thermo Fisher); not specified kit (E.Z.N.A.); Plant/Fungi Total RNA purification kit (Norgen Biotek); QuickPick SML Plant DNA kit (Bionobile); Sbeadex maxi plant (cat. number NAP41602) (LGC group); Spectrum (Sigma).

APPENDIX 3 - REAL-TIME RT-PCR RNA 3-2 (ADAPTED FROM BABU ET AL., 2016)

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 and identification of RRV in leaves.
1.2 The test is adapted from Babu et al. (2016).
1.3 The target sequence is located on the nucleocapsid gene on RNA 3.
1.4 Oligonucleotides:

Primer/probe	Sequence 5′–3′
RRV_3-2For	ACA CTC TTG CAG CTG ATA CTG
RRV_3-2Rev	CTG GGT CCA ATT CTG AAC TCT C
RRV_3-2Probe*	FAM-AGC TTC GGG TCC TCA AGT TGA CAA-BHQ1

* Note that the RRV_3-2Probe is identical to RRV_3-5Probe described in Appendix 2.

1.5 The test has been successfully performed using the TaqMan® RNA-to-Ct™ (Thermo Fisher Scientific) on the Bio-Rad CFX96™ Real-Time PCR system (Bio-Rad).
1.6 Data was analysed using the Bio-Rad CFX Maestro software using an automatic baseline and threshold.

2. Methods

2.1 Nucleic Acid Extraction and Purification
2.1.1 Tissue source: leaves.
2.1.2 RNA extraction procedure is described in Appendix 1A.
2.1.3 Extracted RNA should be stored refrigerated for short-term storage (<8 h), at −20°C (<1 month) or at −80°C for longer periods.
2.2 One-step real-time RT-PCR
2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	6.1	N.A.
TaqMan™ RT-PCR mix (RNA-to-Ct™ 1-Step kit, *Thermo Fisher Scientific*)	2×	10	1×
Forward primer (RRV_3-2For)	10 µM	0.6	0.3 µM
Reverse primer (RRV_3-2Rev)	10 µM	0.6	0.3 µM
Probe (RRV_3-2Probe)	10 µM	0.2	0.1 µM
TaqMan™ RT Enzyme mix (RNA-to-Ct™ 1-Step RT kit, *Thermo Fisher Scientific*)	40×	0.5	1×
Subtotal		18	
RNA extract		2	
Total		20	

2.2.2 Real-time RT-PCR cycling conditions: reverse transcription at 48°C for 15 min; denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 60 s.

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 the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- 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, gBlock). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene, NADH dehydrogenase subunit 5 or eukaryotic 18S rDNA).
- Specific amplification or co-amplification of nucleic acid from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

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. Duplicate of the plant sample material spiked with nucleic acid from the target organism.

3.2 Interpretation of results

Verification of the controls:

- NIC and NAC: should give no amplification.
- PIC and PAC (and if relevant IC and IPC): amplification curves should be exponential.

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 was validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* by NIVIP-NVWA during preparation of the proficiency test organised by the EURL.

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

4.1 Analytical sensitivity data

RRV was detected up to a 10^{-4} dilution in 10-fold serial dilutions of two isolates (MD and 'Knock out'), and the deviating OK-1 isolate was detected up to a 10^{-2} dilution.

4.2 Analytical specificity data

Inclusivity: all three tested RRV isolates were detected.

Exclusivity: no cross reaction observed with prunus necrotic ringspot virus (PNRSV, genus *Ilarvirus*); based on sequence differences at primer and probe positions no cross reactions are expected for other rose-infecting viruses i.e., apple mosaic virus (ApMV; genus *Ilarvirus*), arabis mosaic virus (ArMV; genus *Nepovirus*), cucumber mosaic virus (CMV; genus *Cucumovirus*), strawberry latent ringspot virus (SLRSV; genus *Stralarivirus*), tomato spotted wilt virus (TSWV; genus *Orthotospovirus*), rose cryptic virus-1 (RoCV1; genus *Alphapartitivirus*), and rose spring dwarf-associated virus (RSDaV; genus *Luteovirus*) (Vazquez-Iglesias et al., 2023).

4.3 Selectivity

Leaf material of *Rosa* spp. has been found to cause inhibition, thus a matrix effect can be expected.

4.4 Other information

In the EURL proficiency test 17 out of 25 laboratories used this test and all had 100% conforming results, although Ct values differed considerably. Nevertheless, these results show the robustness of the test, as laboratories performed variations on the test by using different extraction methods. Most laboratories used the RNeasy Plant Mini kit (Qiagen). Other kits used were innuPREP Plant RNA Kit (Analytik Jena); MagMax total RNA Isolation Kit (Thermo Fisher); not specified kit (E.Z.N.A.); QuickPick SML Plant DNA kit (Bio-nobile); Sbeadex maxi plant (cat. number NAP41602) (LGC group).

APPENDIX 4 - REAL-TIME RT-PCR RNA 3-5 (ADAPTED FROM BABU ET AL., 2016)

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 and identification of RRV in leaves.
1.2 The test is adapted from Babu et al. (2016).
1.3 The target sequence is located on the nucleocapsid gene on RNA 3.
1.4 Oligonucleotides:

Primer/probe	Sequence 5′–3′
RRV_3-5For	CTG ATA CTG TTA TCA TCG GAG CTG
RRV_3-5Rev	TCT GAA CTC TCA GGC TTC ACT A
RRV_3-5Probe*	FAM-AGC TTC GGG TCC TCA AGT TGA CAA-BHQ1

* Note that the RRV_3-5Probe is identical to RRV_3-2Probe described in Appendix 3.

1.5 The test has been successfully performed using the TaqMan® RNA-to-Ct™ (Thermo Fisher Scientific) on the Bio-Rad CFX96™ Real-Time PCR system (Bio-Rad).
1.6 Data was analysed using the Bio-Rad CFX Maestro software using an automatic baseline and threshold.

2. Methods

2.1 Nucleic Acid Extraction and Purification
2.1.1 Tissue source: leaves.
2.1.2 RNA extraction procedure is described in Appendix 1A.
2.1.3 Extracted RNA should be stored refrigerated for short-term storage (<8 h), at −20°C (<1 month) or at −80°C for longer periods.
2.2 One-step real-time RT-PCR
2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	6.1	N.A.
TaqMan™ RT-PCR mix (RNA-to-Ct™ 1-Step kit, *Thermo Fisher Scientific*)	2×	10	1×
Forward primer (RRV_3-5For)	10 µM	0.6	0.3 µM
Reverse primer (RRV_3-5Rev)	10 µM	0.6	0.3 µM
Probe (RRV_3-5Probe)	10 µM	0.2	0.1 µM
TaqMan™ RT Enzyme mix (RNA-to-Ct™ 1-Step RT kit, *Thermo Fisher Scientific*)	40×	0.5	1×
Subtotal		18	
RNA extract		2	
Total		20	

2.2.2 Real-time RT-PCR cycling conditions: reverse transcription at 48°C for 15 min; denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 60 s.

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 the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- 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, gBlock). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene, NADH dehydrogenase subunit 5 or eukaryotic 18S rDNA).
- Specific amplification or co-amplification of nucleic acid from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

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. Duplicate of the plant sample material spiked with nucleic acid from the target organism.

3.2 Interpretation of results

Verification of the controls:

- NIC and NAC: should give no amplification.
- PIC and PAC (and if relevant IC and IPC): amplification curves should be exponential.

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 was validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* by NIVIP-NVWA during preparation of the proficiency test organised by the EURL.

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

4.1 Analytical sensitivity data

RRV was detected up to a 10^{-3} dilution in 10-fold serial dilutions of two isolates (MD and deviating OK-1), and the 'Knock out' isolate was detected up to a 10^{-4} dilution.

4.2 Analytical specificity data

Inclusivity: all three tested RRV isolates were detected.

Exclusivity: no cross reaction observed with prunus necrotic ringspot virus (PNRSV, genus *Ilarvirus*); based on sequence differences at primer and probe positions no cross reactions are expected for other rose-infecting viruses, i.e., apple mosaic virus (ApMV; genus *Ilarvirus*), arabis mosaic virus (ArMV; genus *Nepovirus*), cucumber mosaic virus (CMV; genus *Cucumovirus*), strawberry latent ringspot virus (SLRSV; genus *Stralarivirus*), tomato spotted wilt virus (TSWV; genus *Orthotospovirus*), rose cryptic virus-1 (RoCV1; genus *Alphapartitivirus*), and rose spring dwarf-associated virus (RSDaV; genus *Luteovirus*) (Vazquez-Iglesias et al., 2023).

4.3 Selectivity

Leaf material of *Rosa* spp. has been found to cause inhibition, thus a matrix effect can be expected.

4.4 Other information

In the EURL proficiency test 15 out of 25 laboratories used this test and all had 100% conforming results, although Ct values differed considerably. Nevertheless, these results show the robustness of the test, as laboratories performed variations on the test by using different extraction methods. Most laboratories used the RNeasy Plant Mini kit (Qiagen). Other kits used, were, i.e., MagMax total RNA Isolation Kit (Thermo Fisher); not specified kit (E.Z.N.A.); QuickPick SML Plant DNA kit (Bio-nobile).

APPENDIX 5 - REAL-TIME RT-PCR RNA 3 (ADAPTED FROM DOBHAL ET AL., 2016)

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 and identification of RRV in leaves.
1.2 The test is adapted from Dobhal et al. (2016).
1.3 The target sequence is located on the nucleocapsid gene on RNA 3.
1.4 Oligonucleotides:

Primer/probe	Sequence 5′–3′
RRV2F	TGC TAT AAG TCT CAT TGG AAG AGA AA
RRV2R	CCT ATA GCT TCA TCA TTC CTC TTT G
RRV probe-2	FAM-TGC TAG AGA CAT TGG TAC AAC AAG CAA-BHQ1

1.5 The test has been successfully performed using the TaqMan® RNA-to-Ct™ (Thermo Fisher Scientific) on the Bio-Rad CFX96™ Real-Time PCR system (Bio-Rad).
1.6 Data was analysed using the Bio-Rad CFX Maestro software using an automatic baseline and threshold.

2. Methods

2.1 Nucleic Acid Extraction and Purification
2.1.1 Tissue source: leaves.
2.1.2 RNA extraction procedure is described in Appendix 1A.
2.1.3 Extracted RNA should be stored refrigerated for short-term storage (<8 h), at −20°C (<1 month) or at −80°C for longer periods.
2.2 One-step real-time RT-PCR
2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	6.1	N.A.
TaqMan™ RT-PCR mix (RNA-to-Ct™ 1-Step kit, *Thermo Fisher Scientific*)	2×	10	1×
Forward primer (RRV2F)	10 µM	0.6	0.3 µM
Reverse primer (RRV2R)	10 µM	0.6	0.3 µM
Probe (RRV probe-2)	10 µM	0.2	0.1 µM
TaqMan™ RT Enzyme mix (RNA-to-Ct™ 1-Step RT kit, *Thermo Fisher Scientific*)	40×	0.5	1×
Subtotal		18	
RNA extract		2	
Total		20	

2.2.2 Real-time RT-PCR cycling conditions: reverse transcription at 48°C for 15 min; denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 60 s.

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 the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- 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, gBlock). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene, NADH dehydrogenase subunit 5 or eukaryotic 18S rDNA).
- Specific amplification or co-amplification of nucleic acid from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

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. Duplicate of the plant sample material spiked with nucleic acid from the target organism.

3.2 Interpretation of results

Verification of the controls:

- NIC and NAC: should give no amplification.
- PIC and PAC (and if relevant IC and IPC): amplification curves should be exponential.

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 was validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* by NIVIP-NVWA during preparation of the proficiency test organised by the EURL.

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

4.1 Analytical sensitivity data

RRV was detected up to a 10^{-3} dilution in 10-fold serial dilutions of all three tested RRV isolates (MD and 'Knock out' and the deviating OK-1 isolate).

4.2 Analytical specificity data

Inclusivity: all three tested RRV isolates were detected.

Exclusivity: no cross reaction observed with prunus necrotic ringspot virus (PNRSV, genus *Ilarvirus*); based on sequence differences at primer and probe positions no cross reactions are expected for other rose-infecting viruses, i.e., apple mosaic virus (ApMV; genus *Ilarvirus*), arabis mosaic virus (ArMV; genus *Nepovirus*), cucumber mosaic virus (CMV; genus *Cucumovirus*), strawberry latent ringspot virus (SLRSV; genus *Stralarivirus*), tomato spotted wilt virus (TSWV; genus *Orthotospovirus*), rose cryptic virus-1 (RoCV1; genus *Alphapartitivirus*), and rose spring dwarf-associated virus (RSDaV; genus *Luteovirus*) (Vazquez-Iglesias et al., 2023).

4.3 Selectivity

Leaf material of *Rosa* spp. has been found to cause inhibition, thus a matrix effect can be expected.

4.4 Other information

In the EURL proficiency test 16 out of 25 laboratories used this test and all had 100% conforming results, although Ct values differed considerably. Nevertheless, these results show the robustness of the test, as laboratories performed variations on the test by using different extraction methods. Most laboratories used the RNeasy Plant Mini kit (Qiagen). Other kits used, were MagMax total RNA Isolation Kit (Thermo Fisher); not specified (E.Z.N.A.); QuickPick SML Plant DNA kit (Bio-nobile); Spectrum (Sigma).

APPENDIX 6 - REAL-TIME RT-PCR (FROM VAZQUEZ-IGLESIAS ET AL., 2020b)

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 and identification of RRV in leaves.
1.2 The test is based on Vazquez-Iglesias et al., 2020b.
1.3 The target sequence is located on the replicase gene on RNA 1.
1.4 Oligonucleotides:

Primer/probe	Sequence 5′–3′
RRV-F	GAT TAC CTT GTA GCC AAT TAC TTC TAA CTG
RRV-R	CAT CTT CAA TGA TAT GCT CAA TTT AGT TAA
RRV-Pe	FAM-TGT GTT TGC ACT GTT GAC-MGBNFQ

1.5 The test has been validated using iTaq™ Universal Probes One-Step kit (Bio-Rad) using Viia7, ABI7500 or QuantStudio (Applied Biosystems).
1.6 Data was analysed using the ABI Sequence Detection System or QuantStudio Software using an automatic baseline and threshold.

2. Methods

2.1 Nucleic Acid Extraction and Purification
2.1.1 Tissue source: leaves.
2.1.2 RNA extraction was performed by CTAB (Vazquez-Iglesias et al., 2023). Alternative procedures may also be suitable (see Appendix 1).
2.1.3 Extracted RNA should be stored refrigerated for short-term storage (<8 h), at −20°C (<1 month) or at −80°C for longer periods.
2.2 One-step real-time RT-PCR
2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	6.45	N.A.
iTaq Universal Probes One-Step Kit (*Bio Rad)*	2×	10	1×
Forwad primer (RRV-F)	7.5 µM	1	375 nM
Reverse primer (RRV-R)	7.5 µM	1	375 nM
Probe (RRV-Pe)	5 µM	0.50	125 nM
iScript Reverse Transcriptase *(Bio Rad)*	40×	0.05	0.1×*
Subtotal		19	
RNA extract		1	
Total		20	

* Differing from manufacturer's protocol. Validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

2.2.2 Real-time RT-PCR conditions: reverse transcription at 50°C for 10 min; denaturation at 95°C for 2 min; 40 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 60 s.

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 the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- 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 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). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene, NADH dehydrogenase subunit 5 or eukaryotic 18S rDNA).
- Specific amplification or co-amplification of nucleic acid from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

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. Duplicate of the plant sample material spiked with nucleic acid from the target organism.

3.2 Interpretation of results:

Verification of the controls

- NIC and NAC: should give no amplification.
- PIC and PAC (and if relevant IC and IPC): amplification curves should be exponential.

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 was validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* by Fera. The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

4.1 Analytical sensitivity data

RRV was detected up to a 10^{-4} dilution in 10-fold serial dilutions of extracted RNA from RRV-infected rose leaves homogenates (OK-2 isolate) in RNA from healthy rose leaf.

4.2 Analytical specificity data

Inclusivity: OK-2 isolate was detected.
Exclusivity: No cross-reactions occurred with the following non-target viruses: Arabis mosaic virus (ArMV), European mountain ash ringspot-associated virus (EMARaV), fig mosaic virus (FMV), strawberry latent ringspot virus (SLRSV).

4.3 Data on Repeatability

100%.

4.4 Data on Reproducibility

100%.

APPENDIX 7 - CONVENTIONAL RT-PCR (ADAPTED FROM BRATSCH ET AL., 2017)

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 identification of RRV in leaves.
1.2 The test is adapted from Bratsch et al. (2017).
1.3 The targeted sequence is located on the gene that codes for the movement protein on RNA 4.
1.4 Oligonucleotides:

Primer	Sequence 5′–3′	Amplicon size (bp)
RRV4F1	CGT GAC AGG CTC ACT TGA YT	475
RRV4R1	CCT GAC AGT GCA GAG CTT AAT	

1.5 The test has been successfully performed using the OneTaq® One-Step RT-PCR Kit (New England Biolabs) on the TProfessional Basic Gradient thermal cycler (Biometra).

2. Methods

2.1 Nucleic Acid Extraction and Purification
2.1.1 Tissue source: leaves.
2.1.2 RNA extraction procedure is described in Appendix 1B.
2.1.3 Extracted RNA should be stored refrigerated for short-term storage (<8 h), at −20°C (<1 month) or at −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	N.A.	7.5	N.A.
One*Taq* One-Step Reaction mix (*New England Biolabs*)	2×	12.50	1×
Forward primer (RRV4F1)	10 µM	1	0.4 µM
Reverse primer (RRV4R1)	10 µM	1	0.4 µM
One*Taq* One-Step Enzyme mix (*New England Biolabs*)	25×	1	1×
Subtotal		23	
RNA extract		2	
Total		25	

2.2.2 Conventional RT-PCR conditions: reverse transcription at 48°C for 30 min, denaturation at 94°C for 1 min; PCR: 40 cycles consisting of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and elongation at 68°C for 30 s; final elongation at 68°C for 5 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 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 the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- 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 nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene, NADH dehydrogenase subunit 5 or eukaryotic 18S rDNA).
- Specific amplification or co-amplification of nucleic acid from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

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. Duplicate of the plant sample material 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 and IPC) a band of the expected size is visualized (depending if the target, endogenous or exogenous nucleic acid is used). For RRV expected product size is 475 bp.

When these conditions are met:

- A test will be considered positive if a band of the expected size [475 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 was validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* by UKZUZ.

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

4.1 Analytical sensitivity data

RRV was detected up to a 10^{-3} dilution in 10-fold serial dilutions of extracted RNA from RRV-infected rose leaves homogenate in water.

4.2 Analytical specificity data

Inclusivity: two different isolates of RRV (P479a and P479b, both origin USA) were detected.
Exclusivity: no cross reaction observed with PNRSV (genus *Ilarvirus)*.

4.3 Selectivity

No inhibition caused by leaf material of *Rosa* spp. was observed.

4.4 Data on Repeatability

100 %.

4.5 Data on Reproducibility

100 %.

APPENDIX 8 - POSITIVE CONTROLS (GBLOCKS)

Due to the limited amount of available RRV infected material available for use as controls two positive controls (gBlocks) have been developed and are available from NIVIP-NVWA. The sequence of RRV-A has no mismatches with any of the primers and probes described in Appendices 2–5. RRV-B contains sequences of a divergent isolate and has mismatches with some of the primers and probes described in Appendices 2–5 (Table A3).

Sequences

RRV-A:

TGAACAAAAGAAGAAATCATGTTTAGATTACCTTGTAGCC
AATTACTTCTAACTGTGAAATAATTTTGTTTAAAATCAT
TTGTCATTTCTTCATATGATGTGTTTGCACTGTTGACAT
TAACTAAATTGAGCATATCATTGAAGATGTTATCTGTGA
TGTACATGCACCACAGACAGTTGCAGTAGTTGTTAAACA
GCTGAAGCCATCATGAACCTTTTTAAGATACCATGTATT
ATCATTTGTGGTCTTCTTTTTGAGATCTGCTAAGCATTC
AACGGCTCCATCACAACTATTTACAAATCACCATTGCAG
GTTGTTGCATTATAACTCTGCAGCGTGCTGAACAAGGGT
GGACCATTCCACAAAGGTATAAAAAAGCTTTGGAAAGAA
AGGGTAGAGCCAAACTTGATTTGCAGCTAAAGCAGGAGC
AAAGTTCTTGATCAGTTCTTTGAACTTGCCTATAGCTTC
ATCATTCCTCTTTGATTTGCTTGTTGTACCAATGTCTCT
AGCAAGTTCAACATATGCTTCAGATATGTTTTCTCTTCC
AATGAGACTTATAGCATCATCGAAAGTCAATATAAACTGG
GTCCAATTCTGAACTCTCAGGCTTCACTAACTTCTTATT
TTGAACATTTGTCAACTTGAGGACCCGAAGCTTCTGATC
AGCTCCGATGATAACAGTATCAGCTGCAAGAGTGTTGGAT
GTGCTTGTTCCAGGATTTTCGATCTTCG

RRV-B:

TGGACAAAGGAAGAAATCATATTTAAATTACCTTGTA
GCCAGTTACTCCTAACAGTAAAATAATTCTGTTTAAAAT
CATTTGTCATTTCCTCATATGATGTGTTAGCACTATTAA
CATTAACTAGATTAAGCATATCATTGAAAATATTATCTG
TAATGTACAGGCACCACAAACAGTTGCAGTAGTTGTTAA
ACAACTAAAGCCATCATGGACTTTCTTAAGATACCATGT
AGTATCACTAGTCGTTTTCTTTTTAAGATCTGCCAGACA
TTCAACAGCTCCATCACAACTATTTACAAATCACCATTG
CAGGTTGTTGCATTATAACTCTGCAGCGTGCTGAACAAG
GGTGGACCAATCCACAAAGGTATAAAAAAGCTCTGG
AAAGAAAGGGTAGAAACGAACTTGGGATGCAGCTAGAGC
AGGAGCGAAATTCTTGATCAATTCTTTAAACTTGCCTAT
AGCTTCATCATTCCTCTTTGACTTACTTGTTGTACCAAT
GTCTCTAGCAAGTTCAACATATGCTTCAGATATGTTATC
TCTTCCAATGAGACTTATAGCATCATCGAAAGTCAAT
ATAAATTGGGTCCAATTCTGAAGTTTCAGGCTTCACTAG
CTTCTTATTTTGAACATTTGTCAACTTGAGGACCCTCAG
CTTTTGATCAGCTCCGATGATGACAGTATCAGCTGCAAG
AGTGTTAGATGTGCTTGATCCAGGGTTCTCGATCTTCG

TABLE A3 RRV GenBank accessions with corresponding nt regions used in gBlocks.

	RNA1	RNA 2		RNA 3		Length (bp)
gBlock1	MH581213 3306-3462	MH581214 1273-1409	MH581214 134-252	MH581215 510-669	HQ891904 32-191 (RC)*	733
gBlock2	MN106954 3291-3447	MN120642 1254-1390	MN120626 115-233	MN133413 491-650	MN133354 1232-1391	733
gBlock3	KX013764 93-249	MN120603 1254-1390	MN120601 155-233	NC_015300 514-673	KT835651 32-191 (RC)	733
gBlock4	MZ703628 3304-3460	MZ703629 1271-1407	MZ703629 129-247	MZ703630 508-667	MZ703630 1249-1408	733

*RC, reverse complement sequence.