EUROPEAN AND MEDITERRANEAN PLANT PROTECTION ORGANIZATION
ORGANISATION EUROPEENNE ET MEDITERRANEENNE POUR LA PROTECTION DES PLANTES

21-26498 (20-26415, 20-25769, 20-25607, 19-25253, 19-24902, 19-24547, 18-24334, 18- 23648, 18- 23588)[1]

Instruction to authors on the format and content of a diagnostic protocol

Version 2021-03

GENERAL

In general authors should prepare their texts as computer files according to the Instructions for Authors for *Bulletin OEPP/EPPO Bulletin* (https://www.eppo.int/RESOURCES/eppo_publications/eppo_bulletin) from the very first draft. A template is presented in Appendix 1.

Each protocol should contain all the information necessary for the named pest to be detected and positively identified (for some pests the scope may be more specific, i.e. identification of the "named pest" on specific hosts). Protocols for diagnosis (detection and identification) may be used in different circumstances that may require methods with different characteristics. Diagnostic protocols provide the minimum requirements, which may be a single test or a combination of tests, for reliable diagnosis of the relevant pests. Diagnostic protocols also provide additional tests to cover the full range of circumstances. The tests included in diagnostic protocols are preferably selected on the basis of their analytical sensitivity, analytical specificity, repeatability and reproducibility (and where appropriate analytical selectivity). Further information on performance characteristics are given in PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*. These characteristics of the tests should be indicated in the protocol (see section '*General instructions for both detection and identification tests'* page 3). When information on diagnostic sensitivity and specificity is available this should be indicated. Authors are consequently requested to provide this information when available. When tests have undergone a test performance study, this should be indicated, and the results of this test performance study should be included. As a basic requirement, tests should be repeatable. The protocol should describe the necessary information to be recorded (specific guidance for information to be recorded for PCR tests is given in Appendix 3). The protocol should follow the headings (introduction, identity, detection, identification, reference material, reporting and documentation, performance characteristics, further information, feedback on this Diagnostic Protocol, protocol revision, acknowledgements and references) but may include additional sub-headings appropriate to the pest and its diagnosis. The text given under each heading consists of useful suggestions on points which may be addressed. Standard text is indicated where relevant. Related pests should preferably be covered in the same protocol.

Each illustration (figure or photograph) should be sent as a separate file; preferably PNG or TIF (or JPEG for photographs, GIF for drawings). The minimum resolution is 300 dpi. Copyrights for publication of pictures should be checked. Pictures of symptoms or of morphological characteristics of specific bodies can also be uploaded in the EPPO Global Database (https://gd.eppo.int/ instructions on how to upload photos are given in the section *How to submit new photos?*).
As far as possible detailed instructions on materials and on how to perform tests should be given in numbered Appendices at the end of the Standard. When tests already used in other protocols (or in readily available Manuals) are recommended for the detection of a pest, the authors should not describe them again, but cross-refer as appropriate. When the test is adapted from another published test the original reference should be given and the adaptation described.

Note that in EPPO style, the imperative is not used in the main text of a Standard ("Take samples"…). According to the context one can write for example "samples should be taken" or "samples are taken", or "samples may be taken". However, when tests are provided as "recipes" in an annex the imperative may be used, and should then be used consistently. "Must" is not used.

Definitions of terms that should be used in diagnostic protocols are included in PM 7/76.

Use units as shown and use a full stop for decimal points (e.g. 1.5 µL).

[1] Former versions 17-22367 16- 22137, 16-21547, 15-21171, 15- 21171; 15-20820, 14-19833, 13- 19130, 12- 18298 , 12-18078, 12-17367 11- 17244, 11- 17149, 11- 16814, 11- 16544, 10-16428, 10- 15763

When names of expert are used record them as follows "Family name" 'initial' with no dot between the initials e.g. Smith IM

Latin names should be used throughout the protocol. Common names can be given in brackets after first use of the Latin name.

WARNING

Diagnostic protocols should not instruct NPPOs on measures or actions. Terminology used (in particular in flow diagrams should be carefully considered to avoid any confusion with phytosanitary actions taken by the NPPO (in particular the term 'confirmed' has a particular meaning in legislation and should be avoided).

NOTES ON INDIVIDUAL HEADINGS

1. Introduction

A few introductory sentences on the pest and its importance should be provided. Authors of diagnostic protocols should not write a long introduction incorporating information which in any case appears in the EPPO datasheet and **are not of diagnostic significance**. Information on first records, relationship with other organisms, host range, effects on hosts, or geographical distribution should be cross-checked with existing EPPO information such as EPPO Global Database. **If new information is found by the author, he should inform the EPPO Secretariat of the necessity to update the EPPO data package.**

Standard text

'for an updated geographical distribution consult EPPO Global Database'.
when relevant *'a datasheet providing more information on the biology is also available in EPPO Global Database'.*

If several tests can or should be combined (see sections detection and identification), (a) flow diagram(s) should be prepared. This flow-diagram should define the sequence of steps and indicate whether tests are equivalent or not. The following standard sentence should be included at the end of the introduction section:

Standard text "Flow diagrams describing the diagnostic procedure for "*pest*" are presented in Fig. 1 and 2".

2. Identity

Name: correct scientific name, with authority
Synonyms (including former names):
Acronym: for viruses
Taxonomic position:
EPPO Code:
Phytosanitary categorization: A1 or A2 quarantine pest for EPPO countries; EU annex; or equivalent

The EPPO Global Database should be checked for its content related to taxonomy, scientific name and synonyms. Any discrepancy should be reported to the EPPO Secretariat.
In 2014 at the Workshop on EPPO Diagnostic Protocols organised in the framework of the European Mycology Network experts decided that for fungi the Amsterdam Declaration on Fungal Nomenclature 'one fungus one name' should be implemented in EPPO diagnostic protocols. Authors are consequently encouraged to implement this Declaration when preparing diagnostic protocols. Priority is usually given to the oldest name however it is recognized that there are some exceptions where a case by case decision which is made by the International Commission on the Taxonomy of Fungi. Lists of names are being produced. Current names can be found at index Fungorum (www.indexfungorum), Mycobank (www.mycobank.org) and USDA (http://nt.ars.grin.gov/fungaldatabases/), Genera of fungi database (http://www.generaoffungi.org). it is noted that there may be discrepancies between the databases and that it takes some time for these to be updated so it is recommended to check more than one of these databases and to check the most recent literature.

3. Detection

Pest can be detected on growing plants, on consignments of traded commodities or in other situations (e.g. in soil). The following indications should be provided as appropriate.

- Indicate the commodities on which the pest can be found.
- Describe the symptoms (characteristic features, difference with symptoms from other causes, similarities with symptoms from other causes)

- Explain how to discover the pest in the commodity (e.g. visual, hand lens), in particular in which part of the plant (or other matrix) it will be found, and where it will not be found. Indicate which developmental stages of the pest may be encountered. Sampling methods, depending on likely concentration and distribution of pest should be indicated. Sampling of places of production (fields, orchards, forest plots…) is not covered in EPPO Diagnostic protocols.
- Describe methods for extracting, recovering, and collecting the pest from the samples of plants, plant products or other articles or for demonstrating the presence of the pest in the plants, plant products or other articles. This may include tests for demonstrating the presence of the pest in asymptomatic plant material or other materials (e.g. soil or water), such as ELISA tests or culturing on selective media (the information to be provided for media is presented in Appendix 2).
  PM 7/119 *Nematode extraction* provides procedures for nematode extraction and should be cross-referred to in pest specific diagnostic protocols for nematodes.
- Describe procedures to isolate and culture the pest.

For some pests, illustrations of symptoms on the plant and plant product may be helpful.
Provide information on possible confusion with similar signs and symptoms due to other causes.
When a test allowing both detection and identification of a pest is available, it should be mentioned in both sections.
When quick, presumptive indications of identity (which will later need to be confirmed) exist, they should be mentioned.

4. Identification

In this section, the means of identification that leads to an unequivocal conclusion is described; it may be composed of several steps and different tests. As a general rule, the protocol should recommend one or a few particular means of identification which are considered to have advantages (of reliability, ease of use, speed, cost etc.) over other tests. If the recommended tests require equipment and expertise that are not widely available, other tests should be described. In cases where morphological tests can be reliably used but appropriate molecular tests have been developed, the latter are presented as alternative or supplementary tests.

When morphological identification is a recommended method, details should be provided, as appropriate, on:
- procedures to mount and examine the pest (light microscope, electron microscope)
- description of the morphology of the pest or of colonies, with indication of difficulties in seeing particular structures
- identification keys if necessary (to family, genus, species as appropriate)
- illustrations (drawings or photographs, black-and-white or colour) as appropriate, especially of diagnostic morphological characters

Measurements should be given as follows maximum range outside and common range in the middle e.g. (3.5–) 12.5 (–33.5).

The author should also specify if specialized expertise is generally needed for identification of the pest and if confirmation by a specialist is particularly recommended (at least for a first identification or in case of doubt) or if a complementary method should be performed (e.g. PCR, sequencing).

General instructions for both detection and identification tests

- Editorial instructions

The scope of each test should be provided. Each test should be separately described (e.g. ELISA, electrophoresis, PCR, real-time PCR, RFLP, sequencing). Guidelines for information to be included in a Diagnostic Protocol for molecular tests are presented in Appendix 3. Guidelines for information to be included in a Diagnostic Protocol for pathogenicity tests and tests on indicator plants are presented in Appendix 4. Standards describing procedures for performing methods exist such as PM 7/97 on *Indirect Immunofluorescence test for plant pathogenic bacteria;* PM 7/100 on *Rep-PCR tests for identification of bacteria,* PM 7/101 on *ELISA tests for plant pathogenic bacteria. PM 7/125 ELISA tests for viruses, PM 7/126 Electron microscopy in diagnosis of plant viruses, PM 7/129 DNA barcoding as an identification tool for a number of regulated pests*
Authors are requested to refer to these general Standards when appropriate.
When measurements (e.g. temperature, speed, time…) are given when describing a test, these should be affixed with "approximately" when the author considers this is acceptable (e.g. when a given temperature or a range of temperatures is essential for the test to perform correctly this should be specified).
Some methods (e.g. ELISA) require the inclusion of appropriate controls for an unequivocal conclusion. Since quarantine pests are being considered, it may not always be possible to obtain a sample for a positive control, and an alternative may be suggested (e.g. repeated tests, confirmation by other methods). Guidance should also be provided

on possible confusion with similar and related species or taxa. The essential distinguishing morphological characters or test results (or combinations of these) which result in positive diagnosis should be specified.
When relevant, the diagnostic procedure should be briefly described (e.g. extraction from symptomatic material, presumptive diagnostics with a screening test isolation from…" (see recently published protocols for reference).

- Selection of the tests to be included in a protocol

Tests included in this section, should preferably be validated and performance characteristics provided (a summary sheet for validation data is provided in the section 'Validation data for diagnostic tests' of the EPPO Database on Diagnostic Expertise). These performance characteristics should be provided in the relevant appendices describing the test (information on performance characteristics are given in PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*).
When several tests are mentioned, their advantages and disadvantages should be given as well as to what extent they are equivalent.
When several tests using the same method (e.g. PCR on a specific region) are being considered for inclusion in a diagnostic protocol the author should make a judgement of the overall performance characteristics in order to choose the tests which perform better. Normally if one of the tests has undergone a test performance study (assuming the results of the study were adequate) this should be the only one described in full. Nevertheless, reference to other tests could be given. If the author does not feel able to make a judgement between tests they can all be included with a note to the relevant Panel to request assistance in making this judgement. Tests which have not been published in a journal but have undergone a TPS and performed adequately can be considered for inclusion in a diagnostic protocol. For some pest there is little experience in the EPPO region, however, the Panel on Diagnostic and Quality Assurance recognized that diagnostic protocols for such pests is useful. When full description of these tests is not possible because information is not fully available (e.g. to prepare master mix tables for molecular tests) the information available should be provided but this should be flagged in the protocol that a full description is not possible.

5. Reference material

The author should indicate from where reference material (see PM 7/76) can be obtained. The authors are encouraged to deposit reference material in international reference collections where applicable. Reference to sequences in gene banks should be given when the author is confident about species identity verification. For example, Q-bank (https://qbank.eppo.int/) includes sequences for properly documented species and strains present in collections.

6. Reporting and Documentation

Standard text to be included in this section: "Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis"*

7. Performance characteristics

Standard text to be included in this section: 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

Standard text to be included in this section: "Further information on this organism can be obtained from:"

Indicate the name of institutes or individuals with particular expertise on the pest that would be willing to answer questions or to perform a confirmatory diagnosis.

9. Feedback on this Diagnostic Standard

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

10. Protocol revision

Standard text to be included in this section: 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.

11. Acknowledgements

Standard text to be included in this section: "This protocol was originally drafted by:"

Indicate name and address of the expert who wrote the first draft, and of any others who made major contributions (if appropriate).

12. References

Only references cited in the text should be included.

Appendix 1

Template for a diagnostic protocol

Please use this template when preparing a diagnostic protocol. The text included below each section title is a standard text that you must not change except when "if relevant" is mentioned.

Please consult recently published diagnostic protocols in *Bulletin OEPP/EPPO Bulletin* to see other examples.

PM 7/XXX
European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

Diagnostics
Diagnostic

Pest name in latin

Specific scope
This standard describes a diagnostic protocol for *Pest XX*[2].
This Standard should be used in conjunction with PM 7/76 Use of EPPO diagnostic protocols

Specific approval and amendment
Approved in 20XX-XX.

1. Introduction

If relevant: Flow diagrams describing the diagnostic procedure for "*Pest XX*" are presented in Fig. 1 and 2.

2. Identity

Name:
Synonyms:
Acronym:
Taxonomic position:
EPPO Code:
Phytosanitary categorization:

3. Detection

Sub paragraph 'Test sample requirements' recommended

4. Identification

5. Reference material

6. Reporting and Documentation

Guidelines on reporting and documentation are given in EPPO Standard PM7/77 *Documentation and reporting on a diagnosis*.

7. Performance characteristics

[2] *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.*

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:

9. Feedback on this Diagnostic Standard

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

10. 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.

11. Acknowledgements

This protocol was originally drafted by:

12. References

Examples of Appendices

Appendix for buffers and media (if relevant)
See appendix 2 of the instructions to authors for the minimum content and formatting

Appendix for conventional PCR (if relevant)
See appendix 3 of the instructions to authors for the minimum content and formatting

Appendix for real-time PCR (if relevant)
See appendix 3 of the instructions to authors for the minimum content and formatting

Appendix for pathogenicity tests or tests performed with indicator plants (if relevant)
See appendix 4 of the instructions to authors for the minimum content and formatting

Appendix for the description of other tests (if relevant)
See the section "General instructions for both detection and identification tests" of the instructions to authors for the minimum content and formatting

Appendix 2

Instruction to describe buffer and medium preparation

Recipes for buffers and media should be given in an Appendix and presented according to the format presented in the example below.

The recipe should include:

- the name of the buffer or medium and a recent reference, and when a modified medium is used also the reference of the modified version
- Ingredients usually in grams per Liter (L),
- Quantity of distilled or demineralized water (H_2O)
- pH, if applicable

When media are supplemented with antibiotics quantities should be provided (preferably in units of activity, U) as well as how they should be dissolved and when they should be added.

The agar should be indicated as "*microbiological grade agar*" except if a very specific type of agar must be used, in this case it should be specified.

Example of a medium composition

Modified brilliant cresyl blue-starch medium (mBS):

Potato starch	10.0 g
$K_2HPO_4 \cdot 3H_2O$	3.0 g
KH_2PO_4	5.0 g
L-methionine	0.25 g
Nicotinic acid	0.25 g
L-glutamine	0.25 g
Microbiological grade agar	15.0 g
Distilled water to	1 L
Adjust pH to 6.8-7.0	

Duration and conditions of storage should be provided after the media recipe (e.g.: store the prepared medium at 2-8°C; prepared plates should be stored at 2-8°C in the dark; shelf life of stock and of ready to use medium 7 days at 4 +/- 2^0C; use freshly prepared media; store prepared mixture in the dark at <15°C). If applicable, the author should also specify if conditions are different for ready to use plates.

As medium sterilization conditions are often generic, the specification should be given at the beginning of the Appendix with the following standard text:

Standard text: All media are sterilized by autoclaving at 121°C for 15 min, except when stated otherwise (**to be adapted as necessary**)

If special sterilization conditions are required in certain cases e.g. sterilization by filtration, it should be noted after the media recipe.

Appendix 3

Guidelines for information to be included in a Diagnostic Protocol for PCR Testing

Overview

These guidelines are designed to ensure that the Diagnostic Protocols give the requisite information for reliable reproduction of the polymerase chain reaction (PCR) step in molecular analyses. These guidelines do not require details to be given on how to perform analyses of the amplicons produced by the PCR such as gel electrophoresis, except in situation where specific conditions are required to obtain a clear separation of nucleic acid fragments. However, it includes information required for nucleic acid extraction and purification, as this is a prerequisite for PCR. Also, to enable identification for a large range of organisms using basic molecular technology, it includes minimum information for the set up of reverse transcription reactions and restriction enzyme analyses. These guidelines are designed to introduce a strict structure of the presented information with the aim to ease understanding of the test.

Different PCR-based tests may be distinguished, conventional PCR (including RT-PCR, IC-RT-PCR, PCR-RFLP, nested PCR), real-time PCR (probes based Taqman®, SYBR® Green) and other nucleic acid based methods (e.g. LAMP). The minimum information to be provided for the different PCR-based tests are listed.

Please note that the name q-PCR is not used in EPPO Standards as most tests so far are qualitative and not quantitative.

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The information required for each test type is separated in four sections:

Section 1: General Information – general information on the nucleic acid source and preparation, on the gene(s) if applicable/known and amplicon(s) under investigation, and on the reaction constituents, including all details important for reproducibility of results.

Section 2: Methods – methods on nucleic acid extraction and purification, reverse transcription, (real-time) PCR and RFLP, including details on reaction volumes, precise amounts and final concentrations per reaction required for the test as well as PCR run conditions. The guidance on information to be provided on reaction setup is separated in seven sub-sections,

- 2.1) Nucleic acid extraction and purification,
- 2.2) Reverse Transcription (RT; to produce cDNA from RNA),
- 2.3) Conventional PCR,
- 2.4) One step Reverse Transcription PCR,
- 2.5) Real-time Polymerase Chain Reaction – real-time PCR,
- 2.6) One step real-time Reverse Transcription Polymerase Chain Reaction – real-time RT-PCR,
- 2.7) Restriction Fragment Length Polymorphism (RFLP) Reaction.

Consult the relevant sub section for the test that are to be included in the protocol. Amounts of reagents should be indicated as the final concentration in mM, µM or nM. Enzyme amounts should be given in Units. For DNA/RNA the concentration in ng/µL should be indicated in parenthesis; if crude or non-quantified DNA/RNA is used this should be noted. If using ready-made premixes or buffers only the final concentrations have to be indicated where applicable. The information is presented in an order that allows for easy assembly of the reaction.

Section 3: Essential Procedural Information - information that the authors regard as essential and that is not described in the earlier sections. All essential information not contained in the above sections but necessary for successful performance of the reaction according to the authors (especially where the window for a successful reaction is narrow) should be indicated in this section.

Section 4 Data on performance characteristics available

Standard text to be included under the title of the Appendix '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. Scope of the test (detection and/or identification of species X in matrix Y using method Z)

1.2. Date of establishment of the test and if possible later modifications thereof

1.3. Name of targeted gene or other sequence (e.g. internal transcribed spacer region) (accession number of standard organism[1]) if applicable/known

1.4. Amplicon location (first base pair, based on standard organism[1] - including primer sequences), if applicable/known. When many primer sequences are included they should be presented in a table to facilitate reading.

1.5. Oligonucleotides: Forward primer name, sequence (orientation 5'-3'); Reverse primer name, sequence (orientation 5'-3'); probe name (if applicable), sequence (orientation 5'-3'). If applicable labels and purification methods are given. Note: several primers pairs and probes could be used (e.g. PCR that amplify several sequences).

Presentation as Table format

Forward primer name	sequence (orientation 5'-3')	Amplicon size in base pairs (including primer sequences)
Reverse primer name		
Probes…..		

1.6. Cycler or real-time PCR system or other equipment name, producer name

1.7. Software and settings (automatic or manual) for data analysis.

2. Methods

2.1. Nucleic Acid Extraction and Purification (when several molecular tests are included and nucleic acid extraction is common, the author should consider preparing a single Appendix)

2.1.1. Tissue source (e.g., species and/or strain/isolate name [if applicable], number of organisms and developmental stage [if applicable], infected plant material, bacterial colony, mycelium, soil), sampling and/or homogenization method (if applicable), buffer composition and pH, concentration of all constituents (if known), kit producer name(s) (if applicable)

2.1.2. Nucleic acid extraction method, kit producer name and specific instructions (if applicable), buffer composition and pH, concentration of all constituents (if known)

2.1.3. Nucleic acid cleanup procedure, kit producer name and specific instructions (if applicable), buffer composition and pH, concentration of all constituents (if known)

2.1.4. Specify any requirements for nucleic acids used for input to the master mix (e.g. dilutions of extracted nucleic acids)

2.1.5. Storage temperature and conditions of DNA/RNA: **Standard text**
DNA should preferably be stored at approximately -20°C.
RNA should preferably be stored at approximately -20°C for short term (less than one month) or at approximately -80°C for long term storage.

[1] Standard organisms are used to give users of the protocols precise information on the location of the studied gene(s). Use the taxonomically most closely related organism for which the full genome is known, provide its GenBank accession number, the protein and gene names (where applicable) and the range in base pairs (i.e., from base pair number to base pair number) that the amplified fragment covers on the genome of the standard organism

2.2. Reverse Transcription (RT; to produce cDNA from RNA)

2.2.1. Master Mix (**Table to be included and adapted by authors when applicable)**

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	X	N.A.
RT buffer (*producer name*)	X x	X	1x
$MgCl_2$ (or alternatives) (*producer name*)	X mM	X	X mM
dNTPs (*producer name*)	X mM	X	X mM
(if equimolar amounts are used; otherwise specify the final concentrations individually, dATP, dCTP, dGTP and dDTP)	X1 mM dATP X2 mM dCTP, X3 mM dGTP X4 mM dTTP	X X X X	X1 mM dATP X2 mM dCTP, X3 mM dGTP X4 mM dTTP
Other additive(s) or special enzymes if applicable (*name and producer name*)	X X	X	X X
Primer 1	X µM	X	x µM
reverse transcriptase (RT) (*producer name*) *The RT should be added after the denaturation step (see 2.2.2) depending on the RNA structure. Special care should be paid to possible contaminations*	X U/µL	X	X U
Subtotal		X	
RNA extracts*		X	
Total		X	

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* describe any specific requirements for nucleic acids used for input to the master mix (e.g. dilutions of extracted nucleic acids or amplicons).

For routine use authors are asked to provide information in volume and not in nucleic acid concentration.

2.2.2. Reverse Transcription conditions: (e.g. pre-heating, cooling on ice, RT reaction temperature)

2.3. Conventional PCR

2.3.1. Master Mix **Table to be included and adapted by authors when applicable**

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	X	N.A.
PCR buffer (*producer name*)	X x	X	1x
$MgCl_2$ (or alternatives) (*producer name*)	X mM	X	X mM
dNTPs (*producer name*)	X mM	X	X mM
		X	X1 mM dATP
(if equimolar amounts are used; otherwise specify the final concentrations individually, dATP, dCTP, dGTP and dDTP)	X1 mM dATP X2 mM dCTP, X3 mM dGTP X4 mM dTTP	X X X	X2 mM dCTP, X3 mM dGTP X4 mM dTTP
Other additive(s) or special enzymes if applicable (*producer name*)	X X	X	X X
Forward primer (name)	X µM	X	x µM
Reverse primer (name)	X µM	X	x µM
Polymerase (*producer name*)	X U/µL	X	X U
Subtotal		X	
Nucleic acid extract*		X	
Total		X	

** describe any specific requirements for nucleic acids used for input to the master mix (e.g. dilutions of extracted nucleic acids or amplicons).

2.3.2. PCR conditions: Pre-incubation temperature, time (if applicable as, e.g., for single-tube RT-PCR); initial denaturation temperature, time; cycling denaturation temperature, time (other

specifications); cycling annealing temperature, time (other specifications[3]); cycling extension temperature, time (other specifications[3]); heating ramp speed (if appropriate); cooling ramp speed; cycle number; final extension temperature, time

2.4. One step Reverse Transcription PCR

2.4.1. Master Mix **(Table to be included and adapted by authors when applicable)**

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	X	N.A.
RT-PCR buffer (*producer name*)	X x	X	1x
$MgCl_2$ (or alternatives) (*producer name*)	X mM	X	X mM
dNTPs (*producer name*)	X mM	X	X mM
		X	X1 mM dATP
(if equimolar amounts are used; otherwise	X1 mM dATP	X	X2 mM dCTP,
specify the final concentrations	X2 mM dCTP,	X	X3 mM dGTP
individually, dATP, dCTP, dGTP and	X3 mM dGTP	X	X4 mM dTTP
dDTP)	X4 mM dTTP		
Other additive(s) or special enzymes if	X X	X	X X
applicable (*name and producer name)*			
PCR Forward primer (*name*)	X µM	X	x µM
PCR Reverse primer (*name*)	X µM	X	x µM
RT primers (*name*)	X µM	X	x µM
Polymerase (*producer name*)	X U/µL	X	X U
Subtotal		X	
RNA extract		X	
Total		X	

2.4.2. RT-PCR conditions: Pre-incubation temperature, time; initial denaturation temperature, time; cycling denaturation temperature, time (other specifications); cycling annealing temperature, time (other specifications[4]); cycling extension temperature, time (other specifications[3]); heating ramp speed (if appropriate); cooling ramp speed; cycle number; final extension temperature, time

[3] Other specifications relates to specifications such as incremental/decremental time and/or temperature

[4] Other specifications relate to specifications such as incremental/decremental time and/or temperature

2.5. Real-time Polymerase Chain Reaction – real-time PCR

2.5.1. Master Mix **(Table to be included and adapted by authors when applicable)**

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	X	N.A.
Real-time (RT-)PCR buffer (*producer name*)	X x	X	1x
$MgCl_2$ (or alternatives) (*producer name*)	X mM	X	X mM
dNTPs (*producer name*)	X mM	X	X mM
(if equimolar amounts are used; otherwise specify the final concentrations individually, dATP, dCTP, dGTP and dDTP)	X1 mM dATP X2 mM dCTP, X3 mM dGTP X4 mM dTTP	X X X X	X1 mM dATP X2 mM dCTP, X3 mM dGTP X4 mM dTTP
Other additive(s) or special enzymes if applicable (*name and producer name*)	X X	X	X X
Forward Primer (*name*)	X µM	X	x µM
Reverse Primer (*name*)	X µM	X	x µM
Probe 1 (*name*)	X µM	X	x µM
Polymerase (*producer name*)	X U/µL	X	X U
Subtotal		X	
Nucleic acid extract		X	
Total		X	

2.5.2. PCR conditions: Pre-incubation temperature, time (if applicable as, e.g., for single-tube real-time RT-PCR); initial denaturation temperature, time; cycling denaturation temperature, time (other specifications); cycling annealing temperature, time (other specifications[3]); cycling extension temperature, time (other specifications[3]); heating ramp speed (if appropriate); cooling ramp speed; cycle number; final extension temperature, time, step for fluorescence capture. For real-time PCR based on SYBR® Green: melting curve parameters (e.g. Temperature ramp range 65°to 95°C for XX min with XX data acquisitions per °C/ data acquisition for at least each 0.3 K increase).

2.5.3..

Authors should decide which information on PCR conditions is relevant to include.

2.6. One step real-time Reverse Transcription Polymerase Chain Reaction – real-time RT-PCR

2.6.1. Master Mix **(Table to be included and adapted by authors when applicable)**

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	X	N.A.
Real-time RT PCR buffer (*producer name*)	X x	X	1x
$MgCl_2$ (or alternatives) (*producer name*)	X mM	X	X mM
dNTPs (*producer name*)	X mM	X	X mM
(if equimolar amounts are used; otherwise specify the final concentrations individually, dATP, dCTP, dGTP and dDTP)	X1 mM dATP X2 mM dCTP, X3 mM dGTP X4 mM dTTP	X X X X	X1 mM dATP X2 mM dCTP, X3 mM dGTP X4 mM dTTP
Other additive(s) or special enzymes if applicable (*name and producer name*)	X X	X	X X
PCR forward Primer *(name)*	X µM	X	x µM
PCR reverse Primer *(name)*	X µM	X	x µM
Probe *(name)*	X µM	X	x µM
RT enzyme *(name)*	X µM	X	x µM
RT Primer *(name)* if required	X µM	X	x µM
Polymerase (*producer name*)	X U/µL	X	X U
Subtotal		X	
RNA extract		X	
Total		X	

2.6.2. PCR conditions: Pre-incubation temperature, time (if applicable as, e.g., for single-tube real-time RT-PCR); initial denaturation temperature, time; cycling denaturation temperature, time (other specifications); cycling annealing temperature, time (other specifications[3]); cycling extension temperature, time (other specifications[3]); heating ramp speed; cooling ramp speed; cycle number; final extension temperature, time, step for fluorescence capture. For real-time PCR based on SYBR® Green: melting curve parameters (e.g. Temperature ramp range 65°to 95°C for XX min with XX data acquisitions per °C/ data acquisition for at least each 0.3 K increase)

Authors should decide which information on PCR conditions is relevant to include.

2.7. Restriction Fragment Length Polymorphism (RFLP) Reaction

2.7.1. PCR product purification

2.7.1.1. PCR product cleanup procedure, kit producer name and instructions (if applicable), buffer composition and pH, concentration of all constituents (if known)

2.7.1.2. Concentration of amplified DNA and of all nucleic acid solution constituents, pH of nucleic acid solution, storage temperature and conditions

2.7.2.RFLP Reaction

Table to be adapted by authors

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	X	N.A.
restriction enzyme buffer (*producer name*)	X x	X	1x
Other additive(s) or special enzymes if applicable (*name and producer name*)	X X	X	X X
Restriction enzyme(s) (*corresponding enzyme name(s)*)	X U/µL	X	X U
Subtotal		X	
(purified) PCR product		X	
Total		X	

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

2.7.2.1. Incubation temperature, time

2.7.2.2. Denaturation temperature, time (if applicable) or final concentration, name and producer of restriction enzyme inhibitor (if needed).

3. Essential Procedural Information

3.1. Controls

Controls (text to be adapted by authors depending on the matrix but not on the list of controls, and consider if alternative approaches in the last paragraph should be included in PIC and/or PAC sections!):

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 or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include 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 alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of samples spiked with exogenous nucleic acid (control sequence) that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

When generic primers are used on isolated specimens, this could be considered as an alternative to the Positive Isolation Control.

Standard text to add when relevant *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)*

Other possible controls

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

3.2. **Interpretation of results**: in order to assigning results from PCR-based test the following criteria should be followed:

Conventional PCR tests

Verification of the controls

- NIC and NAC: no band is visualized
- PIC, PAC (and if relevant IC) a band of the expected size [xxx] is visualized (depending if the target, endogenous or exogenous nucleic acid is used). **Text to be adjusted by the author according to the type of PIC and PAC or if relevant IC.**

When these conditions are met:

- A test will be considered positive if a band of the expected size [xxx] 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.

Real-time PCR tests

Standard text

Verification of the controls

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

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Additionally, for SYBR® Green based real-time PCR tests: the TM value should be as expected.
- Tests should be repeated if any contradictory or unclear results are obtained.

Note: if the need for a Ct cut-off value has been identified during the validation of the test this should be stated and authors are encouraged to give a range of Ct values observed for true positive samples. The following sentence should appear at the start of this section as a standard text when a Ct cut-off value is mentioned. *The Ct cut-off value given below is as established in [name of the laboratory] As a Ct cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.*

Identification by sequencing

Guidance for sequence analysis is given in Appendices 7 and 8 of EPPO Standard PM 7/129 *DNA Barcoding as an identification tool for a number of regulated plant pests*(EPPO, 2016).

Author to provide specific guidance on sequencing as necessary. For example see guidance given in the IPPC protocol on pine wood nematode section 4.2.8 available at (. Due to the continuously changing data in the reference libraries, the given percentage is only a guiding value

Other nucleic acid based methods (LAMP)

Verification of the controls

- NIC and NAC should produce no turbidity/colour change or no fluorescence
- PIC, PAC (and if relevant IC) should produce: **Text to be adjusted by the author according to the type of PIC and PAC or if relevant IC.**

  the expected turbidity/colour change (details should be given on the expected change e.g. formation of a precipitate, expected colour change, turbidity). Turbidity can also be measured with instruments, if relevant provide the threshold for positivity with the equipment.

  Fluorescence (end point or real-time measurement). For end point measurement a positive reaction is defined by RFU and/or Tm (°C± known variation). For real-time measurement a positive reaction is defined by time of positivity (minutes) and Tm (°C± known variation).

When these conditions are met:

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

4. Performance characteristics available

The author should indicate if validation was carried out in accordance with PM7/98.

4.1. Analytical sensitivity data
4.2. Analytical specificity data
4.3. Data on Repeatability
4.4. Data on Reproducibility

If additional performance characteristics are available (e.g. diagnostic sensitivity or specificity this should also be provided).

Appendix 4

Information needed for the description of pathogenicity tests or tests performed with indicator plants

- For pathogenicity tests, the name of the plant species and cultivar(s) to be used should be given (ideally this should be the same plant species and cultivar on which the pest was isolated, alternatively another plant known to express symptoms may be selected).
- For tests based on indicator plants, the name of the indicator plant species and cultivar(s) should be given (it may be the same plant species and cultivar on which the pest was isolated, or another plant species known to express symptoms).

The following points apply to both types of tests:

- Plant growth stage to be inoculated
- If the plant should be in a specific condition at the time of inoculation (e.g. to increase the uptake of the pathogen), this should be mentioned.
- Number of test plants and controls (positive and negative)
- Conditions of growth for test plants should be described (greenhouse, growth chamber…), including when appropriate temperature, light and humidity conditions. If conditions should differ before, during and after inoculation this should be mentioned. As for other tests, when temperatures are given these should be affixed with "approximately" when the author considers this is acceptable (when a given temperature is essential for the test to perform correctly this should be specified).
- Type and concentration of inoculation material (e.g. dry spores, bacterial suspensions…), type of structure to be used (e.g. conidia, ascospores, …) and mode of preparation.
- Method of inoculation.
- Description of symptom to be observed and whenever relevant minimum number/percentage of plants on which symptoms should be seen.
- Description of the frequency of observation of the test plants to detect symptoms, indication about the time needed for the first symptoms to appear and maximum time period for the observation.