



International Plant Protection Convention
Protecting the world's plant resources from pests

2019-2020

INSTRUCTIONS TO

AUTHORS

DIAGNOSTIC PROTOCOLS FOR REGULATED PESTS

These instructions are based on International Standard for Phytosanitary Measures ISPM 27. Diagnostic protocols for regulated pests and are compiled to provide more specific explanatory guidance for authors of diagnostic protocols.

*(Revised by the Technical Panel on Diagnostic Protocol (TPDP) in August 2019.
Edited by the IPPC Secretariat)*



Food and Agriculture Organization of the United Nations

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CONTACTS

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- **TPDP web page link:** [Technical Panel on Diagnostic Protocols \(TPDP\)](#) ¹
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¹ Full link address: <https://www.ippc.int/core-activities/standards-setting/expert-drafting-groups/technical-panels/technical-panel-diagnostic-protocols>

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DIAGNOSTIC PROTOCOLS FOR REGULATED PESTS - INSTRUCTIONS TO AUTHORS

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These instructions are based on International Standard for Phytosanitary Measures [ISPM27 \(Diagnostic protocols for regulated pests\)](#) and are compiled to provide more specific explanatory guidance for authors of diagnostic protocols (DPs). Authors are encouraged to study ISPM 27 to ensure that the DP is consistent with the standard. A template for DPs and guidelines on their format are also given.

Additional guidance for drafting groups has been developed to satisfy a demand for consistency in content, structure, semantics, terminology and presentation of IPPC standard setting documents. See also [IPPC style guide for standards and meeting documents](#) and [IPPC Procedure Manual for Standard Setting](#).

1. GENERAL CONSIDERATIONS

1.1 Minimum requirements for reliable diagnosis of regulated pests

Under the heading titled “purpose and use of diagnostic protocols”, ISPM 27 states:

Diagnostic protocols may be used in different circumstances that may require methods with different characteristics. Examples of such circumstances grouped according to an increased need for high sensitivity, specificity and reliability are:

- routine diagnosis of a pest widely established in a country
- general surveillance for pest status
- testing of material for compliance with certification schemes
- surveillance for latent infection by pests
- surveillance as part of an official control or eradication programme
- pest diagnostic associated with phytosanitary certification
- routine diagnosis for pests found in imported consignments
- detection of a pest in an area where it is not known to occur
- cases where a pest is identified by a laboratory for the first time
- detection of a pest in a consignment originating in a country where the pest is declared to be absent.

The ISPM also states:

Diagnostic protocols provide the minimum requirements for reliable diagnosis of regulated pests. This may be achieved by a single method or a combination of methods. Diagnostic protocols also provide additional methods to cover the full range of circumstances for which a diagnostic protocol may be used. The level of sensitivity, specificity and reproducibility of each method is indicated where possible. NPPOs may use these criteria to determine the method or combination of methods that are appropriate for the relevant circumstances.

This means that the minimum requirement usually is applicable to one of the first indents (e.g. routine surveillance). Authors should provide information to help the National Plant Protection Organization (NPPO) make decisions on the methodology required for the relevant circumstances.

If necessary, DPs may describe more than one method to take into account the varying capabilities of laboratories and the situations for which the methods are applied. Such situations include diagnosis of different developmental stages of pests, which require different methodologies, as well as the degree of

certainty required by the NPPO. For some purposes a single method may be sufficient, for others a combination of methods may be necessary. This applies both to the minimum requirements for a diagnosis and where additional requirements are necessary (such as where a high degree of certainty in the diagnosis is required). In cases where morphological methods can be reliably used but appropriate molecular methods have been developed, the latter should be presented as alternative or supplementary methods.

1.2 Other general considerations

DPs are published as annexes to ISPM 27 (*Diagnostic protocols for regulated pests*). They describe procedures and methods for the detection and identification of pests that are regulated by contracting parties of the International Plant Protection Convention (IPPC) and relevant for international trade. They are addressed to diagnosticians/diagnostic laboratories performing official tests as part of phytosanitary measures. The DPs provide guidance on the diagnosis of specified pests. Information is provided on the specified pest, its taxonomic status and the methods to detect and identify it. As indicated in section 1.1, DPs contain the minimum requirements for reliable diagnosis of the specified pest and provide flexibility to ensure the methods are appropriate for a range of circumstances of use.

DPs may cover a species, taxa below species level, several species within a genus, or an entire genus, for example where several species within a genus are regulated pests.

Authors should draft DPs in accordance with the requirements given in the main text of ISPM 27.

General guidelines on the format of DPs are appended. By using these guidelines, authors will help ensure consistency between DPs and facilitate processing of draft DPs. These guidelines will be consolidated as more DPs are developed. Authors are also invited to refer, as a model, to DP 01 (for *Thrips palmi*).

- **Appendix 1:** The template that should be used for drafting DPs.
- **Appendix 2:** General guidelines on the formatting of DPs.
- **Appendix 3:** A check list for authors of diagnostic protocols.
- **Appendix 4:** Some general considerations on the concept of combination of methods in diagnostic protocols.
- **Appendix 5:** Template tables for description of polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR) or PCR - restriction fragment length polymorphism (PCR-RFLP) reactions.
- **Appendix 6:** ELISA controls and interpretation of results
- **Appendix 7:** Control options for molecular tests for pest categories and purposes of the tests.
- **Appendix 8:** Best practices for sequencing: using DNA sequences to diagnose a pest.

DPs are drafted by a group of authors called DP drafting groups, which are coordinated by a lead author and overseen by a discipline lead from the Technical Panel on Diagnostic Protocols (TPDP). The DP drafting group, including the lead author, is recommended by the TPDP discipline lead and approved by the entire TPDP. To ensure global coverage of the protocol and to facilitate adoption, authors should consult relevant experts from different regions outside of the DP drafting group prior to submission of final drafts to the TPDP. A cover note giving the list of experts or countries that have written and reviewed the draft, and any main discussion points that have arisen and been resolved should be included (see Appendix 1).

DPs are reviewed on a regular basis (every 5 years unless a specific issue has been raised). Authors should be aware that this will be done.

2. DEFINITIONS²

- **Pest Diagnosis**: The process of detection and identification of a pest.
- **Reproducibility**: Ability of a test method to provide consistent results when applied to aliquots of the same sample tested in different conditions.
- **Sensitivity (also known as analytical sensitivity)**: Smallest amount of the target that can be detected reliably (target may include live organisms, antibodies, nucleic acids).
- **Specificity (also known as analytical specificity)**: Characteristics of a test as concerns its performance with regard to cross-reactions with non-target (false positives) or lack of reaction with target (e.g. subgroups or individuals of the pest) (false negatives).

3. METHODOLOGY

Each DP should contain the methods and guidance necessary for the named pest or pests to be detected and positively identified by an expert (i.e. an entomologist, mycologist, virologist, etc.). Authors should select methods on the basis of their sensitivity, specificity and reproducibility, also taking into account the availability of equipment, the expertise required for these methods and their practicality (e.g. ease of use, speed and cost). Only methods of relevance for diagnostics should be indicated in the protocol.

All methods should be described separately in a consistent manner with sufficient detail (including equipment, reagents and consumables) to enable experts to perform the test without further reference to the literature. However, common laboratory procedures do not need to be detailed in the text. Brand names should not be given unless they are technically necessary and directly affect the result of the diagnosis (see also below). If the method is based on a commercial kit it is not necessary to repeat the manufacturer's instructions, which can be referred to. DPs should not be written in the form of standard operating procedures but should provide sufficient detail to allow NPPOs to develop such procedures. However, the TPDP at its June 2015 meeting decided that tables for the description of PCR, RT-PCR or PCR-RFLP reactions should be included in the draft DPs (See Appendix 5 for templates). Where appropriate, reference may be made to methodology described in other adopted DPs annexed to ISPM 27.

Where units of measurement are indicated (e.g. temperature, pH), a precise value should be indicated only if it is critical to the method (e.g. an analysis has to be performed at exactly 15 °C). In other cases, either a range of values should be given, or the word "approximately" be used before the value.

Validation data

For all methods, information on their sensitivity, specificity and reproducibility, and specifications from multi-laboratory validation trials (when available) should be included (e.g. ring tests). These data, as far as possible, should be quantitative, but in the absence of quantitative data, qualitative information may be provided. For each method, if any element of the validation data is not available (e.g. sensitivity), it should be mentioned in the method description in order to clearly indicate that this element has not simply be omitted.

Brand names

The names of particular brands of chemicals, reagents and equipment should, as far as possible, be avoided and a correct designation or description of the chemical, reagent or equipment shall be given rather than a trade name (brand name).

A standard paragraph under section "Detection and Identification" on the use of brand names should be added to the DPs, before the first mention of a brand name. The TPDP in 2018-02 revised the text as follows:

² To be modified, if definitions are changed in the document on QA terms (under development).

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and reproducibility achieved. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

Brand names should only be included when the brand is considered to affect the level of specificity, sensitivity or reproducibility quoted in the diagnostic protocol. If it is known that only one chemical, reagent and/or equipment is currently available that is suitable for the successful application of the protocol, the brand name may be given in the text but shall be associated with the following footnote:

The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable.

If in the DP there is more than one mention of a brand name, the second mention (and the subsequent mentions) to a brand name shall be associated with the footnote number from the first mention in the text (e.g. if the first mention to a brand name is accompanied by footnote 1, the subsequent mentions to brand names should be accompanied by the same footnote number, i.e. footnote 1).

Controls

A description of all the controls mentioned must be provided, and the minimum requirements for controls should be indicated.

Guidance on positive and negative controls and reference material should be included for each of the tests. Methods where the inclusion of appropriate controls is essential (e.g. enzyme-linked immunosorbent assay (ELISA)) should be indicated (See also Appendix 6: ELISA controls and interpretation of results). Sources and specifications of controls and reference materials (e.g. catalogue numbers of bacterial reference strains) should be provided.

In the case of a high risk of aerosol contamination, and for certain pests, instructions should be provided to monitor possible cross contamination, e.g. through comparison of sequences of positive controls and samples.

The minimum requirements for controls will vary from test to test and from pest to pest. Therefore, by the time the DP drafting group has defined the minimum requirements for which tests should be performed for diagnosis, it should also have decided the minimum requirements for control.

Additional guidance for control options for molecular tests for each pest category (i.e. each discipline) is provided in Appendix 7.

Methods

Authors should provide information and guidance on methods that either singly or in combination lead to diagnosis of the pest. However, DPs should not instruct NPPOs on the methods to use. Guidance should be provided on the interpretation of results, particularly the criteria for the determination of a positive or negative result for each method. In most cases, interpretation of results may be included within the section for each method. In some cases, a specific section may be needed (e.g. for molecular methods). In the case of conventional PCR the sample is considered negative when a band of the expected size is not produced, regardless of other non-specific bands. General elements considering combinations of methods are provided as Appendix 4 for information. When methods are cross-referred to in different parts of the DP, it may be useful to indicate the section number where the method is fully described.

It is not necessary to include all methods which have been reported for a particular pest, only those which are reliable, currently available and considered to be of use for the purposes described in ISPM 27.

If several methods are needed for the diagnosis, or if many alternative methods are included, a flow diagram may be presented. It should show the different alternative methods that allow the minimum requirements for the diagnosis to be reached. Where relevant, it should present the alternative methods

for specific circumstances (e.g. symptomatic fruit, asymptomatic fruit). The diagram should indicate the reliability of each method or combination of methods. It is not intended to be a decision-making tree but is intended to assist NPPOs in determining which method or methods are appropriate for use under different circumstances. It should not refer to different scenarios or situations of use of the DP (e.g. interception). When authors conclude that a combination of methods is needed, the reasons should be provided. The flow diagram should be accompanied by some explanation in the text, indicating the methods available and their advantages. The flow diagram can first be referred to before methods are described. Each method mentioned in the flow diagram should be accompanied by a cross-reference to the section number where this method is described.

When several methods are mentioned, their advantages and disadvantages should be given (e.g. duration of the test, cost, availability of reagents, requirements for specialized knowledge or equipment, limited validation data available such as covering only some populations of an organism) as well as the extent to which the methods or combinations of methods are equivalent.

Since the use of loop-mediated isothermal amplification (LAMP) may require licensing from specific countries, when it is included in the diagnostic protocol, the following footnote has to be included for every mention of LAMP:

When using LAMP on a regular basis in an area which has a patent system such as Japan (Patent Nos. 3,313,358, 3,974,441 and 4,139,424), the United States of America (US6,410,278, US6,974,670 and US7,494,790), the European Union (Nos. 1,020,534, 1,873,260, 2,045,337 and 2,287,338), China (ZL008818262), the Republic of Korea (Patent No. 10-0612551), Australia (No. 779160), and the Russian Federation (No. 2,252,964), it is necessary for users to receive a license from Eiken Chemical Co., Ltd. before use.

Illustrations

If illustrations (e.g. photographs or line drawings) are essential to the diagnosis, they should be included in the protocol (detailed guidance in Appendix 2). Line drawings, if included, should be sufficient for diagnosis. If original illustrations are included, the author should be named. In addition, photographs that provide additional information but are not essential for the diagnosis may be posted on the IPP. In some cases links may be provided to other web sources for photographs. With regards to possible copyrights, the discipline leads are required to submit the information to the IPPC Secretariat, and the Secretariat will contact authors to obtain any relevant permission to use the photographs or other illustrations. This ensures a proper record of any permission granted to use the illustrations.

If figures or photos are not provided in the draft DP, references to external web links (if available) should be provided in a separate section and added to the list of references.

4. STRUCTURE AND CONTENT OF A DIAGNOSTIC PROTOCOL

It is not possible to provide standardized content of DPs. Adopted DPs can be found at <https://www.ippc.int/core-activities/standards-setting/ispms>. DPs should follow the layout of section 2 of ISPM 27 and should be arranged into the following sections, numbered as follows:

- (1) Pest information
- (2) Taxonomic information
- (3) Detection
- (4) Identification
- (5) Records
- (6) Contact points for further information
- (7) Acknowledgements
- (8) References

Each section should be divided into subsections as required (especially the detection and identification sections) and both sections and subsections should be numbered. Appendix 1 provides a template that should be used for drafting DPs.

An index of the sections should be included at the start of the DP and the pages of the DP numbered. As DPs themselves are annexes to ISPM 27, they should not have annexes or appendixes.

Important note: all data in DPs should be publicly available. Authors should in particular be aware that any material that may be developed specifically for the purpose of the DP, for example keys or photos of characters, will be made publicly available during the development process.

4.1 Pest information

Authors should provide brief information on the pest (generally less than one page of type-written text), including, where appropriate, its life cycle, morphology, variation (morphological, biological or both), relationship with other organisms, host range (in general), effects on hosts, present and past geographic distribution (in general, not country-by-country), mode of transmission and dissemination (vectors and pathways). It is not necessary to include specific details about the epidemiology of the disease or its management.

Supplementary information, such as detailed information on the pest's geographical distribution or hosts, should not be included except when directly relevant for diagnosis. The DP is not intended to be a pest data sheet but reference to such data sheets or databases should be provided when publicly available and considered to provide useful background information. For examples see adopted DPs.

All general information on the pest (e.g. biology, hosts) should be in this section, and not in other sections of the protocol.

Authority and dates of taxonomic information should be included. The discipline lead should provide appropriate sources for species authorities for the different pest groups to the authors and editors. Examples for sources for species authorities are:

- Botany: International Plant Name Index (IPNI; <http://www.ipni.org>), noting that due to potential rapid changes in plant taxonomy, the source and date of the authority should be referred to.
- Fungi: Index Fungorum (<http://www.indexfungorum.org/Names/Names.asp>) or Mycobank (<http://www.mycobank.org/>) are considered appropriate.
- Zoology: Zoobank (<http://zoobank.org>) is considered incomplete. When a taxon is not listed in zoobank, the author surname should be cited in full, with forename initials given where necessary to avoid confusion.
- Bacteriology: List of Prokaryotic Names with Standing in Nomenclature (LPSN; <http://www.bacterio.net>) is considered appropriate.
- Virology: International Committee on Taxonomy of Viruses (ICTV; <https://talk.ictvonline.org/>) is considered appropriate.

If the authority has one or two authors, their names should be given using the style of the respective international scientific organization. If more than two authors, the last name of the first author should be followed by "et al.". References for authorities should not be included in the reference section, except for authors of "Candidatus species" of bacteria as these are treated as a reference rather than an authority. The title of the diagnostic protocol once adopted should not include the authority (although historically the authority has been included in some DPs).

4.2 Taxonomic information

Under this section, the correct scientific (Latin) name, authority and date (no authority/date is required for viruses and viroids) should be given and an overview of the relevant taxonomic hierarchy as appropriate to the type of pest (e.g. Domain, Kingdom, Phylum, Order, Family, Genus, Species, relevant below species taxon).

Include synonyms and relevant former names (these may be taxonomically incorrect but relevant in relation to the literature) as appropriate. Only important synonyms should be mentioned, listed in chronological order. If there are other synonyms, a reference to a publication listing them can be added.

For fungi, the teleomorph name should be used; teleomorph synonyms may be included as appropriate. The anamorph name and its synonyms and macro- or micro-conidial states (as relevant) should also be presented under synonyms. For viruses, internationally recognized acronyms should be included.

The English common names widely used in international scientific literature should also be included. If possible and available, indicate a reference giving common names in other languages (but do not include common names in other languages in this section).

For fungi a reference to Mycobank (<http://www.mycobank.org/>) may be included under Reference.

4.3 Detection

As stated in ISPM 27, this section provides information and guidance on:

- the plants, plant products or other articles capable of harbouring the pest
- the signs and/or symptoms associated with the pest (characteristic features, differences or similarities with signs and/or symptoms from other causes), including illustrations, where appropriate
- the part(s) of the plant, plant products or other articles on/in which the pest may be found
- the developmental stages of the pest that may be encountered, together with their likely abundance and distribution on/in the plants/plant products or other articles
- the likely occurrence of the pest associated with developmental stages of the host(s), climatic conditions and seasonality
- methods for discovering the pest in the commodity (e.g. visual, hand lens)
- methods for extracting, recovering, and collecting the pest from the plants, plant products or other articles, or for demonstrating the presence of the pest in the plants, plant products or other articles
- methods for indicating 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
- viability of the pest.

ISPM 27 also states that “guidance is also provided on resolving possible confusion with similar signs and/or symptoms due to other causes”.

Methods for detection may be interpreted differently depending on the type of pest being considered. For example, detection of an insect may relate to observation of individuals or signs of damage in consignments, whereas detection methods for bacteria may involve culturing extracts of suspected plant material on differential or semi-selective medium.

When a detection method may also be used for identification (e.g. in virology), it is recommended that it is described in the Detection section (see 4.4. for the details to be provided for methods) and then cross-referenced in the following Identification section. Any comments about its use for detection or identification should be included in the relevant section. Methods that detect a group of pathogens rather than a specific pathogen should be described in the detection section.

Sampling in protocols refers to sampling for laboratory analysis, not to sampling for inspection of a commodity. For sampling seeds or grain it might be acceptable to give more details. Sampling procedures for inspectors and inspectors’ instructions on recognition of the pest from signs and symptoms should not be included but only essential information for diagnosis should be given. Procedures for inspectors are likely to be covered in an inspection manual. Additional information on the sample that may be relevant for proper diagnosis should be provided (e.g. minimum sample size, storage conditions).

The use of vendor and brand names should be avoided unless essential for test performance. One paragraph at the beginning of the Detection section should be included to cover all mentions of brand names (see previous section “3. Methodology” on “brand names”).

4.4 Identification

In this section, in addition to a description, authors should provide information and guidance on methods that when used alone or in combination lead to the identification of the pest. Methods for quick, presumptive indications of identity (which will later need to be confirmed) may also be included.

Any method that is specific to identification should be described in this section. If methods that might be used for identification are already described in the Detection section, the description should not be repeated, but cross-reference should be made to the relevant subsections.

Methodologies used in DPs are based on morphological, morphometric or biological characteristics of a pest, or on biochemical or molecular properties (see ISPM 27). Morphological characteristics may be investigated directly or may only be examined after isolation or culturing of the pest. This may also be required for biochemical or molecular assays. Where isolation or culturing procedures are necessary components of methods, details should be provided.

Where appropriate, methods for isolation of pests from asymptomatic plants or plant products (such as tests for latent infection) should be given as well as methods for extraction, recovery and collection of pests from plant or other material. Methods should similarly be provided for direct identification of pests using biochemical or molecular tests on asymptomatic material.

ISPM 27 states:

For morphological and morphometric identifications, details are to be provided, as appropriate, on:

- methods to prepare, mount and examine the pest (such as for light microscopy, electron microscopy and measurement techniques)
- identification keys (to family, genus, species)
- descriptions of the morphology of the pest or of its colonies, including illustrations of diagnostic characters, and an indication of any difficulties in seeing particular structures
- comparison with similar or related species
- relevant reference specimens or cultures.

Guidance should be provided on resolving possible confusion with similar and related species or taxa.

For molecular methods, details should be provided, as appropriate, on:

- the target sequence (e.g. target gene, amplicon size and location) and reaction conditions (e.g. oligonucleotide sequence, enzyme source and thermal cycler). See Appendix 5 for template tables for PCR reactions and Appendix 8 for guidance on using DNA sequences in pest diagnosis.
- nucleic acid extraction and purification (e.g. tissue sources, extraction and purification methods, and nucleic acid concentration)
- reverse transcription (e.g. reaction volume, concentration and volume of reagents, denaturation and incubation temperatures; see Appendix 5 for template tables)
- polymerase chain reaction (e.g. reaction volume, concentration and volume of reagents, thermocycling conditions; see Appendix 5 for template tables)
- restriction analysis (e.g. DNA preparation, reaction volume, concentration and volume of reagents, denaturation and incubation conditions; see Appendix 5 for template reaction tables)
- minimum controls (see also Appendix 7: control options for molecular tests for pest categories and test purposes).

Elements regarding the preservation of specimens, especially for entomology, should be included if necessary. Under the section on identification, guidance should be given on short- and long-term preservation (where relevant).

In the case of diagnostic protocols for insects or nematodes, consider presenting the main characters for the diagnosis in a table (see DP 1: *Thrips palmi*: <https://www.ippc.int/en/publications/586/>).

In the case of diagnostic protocols for plants, if there is no specific difficulty in identifying plants of the species concerned using a key, the text may simply give a reference to a suitable key.

4.5 Records

In this section, authors should refer to section 2.5 of ISPM 27 which lists the records required to be kept. There is no need to repeat section 2.5; only records that are required in addition to those detailed in ISPM 27 should be listed in the DP. However, in addition, authors should include a description of appropriate evidence of results where other NPPOs may be adversely affected by the results of the diagnosis and therefore the records and evidence of the results of the diagnosis should be retained for at least one year.

Standard text to be used, with text in square brackets adjusted to the specific needs of the protocol:

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, [in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)) and where [the pest, name of pest] is found in an area for the first time,] the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability: [list of records, e.g. the original sample, larvae and adults, preserved or slide-mounted specimens, culture(s) of the pest, [RNA, DNA] extracts, printed tissue sections and/or spotted plant extracts on paper or nylon membranes, PCR amplicons or test materials (e.g. photographs [of distinctive taxonomic structures, fungal structures, symptoms and signs], ELISA plate results printouts and photographs of gels].

4.6 Contact points for further information

In this section, authors, in cooperation with the discipline lead, should provide contact details (full name, address, email, telephone, facsimile, etc.) of organizations or individuals with particular expertise on the pest or pests, which may be consulted regarding any questions on the DP. These contacts must agree to act in this capacity prior to their inclusion in the DP.

It might be useful to have global coverage when possible, or at least contacts in several regions. However, if the centre of excellence on the subject is in one region, contacts from this region only may be indicated. In general, it is preferable to avoid mentioning two contacts from the same country, except if they have very specific expertise and no contact is available elsewhere. The Secretariat can also be mentioned, in case none of the contact points can be reached.

Wording from ISPM 27 on requests for revision to the DP should also be added (see below and in Appendix 1).

Standard text to be used:

Further information on this protocol can be obtained from:

[name of institutes and contacts in the format: Unit, institute, complete mailing address, country (full name of expert; email; tel.: +XX etc.; fax: +XX etc.)].

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies to the IPPC Secretariat (ippc@fao.org), who will forward it to the Technical Panel on Diagnostic Protocols (TPDP)."

4.7 Acknowledgements

In this section, the names (initials) and addresses of the experts who wrote the first draft of the DP are given, together with those of any others who made major contributions. This list should be finalized in

consultation between the lead author and the discipline lead. The inclusion of names in the acknowledgements should be at the discretion of the discipline lead in consultation with the lead author. In instances where these experts are the same individuals as those listed in the preceding section, the details should be cross-referenced. Only those significantly involved in the development of the draft should be included in this section.

In addition, special contributions may be mentioned here, for example those experts that made extensive comments on the draft or when the draft protocol made extensive use of work done by others (e.g. ring-testing).

Standard text to be used:

The first draft of this protocol was written by [initials, family name (unit, institution, country, (see preceding section))]. In addition, the following experts were significantly involved in the development of this protocol [initials, family name (unit, institution, country, (see preceding section))].

If drawings or illustrations were produced especially for the protocol, they should be acknowledged in the corresponding figure captions.

4.8 References

ISPM 27 states, “

References to accessible scientific publications and/or published laboratory manuals are given that may provide further guidance on the methods and procedures contained in the diagnostic protocol.”

In this section, relevant references to scientific publications and published laboratory manuals cited in the text should be given. The references should be kept to a minimum and should concern the diagnosis of the pest and species with which the pest may be confused, its symptomatology and methods for extraction, detection and identification. It is not necessary to include a complete list of references concerning geographical distribution, host lists, epidemiology and general biology, although reference may be made to key publications which review this information, e.g. pest data sheets. The number of references included will vary between DPs, but preferably the list should include fewer than 40 references. See the guidelines in Appendix 2 to these *Instructions to authors* for the format of references.

At its November 2014 meeting³, the Standards Committee agreed to add a standard paragraph at the beginning of the reference section to refer to the ISPMs cited in the drafts. This has since been modified slightly, as part of a review of general IPPC style, and the current text is as follows:

The present annex may refer to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at <https://www.ippc.int/core-activities/standards-setting/ispm>.

³ 2014 November SC meeting report: <https://www.ippc.int/core-activities/standards-setting/standards-committee>

APPENDIX 1: STANDARDIZED TEMPLATE FOR DIAGNOSTIC PROTOCOLS

(Last revision: 2019-07)

This standardized template is intended to help authors of diagnostic protocols (DPs) when drafting an IPPC diagnostic protocol. The *Instructions to authors* contain information and guidance on the content and formatting of protocols, as well as on the combination of methods in DPs. Required text is provided in black. Text to be completed by the author and guidance on how to complete it is between square brackets with guidance in italics. Text for completion by the Secretariat or TPDP lead is in square brackets and highlighted in grey. Examples are in boxes in pale green. Authors may use this file to write their draft protocol and can then remove all italics, boxed and highlighted text. A checklist for authors is included as Appendix 3 of the *Instructions to authors*, to cross-check the content of the draft once written.

DRAFT ANNEX to ISPM 27 – [Pest name] [(Topic number)] (*Add the scientific name of the pest (but do not include the authority or year of naming). Secretariat will add the topic number of the subject from the List of topics for IPPC standards*)

Examples

DRAFT ANNEX to ISPM 27 – *Striga* spp. (2008-009)

DRAFT ANNEX to ISPM 27 – *Potato spindle tuber viroid* (2006-022)

DRAFT ANNEX to ISPM 27 – *Erwinia amylovora* (2004-009)

Status box

(Include the table below and complete relevant parts. Secretariat and TPDP lead to complete additional parts as appropriate.)

Date of this document	[to be completed by the Secretariat]
Document category	Draft new annex to ISPM 27 (<i>Diagnostic protocols for regulated pests</i>)
Current document stage	[to be completed by the Secretariat]
Origin	Work programme topic: [Topic (date of addition by CPM)] Original subject: [Name (number)]
	Example Work programme topic: Fungi and fungus-like organisms, CPM-1 (2006) Original subject: <i>Tilletia indica</i> / <i>T. controversa</i> (2004-014)
Major stages	[to be completed by the Secretariat]
Consultation on technical level	The first draft of this diagnostic protocol was prepared by: [first name, FAMILY NAME of lead author (unit, institute, city, ISO code of country) and co-authors] (<i>List the lead author and co-authors – complete addresses are not needed, but the unit, institute, city, country should be mentioned</i>)
	Example The first draft of this diagnostic protocol was prepared by: Dominie WRIGHT (Department of Agriculture and Food of Western Australia, Perth, AU); Guiming ZHANG (Laboratory of Plant Inspection and Quarantine, Shenzhen Entry-Exit Inspection and Quarantine Bureau, Shenzhen City, CN).
	(<i>Add, as appropriate, names of all experts who, although not part of the initial DP drafting group, contributed to the drafting or commented on the draft, as follows:</i>) - In addition, [names of experts (first name, family name (unit, institute, city, country))] [was/were] significantly involved in the development of this protocol.

	<p>- This protocol has been commented upon by: [names of experts (first name, FAMILY NAME (unit, institute, city, ISO code of country))]</p> <p>(Also, other relevant information can be mentioned here, for example:)</p> <p>- This draft protocol was adapted from a protocol originally drafted by: [names of experts (first name, FAMILY NAME (unit, institute, city, ISO code of country))]</p> <p>- It was presented at the [e.g. conference/symposium on (name, place), date], and further comments were provided by: [names of experts (first name, FAMILY NAME (unit, institute, city, ISO code of country))]</p>
<p>Main discussion points during development of the diagnostic protocol [to be updated throughout DP development]</p>	<p>[to be completed by the TPDP lead]</p> <ul style="list-style-type: none"> • • <p>(Note: Especially after experts have been consulted at early stages of development, the cover note should indicate substantial comments that were not incorporated in the draft. Include as bullet points.)</p>
<p>Notes</p>	<p>[to be completed by the Secretariat]</p>

Contents

[to be added later]

The IPPC editor and formatter will adjust this section later.

Adoption

This diagnostic protocol was adopted by the Standards Committee on behalf of the Commission on Phytosanitary Measures in ----. [to be completed after adoption]

The annex is a prescriptive part of ISPM 27 (*Diagnostic protocols for regulated pests*).

1. PEST INFORMATION

[Insert pest information text] (*See section 4.1 of the Instructions to authors*)

Example. *Thrips palmi*

Thrips palmi Karny (Thysanoptera: Thripidae) is a polyphagous plant pest, especially of species in the Cucurbitaceae and Solanaceae. It appears to have originated in Southern Asia and to have spread from there during the latter part of the twentieth century. It has been recorded throughout Asia and is widespread throughout the Pacific and the Caribbean. It has been recorded locally in North, Central and South America and Africa. For more general information about *T. palmi*, see EPPO/CABI (1997) or Murai (2002); online pest data sheets are also available from the Pests and Diseases Image Library (PaDIL, 2007) and EPPO (EPPO, 2008).

The species causes economic damage to plant crops both as a direct result of its feeding activity and from its ability to vector tospoviruses such as *Groundnut bud necrosis virus*, *Melon yellow spot virus* and *Watermelon silver mottle virus*. It is extremely polyphagous, and has been recorded from more than 36 plant families. It is an outdoor pest of, amongst others, *Benincasa hispida*, *Capsicum annuum*, *Citrullus lanatus*, *Cucumis melo*, *Cucumis sativus*, *Cucurbita* spp., *Glycine max*, *Gossypium* spp., *Helianthus annuus*, *Nicotiana tabacum*, *Phaseolus vulgaris*, *Pisum sativum*, *Sesamum indicum*, *Solanum melongena*, *Solanum tuberosum* and *Vigna unguiculata*. In glasshouses, economically important hosts are *Capsicum annuum*, *Chrysanthemum* spp., *Cucumis sativus*, *Cyclamen* spp., *Ficus* spp., Orchidaceae and *Solanum melongena*. The thrips may be carried on plants for planting, cut flowers and fruits of host species, as well as on or associated with packing material, and in soil.

Thrips palmi is almost entirely yellow in coloration (Figures), and its identification is hampered by both its small size (1.0–1.3 mm) and its great similarity to certain other yellow or predominantly yellow species of *Thrips*.

2. TAXONOMIC INFORMATION

(Use the standardized text below and see section 4.2 of the Instructions to authors. Note: Species names are always italicized, whereas family and other names are not (apart from family names for viruses and viroids, which are italicized.)

Name:	[Scientific name, authority and date]
Synonym (or) Synonyms: (delete as appropriate)	[Scientific name, authority and date]
Taxonomic position:	[insert taxonomic information]
Common name (or) Common names: (delete as appropriate)	[English common name(s), and reference, where available, to names in other languages]
Reference:	[for fungi a reference to Mycobank may be included]

Examples - Insects

Name:	<i>Thrips palmi</i> Karny, 1925
Synonyms:	<i>Thrips gossypicola</i> Ramakrishna & Margabandhu, 1939
Taxonomic position:	Insecta, Thysanoptera, Terebrantia, Thripidae
Common name:	melon thrips
Name:	<i>Trogoderma granarium</i> Everts, 1898
Synonyms:	<i>Trogoderma khapra</i> Arrow, 1917 <i>Trogoderma koningsbergeri</i> Pic, 1933 <i>Trogoderma afrum</i> Priesner, 1951 <i>Trogoderma granarium</i> ssp. <i>afrum</i> Attia and Kamel, 1965
Taxonomic position:	Insecta: Coleoptera: Dermestidae
Common names:	khapra beetle (English)

Examples – Virus and viroids

Name:	<i>Plum pox virus</i> (acronym PPV)
Synonym:	<i>Sharka virus</i>
Taxonomic position:	Potyviridae, Potyvirus
Common names:	plum pox, sharka

Examples – Bacteria

Name:	' <i>Candidatus Liberibacter solanacearum</i> ' (Liefting et al., 2009)
Synonym:	' <i>Candidatus Liberibacter psyllaurosus</i> ' (Hansen et al., 2008)
Taxonomic position:	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, ' <i>Candidatus Liberibacter</i> '
Common names:	zebra chip (English), zebra complex (English), psyllid yellows (English)

3. DETECTION

[Insert text on detection of the pest] (See sections 3 and 4.3 of the Instructions to authors)

After the main heading, **3. Detection**, insert introductory paragraphs, and organise the methods using the structure below. Subheadings should be used as required (numbering for illustrative purposes only). It is not possible to provide standardized text in this section, but examples can be found in adopted DPs.

Where detection or identification methods are different for plants with symptoms and plants without symptoms, consider separating section 3 into "3.1 Detection in symptomatic plants" and "3.2 Detection in asymptomatic plants", and use the structure below for each of the subsections.

Where brand names are used in the protocol:

a) include the standard paragraph before the first mention, as below:

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and reproducibility achieved. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

b) at the first mention of a brand name, include the following footnote:

The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable.

c) if in the DP there is more than one mention of a brand name, the second mention (and subsequent mentions) of a brand name shall be associated with the footnote number of the first mention with the full text (e.g. if the first mention of a brand name is footnote 1, the subsequent mentions to brand names should be accompanied by the same footnote number, i.e. footnote number 1).

3.1 Symptoms

[Insert text on signs and symptoms of infection] 3.2 Sampling and sample preparation [symptomatic and asymptomatic material]

[Insert text on sampling and sample preparation]

(If methods for preparation of material are generic for all subsequent detection and identification methods, it may be appropriate to include text on preparation of material in a general section at the beginning. Alternatively, if preparation of material relates to a group of methods it may be appropriate to include text associated with each type of methodology. Otherwise, where preparation of material is specific to a method, it should be included in the method description. See also section 4.3 of the Instructions to authors.)

3.3 Isolation [and culturing/growing] [from symptomatic material /from asymptomatic material]

3.3.1 [Name of method] **e.g. Enrichment isolation**

3.3.2 [Name of method] etc.

3.4 Biological detection

3.5 Serological detection

3.5.1 Preparation of material

(If relevant, see note at 3.2)

3.5.2 [Name of method] **e.g. Double-antibody sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA)**

3.5.3 [Name of method] **e.g. Immunofluorescence (IF)**

3.6 Molecular detection

3.6.1 Preparation of material

(If relevant, see note at 3.2)

3.6.2 Nucleic acid extraction

3.6.3 [Name of method] e.g. Conventional reverse transcription-polymerase chain reaction using the primers of Verhoeven *et al.* (2004)

3.6.4 [Name of method] e.g. Immunocapture reverse transcription-polymerase chain reaction

3.6.5 Controls for molecular tests

Appendix 7 provides summary guidance for control options for molecular tests for different pest categories and test purposes.

Insert the following standardized text:

For the test result to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolations and amplification of the target pest or target nucleic acid. For [method name], [name minimum controls, e.g. a positive nucleic acid control, an internal control and a negative amplification control (no template control)] are the minimum controls that should be used.

(The rest of this section should provide a brief description of the controls as outlined below. The minimum controls should be listed first, in the same order as they are named in the standard paragraph. Additional controls, if any, should be at the end. For each control, amend as appropriate, giving additional details as necessary, e.g. specific controls named in individual methods in the protocol, etc.)

Positive nucleic acid control

This control is used to monitor the efficiency of the test method (apart from the extraction) [and with RT-PCR, the amplification]. [Description of the controls, e.g. Pre-prepared (stored) viroid nucleic acid, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product)] may be used.

Internal control

For [method name(s)], plant internal controls [name(s) of gene(s) e.g. House Keeper Gene (HKG) such as COX or NAD] should be included to eliminate the possibility of PCR false negatives due to extraction failure, nucleic acid degradation or the presence of PCR inhibitors. Preferably the internal control primers should be used [*add details, e.g. in a duplex reaction with the pospiviroid/PSTVd primers*].

(Add any qualifying information e.g. difficulties that may be encountered, effects on sensitivity, notes on the part of the assay that the gene acts as a control for (e.g. with RT-PCR assays). Also provide examples of successful use of internal controls if known or relevant and not already referred to in the method descriptions in other sections.)

When an internal control is not mentioned in the description of a PCR method, the laboratory should choose an internal control and validate it.

Negative amplification control (no template control)

This control is necessary for conventional and real-time PCR to rule out false positives due to contamination during the preparation of the reaction mixture. PCR grade water that was used to prepare the reaction mixture is added at the amplification stage.

(A qualifier (e.g. “with the target DNA” or “with other genetic material” may be inserted after “contamination” to differentiate from the ISPM 5 meaning of “contamination”).

Positive extraction control

This control is used to ensure that nucleic acid from the target is of sufficient quantity and quality and that the target is detected. Nucleic acid is extracted from infected host tissue or healthy plant tissue that has been spiked with the target.

The positive control should be approximately one tenth of the amount of [type of material e.g. leaf tissue] used per plant for the [RNA/DNA] extraction. (*Add any other relevant elements, e.g. adjustments to quantity, amounts of control material to use for different bulking rates, and if this control is not detected, provide guidance on repeating tests or adjusting the bulking rate until reliable detection is achieved.*)

In the case of a high risk of aerosol contamination, and for specific pests, consider if instructions should be provided to monitor possible cross contamination, e.g. by comparing the sequences of positive controls and positive samples.

Negative extraction control

This control is used to monitor both contamination during nucleic acid extraction and cross-reaction with host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified. It is recommended that multiple controls be included when large numbers of positives are expected.

(Amend as appropriate, e.g. omit reference to host tissue if not relevant.)

3.6.6 Interpretation of results from [Name of methods]

(Insert as a separate section only if necessary)

4. IDENTIFICATION

[Insert text on identification methods] (*See section 3 and 4.4 of the Instructions to authors*)

It is not possible to provide standardized text in this section, but examples can be found in adopted protocols.

*After the main heading, **4. Identification**, insert introductory paragraphs, and use the structure below. Use the following headings as required (numbering for illustrative purposes only).*

4.1 Morphological identification (Note: for insects, fungi, nematodes, plants)

4.1.1 Preparation of [developmental stage e.g. larvae, adults, seeds, plant material, teliospores] for examination (If necessary, normally for insects.)

4.1.2 Isolation [and culturing/growing] of [name of pest]

4.1.2.1 [Name of method] e.g. Germination of teliospores, Germination of similar Tilletia species

4.1.3 Identification of [developmental stage e.g. larvae, adults of] [family, genus, name of pest]

4.1.4 [Differentiation of / morphological comparison with] [developmental stage e.g. larvae, adults of][family, genus, name of pest] from similar species

[Insert simple key, table or text with relevant details]

4.1.5 Discriminating features of [developmental stage e.g. larvae, adults, name of pest] [of family, genus, name of pest]

[Insert checklist of key diagnostic features]

(Add additional sections (and renumber) depending on the level of discrimination e.g. family, genus, species.)

(For subsequent sections (Biological identification, Serological identification and Molecular identification) follow the same structure as given in section 3. In addition, sections on identification using nutritional and enzymatic tests or biochemical identification methods may be required. If some elements are already described adequately in section 3 (e.g. preparation of material, nucleic acid extraction, specific methods), do not repeat but cross-refer to the relevant subsection number.)

4.2 Biological identification of [name of pest, strains, pathotypes]

4.2.1 Pathogenicity tests

4.3 Serological identification

4.3.1 Preparation of material

(If relevant, see note at 3.2)

4.3.2 [Name of method] (insert new section for each method)

4.4 Molecular identification

4.4.1 Preparation of material

(If relevant, see note at 3.2)

4.4.2 Nucleic acid extraction

4.4.3 [Name of method]

(Insert new section for each method)

4.4.4 Controls for molecular tests

[Insert standardized text from 3.6.5 with appropriate modification] (Insert this section only if necessary i.e. if controls used for identification tests are different to those for detection.)

4.4.5 Interpretation of results from [Name of methods]

(Insert text only if necessary and if interpretation of results is different when methods are used for identification rather than detection.)

Examples of structure of 4. Identification – DP 1: *Thrips palmi*

General introductory paragraphs

- 4.1 Morphological identification of the adult thrips
 - 4.1.1 Preparation of thrips for microscopic examination
 - 4.1.2 Identification of the family Thripidae
 - Table 1: Family Thripidae – shared characteristics
 - Table 2: Genus *Thrips* – shared characteristics, adult specimens
 - 4.1.4 Identification of *Thrips palmi*
 - 4.1.4.1 Morphological characteristics of *Thrips palmi*
 - Table 3: A list of morphological characteristics that collectively distinguish *Thrips palmi* from other species in the genus *Thrips*
 - 4.1.4.2 Comparison with similar species (species that are yellow without darker body markings, or predominantly yellow, or sometimes yellow)
 - Table 4: Simplified checklists of the diagnostic features for quick recognition: (a) the genus *Thrips*; (b) *Thrips palmi* (See Figure 4 for the location of the various features.)
- 4.2 Molecular assays for identifying *Thrips palmi*
 - 4.2.1 SCAR marker-generated sequence-based real-time PCR assay for *Thrips palmi*
 - 4.2.2 COI sequence-based real-time PCR assay for *Thrips palmi*
 - 4.2.3 ITS2 sequence-based PCR-RFLP assay for nine species of thrips including *Thrips palmi*
 - 4.2.4 COI sequence-based PCR-RFLP assay for ten species of thrips including *Thrips palmi*

5. RECORDS

(Include the following standardized text:)

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

(Add additional paragraph(s) as required in individual DPs. For example:)

In cases where other contracting parties may be affected by the results of the diagnosis, [in particular in cases of non-compliance (ISPM 13, *Guidelines for the notification of non-compliance and emergency action*) and where [the pest, name of pest] is found in an area for the first time,] the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability: [list of records, e.g. the original sample, larvae and adults, preserved or slide-mounted specimens, culture(s) of the pest, [RNA, DNA] extracts, printed tissue sections and/or spotted plant extracts on paper or nylon membranes, PCR amplicons or test materials (e.g. photographs [of distinctive taxonomic structures, fungal structures, symptoms and signs), ELISA plate results, printouts or photographs of gels].

(Additional specific text may be added. For example details on preparation of sample and records may be required e.g. storage temperature (at -80°C or freeze-dried and stored at room temperature) or culture conditions (e.g. mycelium from broths or mycelial plugs from agar plates can be stored frozen at -80°C). Guidance may be included on handling isolates shown to have different molecular or biological characteristics compared to previously recorded isolates (e.g. offered to a national pest herbarium). Also, if there is evidence of any of the tests described failing to detect an isolate, authors may propose that details should be sent to the IPPC Secretariat.

In some cases, records of the number of positive subsamples and the estimated number of [telio]spores detected in each positive subsample may need to be kept and, for fungi, records of colony morphology, especially any pigmentation and growth rate under defined conditions, may need to be kept.)

6. CONTACT POINTS FOR FURTHER INFORMATION

(Add the following standardized text. See section 4.6 of the Instructions to authors.)

Further information on this protocol can be obtained from:

[*name of institutes and contacts in the format: Unit, institute, complete mailing address, country (full name of expert; email; tel.: +XX etc.; fax: +XX etc.)*].

Examples

Faculty of Horticultural Science, Department of Plant Pathology, Corvinus University, Villányi út 29-43, H-1118 Budapest, Hungary (Laszlo Palkovics; email: laszlo.palkovics@uni-corvinus.hu; tel.: +36 14825438; fax: +36 14825023).

Department of Agriculture and Food Western Australia, Biosecurity & Research Division, Plant Biosecurity Branch, Entomology Unit, 3 Baron-Hay Court, South Perth, WA 6151, Australia (Andreas Szito; email: aszito@agric.wa.gov.au; tel.: +61 8 9368 3248, +61 8 9368 3965; fax: +61 8 9368 3223, +61 8 9474 2840).

Pest and Disease Identification Team, The Food and Environment Research Agency, Sand Hutton, York YO41 1LZ, United Kingdom. (Dom Collins; email: dom.collins@fera.gsi.gov.uk; tel.: +44 1904 462215; fax: +44 1904 462111).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on

Phyosanitary Measures (CPM) subsidiary bodies to the IPPC Secretariat (ippc@fao.org), who will forward it to the Technical Panel on Diagnostic Protocols TDPD.

7. ACKNOWLEDGEMENTS

(Add the following standardized text indicating the experts that first drafted the text and those that made significant contributions. If the address was already mentioned in section 6, add “(see preceding section)”.)

The first draft of this protocol was written by [first name, family name (unit, institution, country, (see preceding section))]. In addition, the following experts were significantly involved in the development of this protocol [first name, family name (unit, institution, country, (see preceding section))].

(As relevant, use standardized text below – see section 4.7 of the Instructions to authors.)

[Line drawings, Illustrations] for Figure [number] were produced by [name and address of expert]. The methods included in the protocol were ring tested by [names of experts or project and date] financed by [name of country organization and date].

(If relevant add other acknowledgements as necessary – see examples below. Contributors of figures are credited in figure captions, rather than in the Acknowledgements section.)

Example

DP 2: Plum pox virus

This diagnostic protocol was drafted by M. Cambra, A. Olmos and N. Capote (IVIA, Spain (see preceding section)), N.L. Africander (Department of Agriculture, Forestry and Fisheries, Stellenbosch, South Africa), L. Levy (USDA, United States of America (see preceding section)), S.L. Lenardon (Instituto de Fitopatología y Fisiología Vegetal - Instituto Nacional de Tecnología Agropecuaria (IFFIVE-INTA), Córdoba, Argentina), G. Clover (Plant Health & Environment Laboratory, Ministry for Primary Industries, Auckland, New Zealand) and D. Wright (Plant Health Group, Central Science Laboratory, Sand Hutton, York, United Kingdom).

Additional acknowledgements:

DP 13: *Erwinia amylovora*

Most techniques described were ring tested in a DIAGPRO project financed by the European Union in 2003, in an EUPHRESCO project in 2009, and in a Spanish project in 2010.

DP 7: Potato spindle tuber viroid

Thanks are due to S.L. Nielsen (Denmark), L. Seigner, S. Winter, M. Wassenegger (Germany), H. Koenraad (The Netherlands), A. Fox, T. James, W. Monger, V. Mulholland (UK) for helpful comments during development of this protocol.

8. REFERENCES

[Insert references]

The following standard text should be provided before listing the references:

The present annex may refer to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at <https://www.ippc.int/core-activities/standards-setting/ispm>.

(Provide a list of scientific references and other publications referred to in the protocol (see 4.8 in the Instructions to authors)

9. FIGURES

[Insert figures if necessary]

If figures or photos are not provided in the draft DP, references to external web links (if available) should be provided in a separate section before the list of references and added to the list of references. (See section 3 in the Instructions to authors, as well as Appendix 3.)

Examples of figure legends

Figure 1. *Thrips palmi*, female (left) and male (right) (scale bar: 500 μm = 0.5 mm).
Photo courtesy of A.J.M. Loomans, PPS, Wageningen, the Netherlands.

Figure 2. *Trogoderma granarium*: (A) adult, female; (B) comparison of shape of female (left) and male (right); (C) young larva; (D) mature larva. Scale bar: (A), (B), (D) = 2 mm; (C) = 1 mm.
Photos courtesy of (A) Tomasz Klejdysz, Instytut Ochrony Roślin - Państwowy Instytut Badawczy, Poznań, Poland; (B), (D) Ya.B. Mordkovich and E.A. Sokolov, All-Russian Plant Quarantine Centre, Bykovo Russia; and (C) Cornel Adler, Julius Kühn-Institut (JKI) Germany.

Figure 1. Flow diagram showing the process to be used for the detection and identification of *Tilletia indica* in seed and grain samples.

APPENDIX 2: GUIDELINES ON FORMATTING OF DIAGNOSTIC PROTOCOLS

(Last revision: 2019-07)

General guidelines on content and structure of ISPMs are given in the *IPPC style guide* (<https://www.ippc.int/en/publications/132/>). This Appendix partly uses these guidelines but also gives additional recommendations that are specific to DPs. A standardized format for protocols is also under development.

1. First Page

The first page should contain:

- a reference to ISPM 27 (*Diagnostic Protocols for Regulated Pests*) (i.e. “Annex to ISPM 27”)
- the title of the draft protocol
- a status box in the format of Appendix 1, indicating major steps of the development of the DP, experts/countries that have written and reviewed the draft, and any main discussion points that have arisen and been resolved
- a table of contents, listing all numbered headings and subheadings (at the drafting stage, the table of contents should be in the protocol, but it is not necessary to indicate page numbers).

2. Main Text

Numbered headings and subheadings

Individual sections are detailed in the *Instructions to authors*. Headings, subheadings and further subdivisions should be numbered with Arabic numbers, for example: 1.1, 1.2.1, 1.3.2.2.

Titles of level one (1., 2. etc) have a capital letter at the beginning of each word. Other numbered titles have only one capital letter at the beginning of the title.

Use of illustrations and tables

All illustrations (i.e. photographs, line drawings, flow diagrams) and tables should be numbered with Arabic numbers and should be referred to in the text.

Figures/tables and text should match, i.e. all figures/tables should be referred to in the text, or should not be in the protocol. If a figure refers to several separate elements/characters, these elements should also be cross-referred to in the text. The flow diagram should indicate, for each method, the section number under which it is described.

In the interest of file size, full resolution figures should not be in the main text of the protocol if they are too big, but should be provided to the discipline lead as a separate file, with a compressed version together with the caption and any associated text in the protocol itself. Separate Word or PowerPoints files may be provided. Tables should remain with the text of the protocol.

All photographs, or specially drafted or reproduced illustrations should have an attribution. The text may be small type size and oriented vertically at the side of a photograph or it may be included in the figure caption. The discipline leads are required to submit information on copyrights to the IPPC Secretariat, and the Secretariat will contact authors to obtain any relevant permission to use photographs or other illustrations.

Illustrations should be of a sufficient quality for printing. A high quality file of each illustration should be provided, separately from the text, to the IPPC Secretariat. Detailed guidance is provided below:

- (1) Ensure that images (photographs, diagrams, etc.) have a resolution of 300 dpi for sharp printing, and that the printed image is clear, illustrative for the purpose and of sufficiently high quality.

- (2) Reduce images (at 300 dpi) to the smallest final dimensions that convey the necessary information in the image (5–8 cm is considered as a good width for most illustrations). If a full page illustration is needed, maximum width is 16 cm).
- (3) Crop all unnecessary parts of the image.
- (4) Ensure all text concerning the image (explanatory detail with arrows or call-outs etc.) is part of the caption or is linked together. (A lot of separate boxes with details of identification of image number and insect parts poses a great risk of error.)
- (5) At a late stage of DP development (when member comments are integrated and the protocol is being prepared for adoption, i.e. once the figures will not change anymore), also provide all figures/photographs as separate TIF or JPG files, so that they can be further processed to achieve the optimal file size and quality.

Use of footnotes

Use of footnotes should be limited to increase readability of the text. If footnotes are needed, they should be numbered with Arabic numbers. Note: Footnotes are required if the DP uses loop-mediated isothermal amplification (LAMP) as a method in order to include the license disclaimer, or if brand names for equipment or reagents are used (see section 3 of the *Instructions to authors*).

Terminology

- Phytosanitary terms should be used according to the most recent version of ISPM 5: *Glossary of phytosanitary terms* (<https://www.ippc.int/en/publications/622/>).
- The general dictionary reference for English ISPMs is the Oxford English dictionary.
- Use organize, authorize and recognize (and not organise, authorise or recognise).
- Use website and not Web site or Website.

Scientific names

- Taxonomic family names are italicized only for viruses and viroids (not for insects, bacteria, etc.).
- Indicate the authority after the first occurrence (in the text) of the scientific name of a pest.
- The species name should be written in full at its first occurrence (e.g. *Thrips palmi*) and shortened thereafter (*T. palmi*). At the first mention of a new species from the same genus, the genus should be written in full again (except in lists and tables). Scientific names are italicized (but not spp., sp. etc.)
- Use Scientific names for host plants (common names may be indicated between brackets at first occurrence if appropriate).

Measurement units

When measurement units are abbreviated, the standard abbreviation should be used, e.g.:

m	metre
s	second
W	watt
min	minute
L	litre (but write “litre” in full in the main body of the text, where possible)
mL	millilitre
μL	microlitre
M	molar
U	unit

There should be a space between the number and the unit.

In the body text

Other specific formatting

- Gene names are italicized when written in full, except the gene number (e.g. *NADH dehydrogenase 5* gene)
- Acronyms should be written in full at the first mention.

List of references

References should be in alphabetical order.

References to other ISPMs and the IPPC are detailed in the *IPPC procedure manual* and in the *IPPC style guide*, but usually not needed in protocols. The following standard text should be included at the beginning of the reference section:

The present annex may refer to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at <https://www.ippc.int/core-activities/standards-setting/ispms>.

Regarding scientific references and other publications, some examples are given below. Attention is drawn to the fact that the total number of pages should be included for references to books.

Article in a journal

- Bhatti, J.S.** 1980. Species of the genus *Thrips* from India (Thysanoptera). *Systematic Entomology*, 5: 109–166.
- Brunner, P.C., Fleming, C. & Frey, J.E.** 2002. A molecular identification key for economically important thrips species (Thysanoptera: Thripidae) using direct sequencing and a PCR-RFLP-based approach. *Agricultural and Forest Entomology*, 4: 127–136.
- Kox, L.F.F., van den Beld, H.E., Zijlstra, C. & Vierbergen, G.** 2005. Real-time PCR assay for the identification of *Thrips palmi*. *Bulletin OEPP/EPPO Bulletin*, 35: 141–148.
- Mordkovich, Ya.B. & Sokolov, E.A.** 2000. Выявление капрового жука в складских помещениях, *Защита и карантин растений*, 12: 26–27 (in Russian).
- Mound, L.A. & Morris, D.C.** 2007. A new thrips pest of *Myoporum* cultivars in California, in a new genus of leaf-galling Australian Phlaeothripidae (Thysanoptera). *Zootaxa*, 1495: 35–45.

Books or conference proceedings

- Joyce, S.A., Reid, A., Driver, F. & Curran, J.** 1994. Application of polymerase chain reaction (PCR) methods to identification of entomopathogenic nematodes. In A.M. Burnell, R.-U. Ehlers & J.P. Masson, eds. *COST 812 Biotechnology: Genetics of entomopathogenic nematode-bacterium complexes*. Proceedings of Symposium and Workshop. St Patrick's College, Maynooth, Co. Kildare, Ireland, pp. 178–187. Luxembourg, European Commission, DG XII. 277 pp.
- King, A.M.Q., Adams, M.J., Carstens, E.B. & Lefkowitz, E.J.,** eds 2012. *Virus taxonomy: Classification and nomenclature of viruses*. Ninth Report of the International Committee on Taxonomy of Viruses. San Diego, CA, Elsevier Academic Press. 1337 pp.
- Mound, L.A. & Kibby, G.** 1998. *Thysanoptera. An identification guide*. 2nd edition. Wallingford, UK, CABI. 70 pp.

Monograph in a series

- Nakahara, S.** 1994. *The genus Thrips Linnaeus (Thysanoptera: Thripidae) of the New World*. USDA Technical Bulletin No. 1822. 183 pp.
- Sakimura, K., Nakahara, L.M. & Denmark, H.A.** 1986. *A thrips, Thrips palmi Karny (Thysanoptera: Thripidae)*. Entomology Circular No. 280. Division of Plant Industry, Florida Department of Agriculture and Consumer Services. 4 pp.

Section from a book

- Almeida, R.P.P., Coletta-Filho, H.D. & Lopes, J.R.S.** 2014. *Xylella fastidiosa*. In: D. Liu, ed. *Manual of security sensitive microbes and toxins*, pp. 841–850. Boca Raton, FL, CRC Press. 884 pp.
- EPPO/CABI.** 1997. *Thrips palmi*. In: I.M. Smith, D.G. McNamara, P.R. Scott & M. Holderness, eds. *Quarantine pests for Europe*, 2nd edition. Wallingford, UK, CABI. 1425 pp.
- White, T.J., Bruns, T., Lee, S. & Taylor, J.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White, eds. *PCR protocols: A guide to methods and applications*, pp. 315–322. New York, NY, Academic Press. 482 pp.

CD-Rom:

- Moritz, G., Mound, L.A., Morris, D.C. & Goldarazena, A.** 2004. *Pest thrips of the world: visual and molecular identification of pest thrips* (CD-ROM). Brisbane, Australia, Centre for Biological Information Technology (CBIT), University of Brisbane. ISBN 1-86499-781-8.

Article from proceedings

- Murai, T.** 2002. The pest and vector from the East: *Thrips palmi*. In: R. Marullo & L.A. Mound, eds. *Thrips and tospoviruses: proceedings of the 7th International Symposium on Thysanoptera*, Italy, 2–7 July 2001, pp. 19–32. Canberra, Australian National Insect Collection.

Internet documents or websites

- EPPO.** n.d.. EPPO. Available at <http://www.eppo.org/> (last accessed 17 June 2008).
- PaDIL.** n.d. Pests and Diseases Image Library. Available at <http://www.padil.gov.au> (last accessed 18 Oct 2007).
- USDA** (United States Department of Agriculture). 2004. *Minimum sanitation protocols for offshore geranium cutting production*. APHIS-PPQ Pest Detection and Management Programs. 27 pp. Available at http://www.aphis.usda.gov/plant_health/plant_pest_info/ralstonia/downloads/ralstoniaworkplan.pdf (last accessed January 2010).

APPENDIX 3: CHECKLIST FOR AUTHORS OF DIAGNOSTIC PROTOCOLS

(Last revision: 2019-07)

The headings are as used in diagnostic protocols (DPs; see also Appendix 1: Standardized template for diagnostic protocols). Data used for developing the DP are publicly available.

Title

- Use scientific (Latin) name (without the authority), scope indicated.

Status box (First page)

- Detailed information as outlined in Appendix 1 (Template for diagnostic protocols) is provided, and includes a statement at the beginning of the protocol to indicate when it was drafted.

Pest information

- Geographical information is general, not by country; terms are carefully used (present, recorded, established, etc.).
- Scientific name for the pest is used and includes author(s) only after the first occurrence of the name of the pest.
- Scientific names for hosts are used; common names are included in parentheses after the first use of the name of the host.
- *Italics* used for scientific names at or below species level; higher ranks in Roman.
- All general information on the pest (biology, hosts, etc.) is grouped in this section exclusively, max. 1 page. References to data sheets are included, but no details on epidemiology or disease management.
- Subjective terms (e.g. “significant“ economic impact) are avoided.

Taxonomic information

- The authorities for names given in this section are mentioned.
- The scientific name is used throughout the protocol, the common name is indicated only in this section once in English, a reference to common names in other languages is advised.
- The relevant code of nomenclature is followed.
- No journal citations are given after names.
- Synonyms included are the important ones only, listed in chronological order.
- Species names are in *italics*, higher ranks normal (Roman).
- Species are mentioned in full, genus is abbreviated at subsequent occurrences, unless there might be a confusion with other generic names starting with the same letter.

Detection

- Text and flow diagram: both should be in agreement, text includes the steps/methods with their advantages and limitations, whether including a flow diagram is really essential is considered, minimum requirements are clearly indicated, the scope of the diagnoses is clearly defined.
- The flow diagram is not intended to be a decision scheme.
- Section dealing with required controls is included.
- Combined detection/identification methods are described in this section, and referred to in the identification section.

- Sampling information is provided only for laboratory analysis, not for inspection (except for seeds or grain testing, where relevant additional information could be provided).
- The necessity of the use of commercial kits/brand names is checked and brand names avoided where possible (e.g. use microtubes instead of Eppendorf tubes).
- Common laboratory procedures (e.g. handling of samples, quarantine requirements, facilities) are not detailed in the text.
- All methods included are relevant for the diagnosis.
- The reasons for using a combination of methods are provided (for guidance see also Appendix 4 of *Instructions to authors*).
- Addition of a second method is evaluated following ISPM 27.
- When several methods are mentioned, their advantages and disadvantages are given.
- Method descriptions are not written as standard operating procedures.
- References to manufacturer's instructions in method descriptions are provided, the choices of manufacturers are explained or provided with a disclaimer.
- Specificity data are included.
- Controls for the methods used are included.
- Results of test performance studies are referenced.
- pH and temperature required are mentioned as range or exact measurements.

Identification

- Text and flow diagram: both should be in agreement, text includes the steps/ methods with their advantages and limitations, whether including a flow diagram is really essential is considered, minimum requirements are clearly indicated, the scope of the diagnoses is clearly defined.
- The flow diagram is not intended to be a decision scheme.
- Guidance for interpreting sequencing results is provided.
- The reasons for using a combination of methods are provided (for guidance see also Appendix 4 of *Instructions to authors*).
- Results of performance criteria sensitivity, specificity and reproducibility, and data of ring tests or test performance studies are included or referenced.
- Use of tables for morphological characters is considered.
- Controls for the methods used are included.
- Guidance on interpretation of the results is included.

Records

- Reference to section 2.5 of ISPM 27 is included, relevant additional records and evidence that should be maintained in cases where other NPPOs may be involved are mentioned.
- Relevant information on preservation of record samples is provided (kind of material, period).

Contact points for further information

- Contacts are from several regions and appropriate, preference for one contact per country only.
- The standard text provided in section 4.6 of *Instructions to authors* is followed and consistent.

Acknowledgements

- The standard text provided in section 4.7 of *Instructions to authors* is followed and consistent.
- Additional acknowledgements are included as suggested in Appendix 1 of *Instructions to authors*.

References

- The different type of references (journal articles, monographs, books/conference proceedings, CD-Rom, internet documents, websites etc.) are recognized and formatted as indicated in Appendix 2 of *Instructions to authors*.
- References are cross-checked with those mentioned in the text and unambiguous.
- Author citations in text are consistent, for multiple citations they are ordered by year of publication, followed by author in alphabetical order in the same year (e.g. (Smith *et al.*, 1996; Castlebury and Carris, 1999)).

Figures

- All illustrations included are necessary.
- Preference for use of line drawings or photographs has been considered.
- The numbers of illustrations are referred to in the text at the right place.
- Tables are included in the text, and all other illustrations are kept in separate files.
- The size of the illustrations and captions are according to Appendix 2 of *Instructions to authors*.

General

- Appendixes or annexes are not included.
- Phytosanitary terminology is consistent with ISPM 5 (*Glossary of phytosanitary terms*).
- The correct abbreviations of measurement units as indicated in Appendix 2 of *Instructions to authors* are used.
- Are there capabilities for every member country to apply the proposed methods?
- Are limitations of molecular techniques for quarantine pests considered?
- Are the limitations explicitly defined: e.g. about countries, species, hosts (where the investigations were done)?
- Are the limitations of morphometric techniques defined: e.g. as regional keys, immatures, anabiosis cases?
- Are reproducibility, sensitivity and specificity clearly expressed?
- If contacts for organization of validation, proficiency tests, etc. are known, are they provided?

APPENDIX 4: COMBINATION OF METHODS IN DIAGNOSTIC PROTOCOLS – SOME GENERAL CONSIDERATIONS ON THE CONCEPT

(Last revision: 2019-07)

Diagnostic methods are often used in combination with others in order to increase the sensitivity, specificity or reliability of the diagnosis. ISPM 27 provides the following guidance on this:

Diagnostic protocols may be used in different circumstances that may require methods with different characteristics. Examples of such circumstances grouped according to an increased need for high sensitivity, specificity and reliability are:

- routine diagnosis of a pest widely established in a country
- general surveillance for pest status
- testing of material for compliance with certification schemes
- surveillance for latent infection by pests
- surveillance as part of an official control or eradication programme
- pest diagnostic associated with phytosanitary certification
- routine diagnosis for pests found in imported consignments
- detection of a pest in an area where it is not known to occur
- cases where a pest is identified by a laboratory for the first time
- detection of a pest in a consignment originating in a country where the pest is declared to be absent.

For example, in the case of routine diagnosis, the speed and cost of a test method may be more relevant than sensitivity or specificity. However, the identification of a pest by a laboratory or in an area for the first time may require methods with a high level of specificity and reproducibility. The significance of the outcome of a diagnosis is often dependent on proper sampling procedures. Such procedures are addressed by other ISPMs (under preparation).

Diagnostic protocols provide the minimum requirements for reliable diagnosis of regulated pests. This may be achieved by a single method or a combination of methods. Diagnostic protocols also provide additional methods to cover the full range of circumstances for which a diagnostic protocol may be used. The level of sensitivity, specificity and reproducibility of each method is indicated where possible. NPPOs may use these criteria to determine the method or combination of methods that are appropriate for the relevant circumstances.

In particular relevant for “the combination of methods” is the following statement:

Diagnostic protocols provide the minimum requirements for reliable diagnosis of regulated pests. This may be achieved by a single method or a combination of methods.

The core decisions that are required in the case of each protocol are therefore:

What is the minimum requirement for a reliable diagnosis?

Is a combination of methods necessary to achieve this? If yes, which combination?

It is obvious and generally accepted that the combination of methods may only be appropriate if at least one of the core factors “sensitivity, specificity or reliability” are increased by the combination⁴. It is also known, however, that some methods may provide a higher specificity than others (and therefore may be used as a second method), but not necessarily the same sensitivity as the first method (e.g. monoclonal versus polyclonal antibodies; bioassay versus PCR). In particular in such cases the priorities of the applied system (e.g. sensitivity, specificity or reliability) as required by the framework of the diagnosis (see list of examples in quotation from ISPM 27 above) need to be carefully balanced. Depending on the framework in which the diagnosis is applied a certain combination may not be appropriate while in others a combination may be required.

The template on the next page analyses possible situations and provides an indication of whether a combination of methods with certain characteristics may be appropriate in diagnostic protocols (DP). This template may help authors of DPs and the TPDP to follow a consistent approach when the necessity and appropriateness of combinations of methods in DPs are discussed.

In reality when methods are combined all factors are to be considered and the methods are selected according to the needs of the individual situation.

In summary the following conclusions can be drawn:

- (1) The addition of a second method is not recommended if the second method has a **lower sensitivity** or is **less reliable** than the first method. In these circumstances the combination increases the risk of contradicting results. Depending on the mode of interpretation this may include the risk of “false negative results”.
- (2) The addition of a second method is generally not recommended or not appropriate if the second method provides a higher sensitivity, a lower specificity or a higher reliability than the first method unless some other reason supports this combination.
- (3) The addition of a second method is recommended if the second method provides a **higher specificity** than the first method. Such a combination is often used when the first method (screening method) is cheaper or faster than the second one. In general, **high costs and low speed** of methods are good reasons to apply them as a second method only if they also provide some advantages over the first method (e.g. higher sensitivity, higher specificity or higher reliability).

⁴ In some situations it may be decided to apply both or even more tests at the same time in parallel. This paper does not address this situation and the considerations that may lead to such decisions. In general the final characteristics of the parallel application of different methods equates to the “sum” of the best characteristics of the relevant methods applied.

How to apply this template:

1. Consider that the decision on the first method has already been taken. The second method is only applied if the result of the first method is positive. (see also * below).
2. Consider the individual column assuming that the other factors of the methods are equivalent.
3. Focusing on the second method, ask the question: Is the combination recommended?

The classification “**Risk**” is used to express that the combination carries the risk of weakening a result already achieved by method 1. Such a combination should be avoided in all circumstances. The classification “**Not appropriate**” is used to express that in general the combination of factors in the given order is not contributing to the results of a diagnosis. In some specific situations the combination may nevertheless be considered appropriate.

	Sensitivity		Specificity		Reliability		Costs		Speed	
Method 1	higher	Lower	Lower	Higher	Lower	Higher	lower	higher	lower	higher
Method 2	lower	higher	Higher	Lower	Higher	Lower	higher	lower	higher	lower
Combination recommended?	No 	No/yes 	Yes 	No/yes 	No/yes 	No 	Yes 	No 	No 	Yes
Reason	Risk of contradicting results and false negative interpretation	Generally not appropriate, unless sample is already suspected	Appropriate if other factors (speed, cost etc.) suggest this order	Generally not appropriate, unless second method provides some other benefit (isolation)	Generally not appropriate, unless in a situation where a false negative result (of the 1 st method) can be tolerated.	Risk of contradicting results and false negative interpretation	Appropriate if second method provides some other benefit. Typical situation.	Not appropriate unless 2 nd method provides some other benefit (isolation)	Not appropriate unless second method provides some other benefit (isolation)	Appropriate, fast result from first method

* In some situations it may be appropriate that the second method is applied even if the result of the first test was negative. Such situations may occur where most test results are positive and only a few results are negative. This condition does not apply to import situations. Also when consignments for export are tested such situations – if they exist at all – are rare. Such situations may occur in some specific surveillance situations in a heavily infested area. The inclusion of this situation would be very complex and is therefore not addressed in this table.

APPENDIX 5: TEMPLATE TABLES FOR DESCRIPTION OF PCR, RT-PCR OR PCR-RFLP REACTIONS

(Last revision: 2019-07)

Background

Authors are required to use in IPPC diagnostic protocols, and as a quality check tool, tables that describe polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR) or PCR - restriction fragment length polymorphism (PCR-RFLP) reactions.

Table 1. PCR master mix composition, cycling parameters and amplicons

Reagents	Final concentration	Comments
PCR grade water	–†	Quantity for total volume
PCR buffer (or provide individual reagent's concentration)	1 X	
dNTPs	μM	
Other component (e.g. MgCl ₂)		Where relevant, specify
Primer (forward)	μM	
Primer (reverse)	μM	
Probe	μM	if applicable for real time PCR
Enzyme quantity (DNA polymerase)	U	
DNA (quantity/volume)		Specify unit
Cycling parameters		
Initial denaturation	°C min	
Number of cycles		
- Denaturation	°C min	
- Annealing	°C min	
- Elongation	°C min	
Final elongation	°C min	
Expected amplicons		
Description		

† For a final reaction volume of XX μL.

PCR, polymerase chain reaction.

Table 2. Master mix composition, cycling parameters and amplicons for one step real-time PCR

Reagents	Final concentration	Comments
PCR grade water	–†	Quantity for total volume
RT-PCR buffer (or provide individual reagent's concentration)	1 X	
dNTPs	μM	
Other component (e.g. MgCl ₂)		Where relevant, specify
Primer (forward)	μM	
Primer (reverse)	μM	
Probe	μM	if applicable for real time PCR

Enzyme quantity (reverse transcriptase + polymerase)	U
RNA (quantity/volume)	Specify unit
Cycling parameters	
cDNA synthesis	°C min
Initial denaturation	°C min
Number of cycles	
- Denaturation	°C min
- Annealing	°C min
- Elongation	°C min
Final elongation	°C min
Expected amplicons	
Description	

† For a final reaction volume of XX µL.

PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR.

Table 3. Master mix composition and reaction conditions for RFLP/restriction analysis

Reagents	Final concentration	Comments
PCR grade water	–†	Quantity for total volume
Enzyme buffer	1 X	
Other component		Where relevant, specify
Restriction enzyme quantity (name)	U	
PCR product volume	µL	
Reaction condition (temperature and duration)	°C min or h	
Expected fragments		
Description		

† For a final reaction volume of XX µL.

PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

APPENDIX 6: ELISA CONTROLS AND INTERPRETATION OF RESULTS

(Last revision 2019-08)

1. Positive and negative controls for ELISA test

Commercial kits

When using a commercial ELISA kit, the following controls should be added in addition to the positive and negative controls provided in the kit:

- a positive control IDEALLY of the same matrix, inoculated or spiked with the target bacterium/virus for tests used for detection in plant material. For identification of bacterial cultures positive controls can consist of a suspension of the target bacterium.
- a negative control from a healthy host plant for tests used for detection in plant material. For testing bacterial cultures negative controls can consist of suspension buffer only or a suspension of a non-target bacterial species.

These positive and negative controls should be checked (preferably in advance) with the same antibodies following the appropriate ELISA procedure.

In house controls

Positive controls

For bacteria, positive controls of the reference strain of the target organism should be suspended in healthy host plant extract or in an appropriate buffer. It is recommended that reference strains are used as positive controls to avoid misinterpretations due to cross-reactions. Reference strains are available from a number of international culture collections for example, National Collection of Plant Pathogenic Bacteria (NCPBP), FERA, York, UK; Culture Collection of the National Plant Protection Organisation (NPPO NL), Wageningen, the Netherlands; or Collection Francaise de Bacteries Phytopathogenes (CFBP), INRA Station Phytobacteriologie, Angers, France; International Collection of Microorganisms from Plants (ICMP), New Zealand.

Naturally infected tissue (maintained by lyophilization or freezing at below -16°C) should be used whenever possible.

For viruses, naturally infected tissue or extracts (maintained by lyophilization or freezing at below -20°C) should be used whenever possible. Aliquots of positive controls should be prepared to prevent repeated freezing and thawing. Tissue prints can be prepared and stored until use at room temperature and protected from any light (e.g. between paper sheets or in an envelope).

Two wells or tissue prints should be prepared per positive controls.

Negative controls

Healthy plant extract (for detection in plant material) or a suspension of a non-target bacterial species (for identification of bacteria) should be used as negative controls. The healthy plant should whenever possible be the same species/variety and the same plant part at the same growth stage to allow for comparison with tested samples. Aliquots / extracts of the same host plant which previously tested negative for the target bacterium/virus can also be used as negative controls. For Tissue print-ELISA, healthy controls previously immobilized on membranes can be used.

At least two wells or tissue prints should be prepared per negative control.

Blank or buffer controls

A further negative control consisting of extraction or suspension buffer can be included. These wells do not receive any sample and these blank wells control for any variation (or contamination) due to the plate and test reagents to the measured OD.

2. Interpretation of ELISA tests results

When using a commercial kit, the manufacturer's instructions should be followed.

Verification of the controls:

Negative ELISA readings in positive control wells, prints or dots indicate that the test has not been performed correctly or that it has been inhibited. Positive ELISA readings in negative control wells, prints or dots indicate that cross-contamination or non-specific antibody binding has occurred. In such cases, the test should be performed again with the appropriate modifications.

Interpretation of ELISA tests results for detection of bacteria (plant material)

For detection in plant material the interpretation of the optical density (OD) value of the negative sample extract well should be the basis for determining the thresholds of detection (background) minus the OD of the substrate well. The positive result is determined on a case-by-case basis depending on the pest and the matrix. It is recommended that the test be repeated for samples just below the limit of the threshold.

Usually, the ELISA test is considered positive if the OD value is > 2 times that of the healthy plant extract control.

Interpretation of ELISA tests results for identification (pure cultures of bacteria)

The ELISA test is considered negative if:

- the average absorbance or OD reading from duplicate sample wells is $< 2 \times$ OD of that in the negative sample control well,
- and the OD for the positive controls are all above 1.0 (after 120 min incubation with the substrate) and are greater than twice the OD obtained for negative sample extracts.

The ELISA test is considered positive if:

- the average OD reading from each of the duplicate sample wells, and the positive control wells are $\geq 2 \times$ OD in the negative sample extract well.

It is recommended that the test be repeated for samples that give a reaction just below the limit of the threshold.

Interpretation of ELISA tests results for detection / identification of viruses

There are different options for interpreting ELISA test results and in particular to establish a threshold. It is also recommended that the manufacturer's instructions to be checked for interpretation of test results. Further information is provided in Sutula *et al.* (1986). The following procedure is recommended, however it is recognized that in particular when the negative control of healthy plant material is not the same as the plant to be tested, the laboratory should adjust and validate the calculation of the threshold, or confirm positive results by another method.

The ELISA test is considered negative if:

- the average OD value from duplicate sample is less than 0.1 or is $< 2 \times$ OD of that in the negative control of healthy plant extracts.

Usually the ELISA test is considered positive if the average OD value from each of the duplicate sample wells is $\geq 2 \times$ OD of that in the negative control of healthy plant extracts.

Note that when using polyclonal antibodies, it is essential that the negative controls are as similar as possible to the matrix tested (e.g. plant species, cultivar, tissue type) in the same plate.

The test should be repeated when duplicate wells differ by more than 50% OD value. In critical cases, for samples that give a reaction close to the threshold of e.g. $2 \times$ OD of that in the negative control of healthy plant extracts or when matrix effects cannot be excluded, it is recommended that another test (different source of antibody or another method) be used.

Other procedures for interpretation are in use involving consideration of standard deviations (average of healthy controls + $3 \times$ standard deviation).

Interpretation for tissue print, squash or dot ELISA tests results

The ELISA test is negative if there is no coloured precipitate in the sample print or dot, provided that the positive control is positive and the negative control is negative. The test is positive, if there is purple–violet-coloured precipitate in the sample print or dot, provided that the positive control is positive and the negative control is negative.

For some viruses restricted to the phloem tissues, the observation of precipitates should occur in the vascular area only.

References

Sutula C.L., Gillett J.M., Morrissey S.M. & Ramsdell D.C. 1986. Interpreting ELISA data and establishing the positive–negative threshold. *Plant Disease*, 70: 722–726.

EPPO 2010. PM 7/101 (1): ELISA tests for plant pathogenic bacteria. *EPPO Bulletin*, 40: 369-372. doi:[10.1111/j.1365-2338.2010.02420.x](https://doi.org/10.1111/j.1365-2338.2010.02420.x)

EPPO 2015. PM 7/125 (1) ELISA tests for viruses. *EPPO Bulletin*, 45: 445-449. doi:[10.1111/epp.12259](https://doi.org/10.1111/epp.12259)

APPENDIX 7: CONTROL OPTIONS FOR MOLECULAR TESTS FOR PEST CATEGORIES AND PURPOSES OF THE TESTS

(Last revision: 2018-02)

The table provides guidance on the need (obligatory, recommended, optional or not needed) to include different controls (negative amplification control, positive amplification control, negative extraction control, positive extraction control, internal control) during molecular tests for combinations of pest categories (bacteriology, botany, entomology, mycology, nematology, phytoplasmas and virology) and purposes of testing (detection or identification).

Discipline	Purpose of the test	Negative extraction control (NEC)	Positive extraction control (PEC)	Negative amplification control (NAC)	Positive amplification control (PAC)	Internal control
Bacteriology	Detection	<p>Obligatory</p> <p>Include this control for each series of extractions</p> <p>When negative samples are expected in the area and if an internal control is in place, this control can be replaced by samples detected as negative in the same PCR run.</p>	<p>Obligatory</p> <p>Include this control for each series of extractions</p> <p>Where the pest is present in the area, this control can be replaced by samples detected as positive in the same PCR run.</p>	Obligatory	<p>Obligatory</p> <p>If several species are to be detected, PAC for each species should be included</p>	<p>Not needed if a universal primer set that amplifies pest and matrix (e.g. generic primers, which amplify target regions in 16S rDNA) is used.</p> <p>Recommended in other cases the use of primers targeting a plant housekeeping gene such as Actin, COX, 18S rDNA or <i>GAPDH</i></p>
	Identification (pure culture)	Optional	Optional	Obligatory	<p>Obligatory</p> <p>If several species are to be identified, PAC for each species should be included</p>	<p>Not needed if a universal primer set (that amplifies pest) is used</p> <p>Recommended in other cases</p>
Botany	Identification (isolated plant part / seed)	Obligatory	Optional	Obligatory	<p>Obligatory</p> <p>If several species are to be identified, PAC for each species should be included</p>	<p>Recommended use of primer sets to detect either plant housekeeping gene (e.g. Actin, 28S rDNA or COX) or host specific sequence.</p>

Discipline	Purpose of the test	Negative extraction control (NEC)	Positive extraction control (PEC)	Negative amplification control (NAC)	Positive amplification control (PAC)	Internal control
Entomology	Identification (isolated insect/acari)	Obligatory	Optional	Obligatory	Obligatory If several species are to be identified, PAC for each species should be included	Not needed if a universal primer set is used (e.g. 18S rDNA or ITS gene target) Recommended in other cases, e.g. <i>COI</i> (<i>CoxI</i>) primers LCO1490/ HCO2198 (Folmer <i>et al.</i> Molecular Marine Biology and Biotechnology 1994:3(5) 294-299).
	Detection	Obligatory Include this control for each series of extractions When negative samples are expected in the area and if an internal control is in place, this control can be replaced by samples detected as negative in the same PCR run.	Obligatory Include this control for each series of extractions Where the pest is present in the area, this control can be replaced by samples detected as positive in the same PCR run.	Obligatory	Obligatory If several species are to be detected, PAC for each species should be included	Not needed if a universal primer set that amplifies pest and matrix, e.g. 18S rDNA gene or a fungal housekeeping gene such as mitochondrial <i>nad5</i> (NADH dehydrogenase 5), is used Recommended in other cases the use of primers targeting a plant housekeeping gene such as Actin, <i>COX</i> , 18S rDNA or <i>GAPDH</i> .
Mycology	Identification (pure culture)	Optional	Optional	Obligatory	Obligatory If several species are to be identified, PAC for each species should be included	Not needed if a universal primer set (that amplifies pest e.g. 18S rDNA) is used Recommended in other cases (e.g. 18S rDNA primers)

Discipline	Purpose of the test	Negative extraction control (NEC)	Positive extraction control (PEC)	Negative amplification control (NAC)	Positive amplification control (PAC)	Internal control
Nematology	Detection	<p>Obligatory</p> <p>Include this control for each series of extractions</p> <p>When negative samples are expected in the area and if an internal control is in place, this control can be replaced by samples detected as negative in the same PCR run.</p>	<p>Obligatory</p> <p>Include this control for each series of extractions</p> <p>Where the pest is present in the area, this control can be replaced by samples detected as positive in the same PCR run.</p>	Obligatory	<p>Obligatory</p> <p>If several species are to be detected, PAC for each species should be included</p>	<p>Not needed if a universal primer set (that amplifies pest and matrix, e.g. 18S gene or ITS region) is used</p> <p>Recommended in other cases the use of primers targeting a plant housekeeping gene such as Actin, COX, 18S rDNA or GAPDH.</p>
	Identification (isolated nematodes)	Optional	Optional	Obligatory	<p>Obligatory</p> <p>If several species are to be identified, PAC for each species should be included</p>	<p>Not needed if a universal primer set (that amplifies pest, e.g. 18S gene) is used.</p> <p>Recommended in other cases (e.g. 18S rDNA or COI gene)</p>
Phytoplasmas	Detection / identification	<p>Obligatory</p> <p>Include this control for each series of extractions</p> <p>When negative samples are expected in the area and if an internal control is in place, this control can be replaced by samples detected as negative in the same PCR run.</p>	<p>Obligatory</p> <p>Include this control for each series of extractions</p> <p>Where the pest is present in the area, this control can be replaced by samples detected as positive in the same PCR run.</p>	Obligatory	<p>Obligatory</p> <p>If several species are to be detected, PAC for each species should be included.</p>	<p>Not needed if a universal primer set that amplifies pest and matrix (e.g. generic primers, which amplify target regions in 16S rDNA) is used</p> <p>Recommended in other cases the use of primers targeting a plant housekeeping gene such as Actin, COX, 18S rDNA or GAPDH</p>

Discipline	Purpose of the test	Negative extraction control (NEC)	Positive extraction control (PEC)	Immunocapture control (ICC)	Negative amplification control (NAC)	Positive amplification control (PAC)	Internal control
Virology	Detection / identification	<p>Obligatory (not applicable for IC PCR) Include this control for each series of extraction When negative samples are expected in the area and if an internal control is in place, this control can be replaced by samples detected as negative in the same PCR run.</p>	<p>Obligatory (not applicable for IC PCR) Include this control for each series of extraction Where the pest is present in the area, this control can be replaced by samples detected as positive in the same PCR run.</p>	<p>Obligatory In the case of IC-RT-PCR where no nucleic extraction is performed, plant sap from positive material should be used as a positive control, and plant sap from a healthy plant should be used as a negative control.</p>	Obligatory	<p>Obligatory If several species are to be detected, PAC for each species should be included</p>	<p>Recommended is the use of primers targeting a plant housekeeping gene such as Actin, COX, 18S rDNA or GAPDH.</p>

COI/COX, cytochrome c oxidase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; IC, immunocapture; ITS, internal transcribed spacer; NADH, nicotinamide adenine dinucleotide; PCR, polymerase chain reaction; rDNA, ribosomal DNA; RT-PCR, reverse transcription polymerase chain reaction;

APPENDIX 8: BEST PRACTICES FOR SEQUENCING: USING DNA SEQUENCES TO DIAGNOSE A PEST

(Last revision: 2019-08)

I. Purpose

The purpose of this document is to outline the technical and scientific expectations for a diagnostic method that compares similarity or dissimilarity of DNA sequences generated from relatively short fragments (<2,000 bases) of an organismal genome. This includes the technique of DNA barcoding that is based on fragments of the mitochondrial cytochrome oxidase I gene for animals and combinations of other short gene fragments such as internal transcribe spacer regions of other taxa. The document does not consider diagnostic processes that involve comparison of large genome and transcriptome data sets.

II. Criteria for ensuring the DNA sequence data to be queried in an analysis is appropriate

The protocol should state that controls are needed during generation of DNA sequence from the suspect organism (as detailed in TPDP instructions to authors).

The protocol should define a measure of data quality using an algorithm such as phred scores or comparison of results from multiple reactions using distinct primers to confirm base calls.

Translation of coding sequences should be used to detect pseudogenes that would compromise interpretation.

III. Criteria for ensuring that the DNA sequence library used to diagnose a query is fit for purpose

The protocol should identify the DNA sequence resource (the record, bank or library) to be used for the desired comparison and diagnosis. This resource could be:

- (1) A single sequence record on a public database (e.g., GenBank accession record), but it should be readily accessible and monitored regularly for changes over time.
- (2) One or more DNA sequence records stored in a static repository or file that cannot be altered without controlled permissions; (this is static because changes to the library effectively creates a new resource that can be verified for being fit for purpose).
- (3) A dynamic database that is quality controlled to ensure that new records do not alter the outcome of each comparison; (dynamic means that new records are entered and old records are removed over time to a data base).

The protocol should provide a **published reference** as evidence that the library (or part of it) meets the sampling expectations for generating the expected diagnosis. The reference should provide an explicit recommendation for use of DNA sequence data for diagnosis of a targeted pest or pathogen. This includes:

- (4) States that the DNA sequence library includes the appropriate taxonomic sampling (i.e. sequence records of species/variants other than the targeted species) to enable biologically relevant diagnosis. If too few species are included in the database it might not function as a replacement of the morphological methods.
- (5) States that the DNA sequence library includes the appropriate intraspecific sampling to enable biologically relevant diagnosis. If the ecological and geographical range of a species is sampled with too few collections, it is possible that the dissimilarity reported between species is a sampling artifact.

- (6) States that the DNA records are derived from specimens that are retained as reference specimens (also known as vouchers), when appropriate for the taxonomic group, have been expertly identified, and are traceable for future investigation.

IV. Required information for proper interpretation of the comparison

The protocol should indicate the alignment strategy for the method and indicate if it uses a global strategy (i.e. alignment of entire sequence length using clustal), a local alignment method (such as BLAST), or other so that labs can select the appropriate technique.

The protocol should indicate the method of sequence comparison and include a reference on how to perform that analysis: genetic distance values, character state at set nucleotide sites, perfect match criteria, or phylogenetic analysis

The protocol should provide clear interpretation rules to identify a suspect sample.

- (1) It should state how similar the query sequence and the reference sequence for the pest must be in order to determine a match.
 - a. To confirm the match is not the result of missing information⁵ in the edited sequence or rare genotypes⁶ in the population, the protocol should indicate how dissimilar the query sequence should be from the next most genetically similar species in the reference library.
- (2) When appropriate, it should state how dissimilar the query sequence and the reference sequence for the pest must be in order to generate a mismatch.
 - a. To confirm the mismatch is not the result of pseudogenes or other genetic variants and contaminants⁷, the protocol should indicate how similar the query sequence should be to other species in the reference library.

⁵ If the quality control measures for the protocol are not stringent it is possible for a query sequence to match more than one species in a library.

⁶ If a new population or species is sampled it could exhibit affinity to two or more species in the database. For example match species 1 and species 2 by >98%.

⁷ If the sequence is of high quality but does not match with any of the taxa in the library it is possible that it is a contaminant during the extraction or PCR steps and should not be used in a diagnosis.