EUROPEAN AND MEDITERRANEAN PLANT PROTECTION ORGANIZATION

ORGANISATION EUROPEENNE ET MEDITERRANEENNE POUR LA PROTECTION DES PLANTES

**25-xxxxx (24-29614)**

**Template for the drafting of pest specific EPPO diagnostic Standards**

|  |
| --- |
| This template should be used in conjunction with Instruction to authors on the format and content of a diagnostic protocol.  In the following template:   * Comments in red correspond to instructions and should be deleted. * Text in blue corresponds to examples and should be changed/adapted. * Text in black corresponds to Standard text and should be kept and should not be changed. |

**Diagnostics**

**PM 7/XXX *Scientific name of the pest***

**Specific scope**

This Standard describes a diagnostic protocol for ***Scientific name of the pest*** ***[[1]](#footnote-2)***.

**This Standard should be used in conjunction with PM 7/76 Use of EPPO Diagnostic Standards**

Terms used are those in the EPPO Pictorial Glossary of Morphological Terms in Nematology[[2]](#footnote-3).

**Specific approval and amendment**

Approved in 20XX-XX. If relevant: Revised in 20XX-XX. Main revisions include xxx.

Authors and contributors are given in the Acknowledgements section.

1. **Introduction**

For an updated geographical distribution consult EPPO Global Database (EPPO, year).

A datasheet providing more information on the biology is also available in EPPO Global Database (EPPO, year).

Flow diagrams describing the diagnostic procedure for “*Pest XX*” are presented in Figure 1 and 2.

**Figure X**: Flow diagram describing the diagnostic procedure for XXX. This flow diagram is intended to provide an overview of the diagnostic process and may not cover all possible scenarios.

1. **Identity**

**Name:**

**Other scientific names:**

**Acronym:**

**Taxonomic position:**

**EPPO Code:**

**Phytosanitary categorization:** EPPO A1/A2 no. XX, EPPO Alert list XXX (year) n° XXX; EU Quarantine pest (Annex XX, Part X[[3]](#footnote-4)); EU emergency measures (year); EU RNQP (Annex IV3).

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

1. **Detection**
   1. **Symptoms**

* 1. **Detection in (symptomatic/asymptomatic) plant material / seeds** 
     1. Test sample requirements
     2. Extraction
     3. Isolation
     4. Serological/molecular/baiting… tests

The following tests are recommended for the detection of xxx in xxx:

* Conventional PCR for *Dickeya* spp. from Nassaret al. (1996) described in Appendix X
* YYY
  + 1. Other tests

1. **Identification**
   1. Morphological characteristics/identification
      1. Morphological characteristics of (at genus level)
      2. Morphological characteristics of (at species level)
      3. Possible confusion with similar species
   2. Molecular tests
      1. (Real-time) PCR tests

The following tests are recommended for the identification of xxx in xxx:

* Conventional PCR for *Dickeya* spp. from Nassaret al. (1996) described in Appendix X
* YYY
  + 1. Barcoding

A protocol for DNA barcoding based on *[locus]* is described in Appendix 1 of PM 7/129 (2) *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2021).

Guidance for sequence analysis is given in Appendices 7 and 8 of EPPO Standard PM 7/129 *DNA Barcoding as an identification tool for a number of regulated plant pests* (EPPO, 2021).

* 1. Serological / pathogenicity… tests
  2. Other tests

1. **Reference material**

Reference material can be obtained from xxx.

Sequences are available in EPPO-Q-bank (https://qbank.eppo.int/).

1. **Reporting and Documentation**

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

1. **Performance characteristics**

When performance characteristics are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

1. **Further information**

Further information on this organism can be obtained from:

Nom J, Institute, Postal address (ISO code country); mail.

1. **Feedback on this Diagnostic Standard**

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

1. **Standards revision**

A regular review process is in place to identify the need for revision of Diagnostic Standards. Standards identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

1. **Acknowledgements**

This Standard was originally drafted by: Name X, institute (ISO code country)…

The revision was prepared by: Name X, institute (ISO code country)…

The Standard was reviewed by the Panel on Diagnostics in XX.

1. **References**

Gargouri S, Hajlaoui MR, Guermech A & Marrakchi M (2001) Identification des espèces fongiques associées à la pourriture du pied du blé et leur répartition selon les étages bioclimatiques. *EPPO Bulletin* **31**, 499-503.

EPPO (2024) EPPO Global Database. https://gd.eppo.int [accessed 10/Apr/2024]

**Appendix XX Buffers and media**

All media are sterilized by autoclaving at 121°C for 15 min, except when stated otherwise.

1. **Buffers**

*Phosphate buffer saline (10 mM PBS buffer, pH 7.2)*

|  |  |
| --- | --- |
| NaCl | 8.0 g |
| KCl | 0.2 g |
| Na2HPO4·12H2O | 2.9 g |
| KH2PO4 | 0.2 g |
| Distilled water to 1 L |  |
| Adjust pH to 7.2 |  |

1. **Media**

*PARPNH-V8 selective agar* (Tsao, 1983, modified by Jung *et al.*, 1996)

|  |  |
| --- | --- |
| V8 juice | 100mL |
| Microbiological grade agar | 16 g |
| CaCO3 | 2 g |
| Distilled water | 900 mL |
| Pimaricin \* (CAS: *xxxx*) | 10 mg |
| Ampicillin (CAS: *xxxx*) | 200 mg |
| Rifampicin \* (CAS: *xxxx*) | 10 mg |
| Pentachloronitrobenzene (PCNB) \* (CAS: *xxxx*) | 25 mg |
| Nystatin (CAS: *xxxx*) | 50 mg |
| Hymexazol (CAS: *xxxx*) | 50 mg |

\* Does not dissolve well in water (ethanol or DMSO may be used).

Adjust pH to 6.8-7.0

All antibiotics are added to the V8-Agar when it has cooled down to approximately 45 °C.

**Appendix XX Conventional/Real-time/Nested PCR (author, year)**

*The test below differs from the one described in the original publication (see 1.2).*

*The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used, provided that a verification (see PM 7/98) is carried out.*

1. **General Information**
   1. This test can be used for the detection and/or identification of species X in matrix Y.
   2. The test was developed by XXX in 20XX and adapted by XXX in XXX.
   3. The target sequence is located on the XX gene coding for the XX protein.
   4. Amplicon location (first base pair, based on standard organism - including primer sequences), if applicable/known.
   5. Oligonucleotides: Forward primer name, sequence (orientation 5’-3’); Reverse primer name, sequence (orientation 5’-3’); probe name (if applicable), sequence (orientation 5’-3’). If applicable labels and purification methods are given. Note: several primers pairs and probes could be used (e.g. PCR that amplify several sequences).

|  |  |  |  |
| --- | --- | --- | --- |
|  | Name | Sequence | Amplicon size (including primer sequences) |
| Forward primer | xxxx | 5’- XXX XXX XXX XXX XXX XXX -3’ | XX bp |
| Reverse primer | xxxx | 5’- XXX XXX XXX XXX XXX XXX -3’ |
| Probe….. | xxxx | 5’- FAM- XXX XXX XXX XXX XXX XXX -BHQ1-3’ |  |

* 1. Cycler or real-time PCR system or other equipment name, producer name
  2. Software and settings (automatic or manual) for data analysis.

1. **Methods**
   1. Nucleic Acid Extraction and Purification
      1. Tissue source (e.g., species and/or strain/isolate name [if applicable], number of organisms and developmental stage [if applicable], infected plant material, bacterial colony, mycelium, soil), sampling and/or homogenization method (if applicable), buffer composition and pH, concentration of all constituents (if known), kit producer name(s) (if applicable)
      2. Nucleic acid extraction method, kit producer name and specific instructions (if applicable), buffer composition and pH, concentration of all constituents (if known)
      3. Nucleic acid cleanup procedure, kit producer name and specific instructions (if applicable), buffer composition and pH, concentration of all constituents (if known)
      4. Specify any requirements for nucleic acids used for input to the master mix (e.g. dilutions of extracted nucleic acids)
      5. DNA should preferably be stored at approximately -20°C.

RNA should preferably be stored at approximately -20°C for short term (less than one month) or at approximately -80°C for long term storage.

* 1. Reverse Transcription (RT; to produce cDNA from RNA)
     1. Master Mix

|  |  |  |  |
| --- | --- | --- | --- |
| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
| Molecular gradewater | N.A. | X | N.A. |
| RT buffer (*producer name*) | X x | X | 1x |
| MgCl2 (or alternatives) (*producer name*) | X mM | X | X mM |
| dNTPs (*producer name*)  (if equimolar amounts are used; otherwise specify the final concentrations individually, dATP, dCTP, dGTP and dTTP) | X mM  X1 mM dATP  X2 mM dCTP,  X3 mM dGTP  X4 mM dTTP | X  X  X  X  X | X mM  X1 mM dATP  X2 mM dCTP,  X3 mM dGTP  X4 mM dTTP |
| Other additive(s) or special enzymes if applicable (*name and* *producer name*) | X X | X | X X |
| Reverse primer (name) | X µM | X | x µM |
| Reverse transcriptase (RT) (*producer name*)  *The RT should be added after the denaturation step (see 2.2.2) depending on the RNA structure.* | X U/µL | X | X U |
| Subtotal |  | X |  |
| RNA extracts\* |  | X |  |
| Total |  | X |  |

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

* + 1. Reverse Transcription conditions: (e.g. pre-heating, cooling on ice, RT reaction temperature)
  1. Conventional PCR
     1. Master Mix

|  |  |  |  |
| --- | --- | --- | --- |
| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
| Molecular grade water | N.A. | X | N.A. |
| PCR buffer (*producer name*) | X x | X | 1x |
| MgCl2 (or alternatives) (*producer name*) | X mM | X | X mM |
| dNTPs (*producer name*)  (if equimolar amounts are used; otherwise specify the final concentrations individually, dATP, dCTP, dGTP and dTTP) | X mM  X1 mM dATP  X2 mM dCTP,  X3 mM dGTP  X4 mM dTTP | X  X  X  X  X | X mM  X1 mM dATP  X2 mM dCTP,  X3 mM dGTP  X4 mM dTTP |
| Other additive(s) or special enzymes if applicable (*producer name*) | X X | X | X X |
| Forward primer (name) | X µM | X | x µM |
| Reverse primer (name) | X µM | X | x µM |
| Polymerase (*producer name*) | X U/µL | X | X U |
| Subtotal |  | X |  |
| Nucleic acid extract\* |  | X |  |
| Total |  | X |  |

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

* + 1. PCR conditions: Pre-incubation temperature, time (if applicable); initial denaturation temperature, time; cycling denaturation temperature, time (other specifications); cycling annealing temperature, time (other specifications); cycling extension temperature, time (other specifications3); heating ramp speed (if appropriate); cooling ramp speed; cycle number; final extension temperature, time.
  1. One step Reverse Transcription PCR
     1. Master Mix

|  |  |  |  |
| --- | --- | --- | --- |
| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
| Molecular grade water | N.A. | X | N.A. |
| RT-PCR buffer (*producer name*) | X x | X | 1x |
| MgCl2 (or alternatives) (*producer name*) | X mM | X | X mM |
| dNTPs (*producer name*)  (if equimolar amounts are used; otherwise specify the final concentrations individually, dATP, dCTP, dGTP and dTTP) | X mM  X1 mM dATP  X2 mM dCTP,  X3 mM dGTP  X4 mM dTTP | X  X  X  X  X | X mM  X1 mM dATP  X2 mM dCTP,  X3 mM dGTP  X4 mM dTTP |
| Other additive(s) or special enzymes if applicable (*name and producer name)* | X X | X | X X |
| PCR Forward primer (*name*) | X µM | X | x µM |
| PCR Reverse primer (*name*) | X µM | X | x µM |
| RT primers (*name*) | X µM | X | x µM |
| Polymerase (*producer name*) | X U/µL | X | X U |
| Subtotal |  | X |  |
| RNA extract |  | X |  |
| Total |  | X |  |

* + 1. RT-PCR conditions: Pre-incubation temperature, time; initial denaturation temperature, time; cycling denaturation temperature, time (other specifications); cycling annealing temperature, time (other specifications3); cycling extension temperature, time (other specifications); heating ramp speed (if appropriate); cooling ramp speed; cycle number; final extension temperature, time.
  1. Real-time PCR
     1. Master Mix

|  |  |  |  |
| --- | --- | --- | --- |
| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
| Molecular grade water | N.A. | X | N.A. |
| Real-time (RT-)PCR buffer (*producer name*) | X x | X | 1x |
| MgCl2 (or alternatives) (*producer name*) | X mM | X | X mM |
| dNTPs (*producer name*)  (if equimolar amounts are used; otherwise specify the final concentrations individually, dATP, dCTP, dGTP and dTTP) | X mM  X1 mM dATP  X2 mM dCTP,  X3 mM dGTP  X4 mM dTTP | X  X  X  X  X | X mM  X1 mM dATP  X2 mM dCTP,  X3 mM dGTP  X4 mM dTTP |
| Other additive(s) or special enzymes if applicable (*name and* *producer name*) | X X | X | X X |
| Forward Primer (*name*) | X µM | X | x µM |
| Reverse Primer (*name*) | X µM | X | x µM |
| Probe (*name*) | X µM | X | x µM |
| Polymerase (*producer name*) | X U/µL | X | X U |
| Subtotal |  | X |  |
| Nucleic acid extract |  | X |  |
| Total |  | X |  |

* + 1. PCR conditions: Pre-incubation temperature, time (if applicable as, e.g., for the activation of the polymerase); initial denaturation temperature, time; cycling denaturation temperature, time (other specifications); cycling annealing temperature, time (other specifications); cycling extension temperature, time (other specifications[[4]](#footnote-5)3); heating ramp speed (if appropriate); cooling ramp speed; cycle number; final extension temperature, time, step for fluorescence capture.  
       For real-time PCR based on intercalating dyes: melting curve parameters (e.g. Temperature ramp range 65°to 95°C for XX min with XX data acquisitions per °C/ data acquisition for at least each 0.3 K increase).
  1. One step real-time Reverse Transcription PCR
     1. Master Mix

|  |  |  |  |
| --- | --- | --- | --- |
| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
| Molecular grade water | N.A. | X | N.A. |
| Real-time RT PCR buffer (*producer name*) | X x | X | 1x |
| MgCl2 (or alternatives) (*producer name*) | X mM | X | X mM |
| dNTPs (*producer name*)  (if equimolar amounts are used; otherwise specify the final concentrations individually, dATP, dCTP, dGTP and dTTP) | X mM  X1 mM dATP  X2 mM dCTP,  X3 mM dGTP  X4 mM dTTP | X  X  X  X  X | X mM  X1 mM dATP  X2 mM dCTP,  X3 mM dGTP  X4 mM dTTP |
| Other additive(s) or special enzymes if applicable (*name and* *producer name*) | X X | X | X X |
| PCR forward Primer *(name)* | X µM | X | x µM |
| PCR reverse Primer *(name)* | X µM | X | x µM |
| Probe *(name)* | X µM | X | x µM |
| RT enzyme *(name)* | X µM | X | x µM |
| RT Primer *(name)* if required | X µM | X | x µM |
| Polymerase (*producer name*) | X U/µL | X | X U |
| Subtotal |  | X |  |
| RNA extract |  | X |  |
| Total |  | X |  |

* + 1. PCR conditions: Pre-incubation temperature, time (if applicable as, e.g.,); initial denaturation temperature, time; cycling denaturation temperature, time (other specifications); cycling annealing temperature, time (other specifications); cycling extension temperature, time (other specifications4); heating ramp speed (if appropriate); cooling ramp speed; cycle number; final extension temperature, time, step for fluorescence capture.

For real-time PCR based on SYBR® Green: melting curve parameters (e.g. Temperature ramp range 65°to 95°C for XX min with XX data acquisitions per °C/ data acquisition for at least each 0.3 K increase)

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

* 1. Restriction Fragment Length Polymorphism (RFLP) Reaction
     1. PCR product purification
        1. PCR product cleanup procedure, kit producer name and instructions (if applicable), buffer composition and pH, concentration of all constituents (if known)
        2. Concentration of amplified DNA and of all nucleic acid solution constituents, pH of nucleic acid solution, storage temperature and conditions
     2. RFLP Master mix

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

* + 1. Reaction conditions
       1. Incubation temperature, time
       2. Denaturation temperature, time (if applicable) or final concentration, name and producer of restriction enzyme inhibitor (if needed).
  1. LAMP tests
     1. Master Mix

|  |  |  |  |
| --- | --- | --- | --- |
| Reagent | Working concentration | Volume per reaction [µl] | Final concentration |
| Molecular grade water | N.A. | XX | N.A. |
| LAMP buffer/ ISO-Master Mix | 2x | XX | 1x |
| F3/B3 (Forward/Backward=Reverse outer primer) | X1/X2 µM | XX | X1/X2 µM |
| FIP/BIP (Forward/Backward=Reverse inner primer) | X3/X4 µM | XX | X3/X4 µM |
| F-loop/B-loop (when applicable, not in all LAMP assays included) | X5/X6 µM | XX | X5/X6 µM |
| Subtotal |  | XX |  |
| Nucleic acid extract |  | XX |  |
| Total |  | XX |  |

* + 1. LAMP reaction conditions and analysis of LAMP product

Amplification: 60-65°C; 20-50 min

Anneal/Melting curve: 95-75°C at 0.05°C/s

The analyses of the fragments produced by LAMP can be performed using different methods e.g. gel electrophoresis, lateral flow, observation of colour or turbidity development by naked eye, or by fluorescent DNA-intercalator.

1. **Essential Procedural Information** 
   1. **Controls**

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

* Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
* Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
* Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
* Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be close to the limit of detection.

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

These can include:

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

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

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

Laboratories should take additional care to prevent risks of cross contamination when using high concentration positive controls (e.g. cloned products, gBlocks, and whole genome amplicons) directly or when preparing dilutions of them.

*Other possible controls*

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

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

**Conventional PCR tests**

*Verification of the controls*

* NIC and NAC: no band is visualized
* PIC, PAC: a band of the expected size [xxx] is visualized. If relevant, a band of the expected size is visualized for the IC and IPC.

*When these conditions are met:*

* A test will be considered positive if a band of the expected size [xxx] bp is visualized. If relevant, a band of the expected size is visualized for the IC and IPC.
* A test will be considered negative, if no band or a band of a different size than expected is visualized.
* Tests should be repeated if any contradictory or unclear results are obtained.

*It should be noted that in virology bands of different sizes may correspond to strains of the target organism and care should be taken when interpreting conventional PCR products.*

**Real-time PCR tests**

*The Ct cut-off value given below is as established in [name of the laboratory]. As a Ct cut-off value is equipment, material and**chemistry dependent, it needs to be verified in each laboratory when implementing the test.*

*The T*M *could be equipment, material and chemistry dependent, it needs to be checked in each laboratory when implementing the test.*

*Verification of the controls*

* NIC and NAC: should give no amplification
* PIC and PAC (and if relevant IC and IPC): amplification curves should be exponential with a Ct value below XX.
* The TM value should be as expected (XX°C ± 1°C for XX).

*When these conditions are met:*

* A test will be considered positive if it produces an exponential amplification curve with a Ct value below XX.
* A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
* A melt curve analysis is performed and the obtained TM value is as expected (XX°C ± 1°C for XX).
* Tests should be repeated if any contradictory or unclear results are obtained.

**Other nucleic acid based methods (LAMP)**

*Verification of the controls*

* NIC and NAC: should produce no turbidity/colour change or no fluorescence
* PIC, PAC (and if relevant IC): should produce:

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

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

*When these conditions are met:*

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

1. **Performance characteristics available**

Validation was carried out by XXX in accordance with PM7/98. The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data <https://dc.eppo.int/validation_data/validationlist> ).

* 1. Analytical sensitivity

Analytical sensitivity evaluated using *strains / isolates / dilutions / matrix*… was *nb of specimen, cfu/mL, cell/mL, copy numbers, quantity of DNA/RNA….*.

* 1. Analytical specificity

Inclusivity: XX% evaluated on [number of] target specimens/strains/isolates/populations of XX.

Exclusivity: XX% evaluated on [number of] non-target specimens/strains/isolates/populations covering the following species: provide the list of species

* 1. Selectivity
  2. Repeatability

XX% (evaluated on X replicates atX 103 cfu/mL).

* 1. Reproducibility

XX% (evaluated with X replicates at X cfu/mL by X operators on Xdifferent days and with X different PCR equipment)

**Appendix XX ‘Technique’ HTS test (author, year)**

*The test below differs from the one described in the original publication (see 1.2).*

*The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used, provided that a verification (see PM 7/98) is carried out.*

1. **General Information**
   1. This test can be used for the detection and/or identification of species X in matrix Y.
   2. The test was developed by XXX in 20XX and adapted by XXX in XXX.
   3. Generic description of the test (e.g. rRNA depleted total RNA whole genome shotgun sequencing (Illumina), blast-based identification of putative viral contigs from de novo/reference assembly and manual verification of viral species identity)
   4. Targets: e.g. complete genomes/partial genomes/sequence reads of regulated plant viruses and viroids.
   5. Sequencing equipment, software(s) for data analysis, Computational (RAM/CPU, clusters, nodes, etc...) and storage requirements
2. **Methods**
   1. Sample preparation
      1. Bulk or individual samples
      2. Matrix (leaves, stems, fruits, …)
      3. Maceration/homogenization/grinding...
      4. Back-up sample for critical cases –80 °C
   2. Nucleic Acid Extraction and Purification
      1. Nucleic acid extraction method, kit producer name and specific instructions (if applicable), buffer composition and pH, concentration of all constituents (if known)
      2. Nucleic acid cleanup procedure and/or DNAse treatment (if applicable), kit producer name and specific instructions, buffer composition and pH, concentration of all constituents (if known)
      3. Specify any requirements for nucleic acids used for input to the library preparation (e.g. dilutions of extracted nucleic acids, PolyA addition for MinIon sequencing)
      4. Storage temperature and conditions of DNA/RNA:

DNA should preferably be stored at approximately -20°C.

RNA should preferably be stored at approximately -20°C for short term (less than one month) or at approximately -80°C for long term storage.

* 1. Library preparation

Indicate if the library preparation is performed in house or if it is outsourced. Even if it is outsourced provide as much information as possible on the workflow.

* + 1. For RNA, quality of input RNA as RNA integrity number (RIN) with tapestation or bioanalyzer (hard cut-off or indicative)
    2. Nucleic acid concentration: name of the kit (manufacturer) and equipment
    3. Library preparation: name of the kit (manufacturer) with protocol X or according to manufacturer’s instructions
    4. If applicable, Ribo depletion or polyA enrichment: name of the kit (manufacturer) and equipment
    5. Dual indexing (or barcoding) Illumina vs MinIon
    6. Amplification of library: for Illumina refer to general information and manufacturer’s protocol, for MinIon cite protocol if published or describe it.
    7. Checking the quality of the library(ies): fragment size and concentration expected.
    8. Pooling of libraries in equimolar amount
  1. Sequencing

Indicate if the sequencing is performed in house or if it is outsourced. Even if it is outsourced provide as much information as possible on the workflow.

* + 1. Sequencing platform (e.g. NextSeq V1, V2 or V3 flowcells; MiSeq P2 or P3)
    2. Read length (e.g. 150 PE, 300 PE, MinIon)
    3. Output per sample requirements
    4. Quality per sample requirements
    5. Demultiplexing (stringency: quality, index fit, read length)
  1. Bioinformatic analysis
     1. How to install/implement bio-informatic pipeline (refer to papers when previously published)
     2. Description of principles of the bioinformatic pipeline, e.g. minimal coverage, etc
     3. Refer to the settings for individual analysis steps e.g. to technical documentation, GitHub repository or (a) scientific paper(s) to allow implementation and repetition of the pipeline
     4. What type of output is to be expected: e.g. graphical representation of blast (e.g. Krona plots). Distinguish essential output and output for troubleshooting.
     5. Step by step description to go over the different output and which conclusions can be drawn to continue with the analysis of produced output
     6. List the information that need to be documented (including version control of scripts and databases used).

1. **Essential Procedural Information** 
   1. **Controls**

For a reliable test result to be obtained, the following controls should be included for each HTS run, respectively.

* Negative control to monitor contamination
* Positive control to monitor contamination, to ensure the detection of specific targets and/or to ensure the detection of low-levels of target when used at low concentration
* Alien control to monitor contamination, to ensure the detection of specific targets when used at high concentration and/or to ensure that the detection of low-levels of target when used at low concentration
* Internal control to ensure that low-levels of target can be detected.

Summary of the controls used to ensure the technical performance of the tests

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Step in the procedure** | **NAD5** | **PvEV** | **ERCC** | **RCS** |
| Sample preparation |  |  |  |  |
| Nucleic Acid Extraction and Purification | x |  |  |  |
| Library preparation |  | x | x |  |
| Sequencing |  | x | x | x |
| Bioinformatic analysis |  |  | x | x |

* 1. **Interpretation of results**: in order to assigning results from the HTS test, the following criteria should be followed:

*Verification of the controls*

List the criteria/threshold of acceptance for the different controls: e.g. being able to identify the host based on rbcL sequence (is identity on genus level enough?), criteria for % rRNA reads, number of ERCC reads in samples, etc.

*When these conditions are met:*

* Describe the conditions under which a test will be considered positive or negative
* Tests should be repeated if any contradictory or unclear results are obtained.

1. **Performance characteristics available**

Validation was carried out by XXX in accordance with PM7/98.

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

* 1. Analytical sensitivity

e.g. Relative dilution of RNA from an infected host plant in RNA from a host plant not infected by the target or infected plant material in plant material not infected by the target; when relevant: the bio-informatic analytical sensitivity, e.g. lowest number of sequence reads (when not a fixed number)

* 1. Analytical specificity

e.g. Analytical specificity of wet laboratory steps or the bio-informatic and data-analysis steps of the obtained consensus sequences (i.e. diagnostic resolution = the ability to identify the target at the relevant taxonomical level).

Inclusivity and exclusivity: indicate the list of species for which the identity was confirmed using another tests or by provider (when obtained from external collections).

* 1. Selectivity

e.g. Dilution of RNA of plants with selected target(s) in healthy host RNA of difficult matrices: effect on average read coverage

* 1. Repeatability

e.g. Within validation study: different preparations of the same/similar biological sample, analysed by the same person at the same moment.

* 1. Reproducibility

e.g. Within validation study: different preparations of the same/similar biological sample, analysed by different persons at different moments in time, , but also trend analysis of controls such as External RNA Controls Consortium (ERCC) or alien controls.

* 1. Additional information

e.g. The test was used to demonstrate the detection and identification of other viruses and viroids (refer to publication and/or to the EPPO database on diagnostic expertise (validation section)).

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

1. *Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.* [↑](#footnote-ref-2)
2. <http://www.eppo.int/QUARANTINE/diag_activities/EPPO_TD_1056_Glossary.pdf> [↑](#footnote-ref-3)
3. Commission Implementing Regulation (EU) 2019/2072 [↑](#footnote-ref-4)
4. [↑](#footnote-ref-5)