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Validation of diagnostic tests to support plant health

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Abstract:
A general standard operating procedure (SOP) for the production of reference material (RM) for use in plant health diagnostics was developed within the VALITEST project. The general SOP was designed based on information on existing SOPs and guidelines available with the consortium partners (Deliverable 3.2) and partly incorporates the quality criteria for reference material (Deliverable 3.1). The general SOP describes the different steps required in the production process, ranging from the different possible sources of the reference material, tests to confirm its identity, possibly required multiplication steps to the actual production process. For each step in the process, criteria and critical points can be identified, but they will depend on the nature of the RM and on the target test in which the RM will be used.

The original Deliverable 3.2 (February 2019) was evaluated by all partners (Milestone 3.4 Evaluation report) and updated towards this new version. The updated general standard operating procedure (SOP) for the production of reference material (RM) includes a stand-alone version of a template for the production of RM (Annex I) and an example of a full SOP for the production of RM of *Fusarium circinatum* to be used in (Real-Time) PCR assays (Annex II).

Partners involved Task that finally led to this SOP are WR, UNITO, WBF, EPPO, NIB, NVWA, ANSES, IPADLAB, SEDIAG

The content of this SOP represents the views of the author only and is his/her sole responsibility; it cannot be considered to reflect the views of the European Commission and/or the Research Executive Agency or any other body of the European Union. The European Commission and the Agency do not accept any responsibility for use that may be made of the information it contains.

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1 Purpose

As described in the VALITEST project proposal **WP3** aims at developing recommendations for the quality of reference materials for validation purposes. An essential condition for proper validation of methods and execution of test performance studies is the availability of well-defined and characterized reference material. The actual production of reference material involves many steps for which currently no common guidelines are shared by the different producing laboratories and companies.

There is clearly a growing market demand for more differentiation and better and more uniform quality assurance for the reference material used in tests for plant pests. In this deliverable the minimum criteria are defined for the production of reference material (RM) production for non-certified suppliers such as research laboratories and suppliers including commercial SMEs (international collections, specific suppliers). For the production of certified reference materials (CRM) producers will have to meet the ISO 17034:2016 guidelines.

This deliverable describes a standard operating procedure (SOP) for the production of different types of reference material (RM) for use in validation studies for diagnostic tests. Metrological sound reference material needs not just to be representative from the biological point of view but it should also have well defined cell count or DNA/RNA copy number (if applicable), homogeneity, stability and purity. However, suitable validation tests to check the RM itself will vary with the nature of the RM and this SOP can by no means be exhaustive for all possible applicable tests. Therefor in text boxes throughout the deliverable specific examples are given, while in Annex II a full example SOP is given. Used terminology and procedures in this SOP are where possible in line with those described in ISO (especially ISO17034:2016) and EPPO Standards and references are given to those guidelines. Annex I is a document containing a format to aid laboratories in producing their own RM production procedures.

2 Scope

The general standard operating procedure (SOP) developed in this deliverable is intended for use in the production of different types of reference material by different plant health laboratories and by producers of plant health diagnostics tests for use in test performance studies or as controls when performing plant pest diagnostics. For the production of certified reference materials (CRM) producers must meet the ISO 17034:2016 guidelines.

This SOP has been evaluated by the participants of the VALITEST Test Performance Study (TPS) 1 and is intended to be used in VALITEST TPS2.

3 Terms, abbreviations and definitions

Generally, reference material used in plant diagnostic testing is used to provide essential traceability in calibration and testing. Reference materials are used to monitor the performance of detection and identification tests, to demonstrate the accuracy of results, to calibrate or verify equipment and tests, to monitor lab performance, and to enable the comparison of tests (PM 7/98, *in press*).

Reference material may be derived from a collection which can either be a working collection, a reference collection or a certified reference collection. It may also be derived from plant material from the field or collected at import (e.g. for a newly discovered pest for which no collection material is

available yet). In addition, when working with reference materials users may have different ideas and expectations on their use and performance. It is therefore important to first define what reference materials are, where they come from and in what context they can and should be used.

For the definitions those in ISO 17034:2016; *Guidelines on the general requirements for the competence of reference material producers* and EPPO Standards PM 7/76 (5); *Use of EPPO diagnostic protocols* (2018), were used -where applicable. Other definitions were prepared in the framework of the FP7 EU-project Q-collect 'Coordination and Collaboration between reference collections of plant pests and diseases for EU Plant Health' (Grant Agreement number:612712), that focussed on coordination and collaboration between reference collections of plant pests and diseases within the EU. These are also very relevant for this current VALITEST project.

Assigned Value:

EPPO Standard PM 7/122: Assigned quantity values correspond to the expected result of the test (pest present or absent, concentration of the pest, morphological characteristics of the specimen, etc.).

Certified reference material (CRM):

EPPO PM 7/76 (5): Reference material derived from a source that certifies the authenticity of the material. The material should preferably come from an internationally recognized source such as a national reference collection. The material should have a unique identification code allowing traceability and the name of the person who certified its authenticity. Details of how the material was authenticated should also be supplied. If appropriate, information about its activity (e.g. pathogenicity, antigenic properties) under specified conditions should also be supplied along with any related uncertainty at a stated level of confidence.

ISO 17034**:**2016: Reference material characterized by a metrological valid procedure (ISO Guide 35) for one or more specified properties, accompanied by a reference material certificates (ISO Guide 31) that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability .

For clarification: The ISO 9000 family of quality management systems standards is designed to help organizations ensure that they meet the needs of customers and other stakeholders while meeting statutory and regulatory requirements related to a product or service. ISO 9001 is the only standard in the ISO 9000 series to which organizations can certify. ISO 17034:2016 defines the qualifications for reference material producers. While ISO Guide 35 gives details on metrological valid procedures for properties of RM, ISO Guide 31 provides information for reference material certificates. Per definition only ISO 17034:2016 certified reference material producers can produce certified reference material according to the other mentioned guidelines.

Certified value:

ISO Guide 30: 2015, section 2.2.3 Value assigned to a property of a (certified) reference material that is accompanied by an uncertainty statement and a statement of metrological traceability, identified as such in the reference material certificate.

Commutability (RM):

Characteristic describing the extent to which the reference material resembles actual samples. A reference material is considered commutable when a test produces the same result as it does for an authentic sample that contained the same analyte concentration.

While naturally contaminated samples are often considered as the most appropriate material for reference material, this is not always available. We recognise seven classes of commutability, ranging

from naturally infested plant material to completely artificial synthetic nucleic acids (see paragraph 4.1; sources of reference material).

Field material:

Material collected directly from the field or at import, usually plant parts. These may or may not show typical symptoms of the pest. The material may (or may not) contain the target pest and it may contain other organisms which may interfere in one or more of the steps later in the procedure. As working collection material, most likely it will not meet all requirements for proper form and/or documentation and should therefore go through identification steps (and possibly multiplication steps).

Homogeneity (RM):

EPPO PM 7/122 section 3.5.1: the assessment of homogeneity should generally be performed after the samples have been packaged in the final form and before distribution to participants. Homogeneity can be demonstrated prior to packaging where no influence of packaging is reasonably expected. The EPPO guideline provides further information on the assessment of homogeneity for different types of material also stating: it recommends to test a minimum of 10 randomly chosen samples (for each pest/ matrix/infestation level, including negative samples) in duplicate.

ISO Guide 35:2017 gives further guidance on the assessment of within unit and between unit homogeneity and suggests a number of test samples between 10 and 30 when testing for homogeneity.

In cases where it is not technically possible -for example when limited material is available- less samples may be checked. The following numbers of samples for the homogeneity test samples are recommended, depending on the number of actually produced samples:

Number of samples prepared Per production lot	Number of samples to be tested
≤19	3
20-39	4
40-49	5
50-59	6
60-69	7
70-79	8
80-95	9
≥95	10

Depending on the type of material and intended use of the RM, properties and desired levels of the RM should be defined before the homogeneity testing (ANSES: Method for undertaking and interpreting homogeneity and stability studies).

Identity (RM):

The reference material should be clearly identified and characterized to ensure its correct identification. Following the recommendation this should be done if possible by at least two preferably validated and independent tests based on different physical characteristic of the RM. If the specimen used originates from and is available in several reference collections working according to commonly agree quality standards this provides additional guarantee for its identity. At minimum, the material should be thoroughly identified to the level of international accepted diagnostic protocols (when available) to ensure it is properly identified. The list of tests used for its identification should be clear from the RM documentation.

Material traceability (RM):

Material traceability of the RM covers the origin of the material including prior handling and multiplication (if applicable). If the material is derived from a collection, it should be traceable to a specific specimen in that collection including its history of maintenance and handling in that collection. If it is derived from field material metadata should be documented; i.e. when and where the material was collected, from what plant species and part and whether or not it was showing which symptoms.

Multiplication (of RM):

If more or a different type of (biological) material of a certain candidate reference material is needed, the material may be used for multiplication/ amplification. This may for instance mean multiplication in or on plants or specific substrates, but also be an amplification of specific target genes.

NAC:

Negative amplification control (in molecular or other tests).

NC:

Negative control.

NIC:

Negative isolation control (in molecular or other tests).

PAC:

Positive amplification control (in molecular or other tests).

PC:

Positive control.

Pest (IPPC, 2017):

Any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products.

PIC:

Positive isolation control (in molecular or other tests).

Purity (RM):

Purity is defined as a ratio of target pest versus non-targets, particularly non-targets interfering with a test.

Presence of non-target material cannot always be avoided and does not need to be a problem however, this depends on the exclusivity specificity of the test used i.e. the performance of a test with regard to cross-reaction with a range of non-targets (e.g. closely related organisms, contaminants) (EPPO PM7/76 (5).

Quality assurance:

Part of quality management, focussed on providing confidence that quality requirements will be fulfilled.

Dependent on the laboratories own quality management system, samples and materials may be rejected if basic quality requirements are not met. For example, samples clearly contaminated with non-target organisms or (spoiled) samples transported under sub-optimal conditions may be discarded.

Reference collection:

Q-Collect: A collection of individuals maintained for the purpose of study and authentication. Reference collections are generally large undertakings maintained by institutions; instead of having a single representative of each species, they will typically have multiples, so as to illustrate variations and, be able to provide samples externally for comparisons and research. Reference collections are an important source of information about variations of populations within a species. They are also the repository of type strains or holotypes used as the official definition of a particular species.

Examples of reference collections are microbial culture collections focussing on acquisition, authentication, production, cataloguing and distribution on microbial cultures.

Reference material (RM):

EPPO PM 7/76 (5) Material appropriate to the test and diagnosis being performed such as live cultures, infested plant material, DNA/RNA preparations, images of a diagnostic quality or mounted specimens. The reference material used should be documented and appropriate for the test and diagnosis being performed. It should be ensured that it has the features for which it was selected, for example expressing a desired antigen for use in serological diagnosis or display specific physical features (e.g. sporulation) if used for morphological diagnosis ().

Reference material document (RM document):

ISO17034:2016 Document containing all the information that is essential for using any reference material.

The RM document covers both the product information sheet and reference material certificate. It should contain certified values: values assigned to a property of a reference material (*e.g.* homogeneity or stability) that is accompanied by an uncertainty statement and a statement of metrological traceability, identified as such in the reference material certificate. For more information ISO Guide 31:2015 gives more data on the contents, labels and accompanying documentation needed for reference materials.

Stability (RM):

Samples should be demonstrated to be sufficiently stable to ensure that they will not undergo any significant change, including storage and transport conditions. The number of aliquots to be tested for stability depends on the quantity of the reference material produced (recommended minimum 3 random chosen samples). ISO Guide 35:2017 gives further guidance on the assessment of stability and the management of the risks associated with possible stability issues related to the properties of interest. The ISO Guidelines state that RM should be sufficiently stable for its intended use, defining stability further in stability under long-term storage conditions, transport conditions and where applicable storage conditions at the RM user's laboratory. It can include considerations of stability after opening of RM, if re-use is permitted.

EPPO PM 7/122(1) recommends testing a minimum of 3 randomly chosen samples in duplicate. However, as this may not always be feasible, testing may be reduced if suitable data are available from previous stability testing or according to the expertise of the organizer. The choice of the number of tested samples should be documented (EPPO 7/122 (1)/ ISO13528). Further information regarding the assessment of stability for different type of material is provided in EPPO PM 7/122(1). ISO Guide

35:2017 allows less experimental stability studies if the RM producer has prior information on stability from previous materials held for an extended period under the same planned storage conditions and which were characterised for the same properties and share the same matrix composition.

Test:

EPPO PM 7/76 (5): the application of a method to a specific pest and a specific matrix.

Test performance study (also referred to as ring tests or collaborative trials):

EPPO PM 7/76 (5): Evaluation of the performance of one or more tests by two or more laboratories using defined samples.

Traceability (RM):

Traceability can be considered as an aspect of both identity and origin of the material in the sense that it may provide some additional guarantees to the correct identity of the materials used to prepare the reference material and its future availability. To ensure traceability it is important to properly identify and describe the matrix as well and ensure its proper identification. Provide any relevant meta-data available for the RM.-see material traceability.

Traceability can also refer to how certain values of the reference material were determined, the actual determined values and uncertainties in them.

Working collection:

Q-collect: Collections, usually of individuals belonging to a single organism or group of related organisms, maintained for the purpose of scientific investigation by experts. Working collections are usually maintained by individual researchers or research groups with recognized knowledge of the organism(s) in question. They do not usually provide samples externally other than to deposit individuals of interest into one or more reference collections for safe keeping and/or protection of intellectual property.

4 Methodology

Partners within WP3 as well as commercial laboratory partners within the VALITEST consortium were approached to supply already existing SOPs for use in the production of specific reference material. None of the partners who replied, had such specific SOPs available. Some partners supplied general SOPs on i.e. management and maintenance of (reference) material in collections (ANSES, NVWA, and FERA).

Based on these more generic SOPs and personal expertise within WP3 a flow chart was designed to serve as the basis for a SOP for the general production of reference materials (see Figure 1). This flow chart provides an overview of steps to follow for the production of reference material specific for one or more target organism(s) and one or more target test(s). Competence of laboratories and personnel as well as traceability of materials, methods, and test results involved in the different production steps is essential. The EPPO standard PM 7/84 (2) describes basic requirements for quality management in plant pest diagnosis and the general management and technical requirements described in this Standard also apply to the establishment and running of reference collections of plant pests and should be met.

The different steps identified within the SOP are discussed below:

4.1 Sources of (candidate) RM

It is acknowledged that both the physical nature as well as the source of the material intended as positive of negative RM can be quite diverse. Plant health diagnostic deals with a variety of different pests each with their own characteristics and their own specific tests. The majority of pests are not available in pure or cultured form and different biological types of RM were defined in Deliverable 3.1 in decreasing order of commutability):

- naturally infested plant material
- artificially infested plant material
- spiked plant material
- purified or isolated organisms
- total nucleic acids from a sample (target organism in background)
- purified nucleic acids
- synthetic nucleic acids

The (candidate) reference material may come from different sources and in different forms. The source and form do determine the steps necessary in the actual production of the reference material. The first step in the process is therefor to distinguish between the possible sources of the candidate material. The SOP distinguishes four different possible sources: CRM, RM, working collection material and field material (for definitions see above). CRM comes in a specific form and with the defined use for one or more specified tests, when using it for other purposes it loses its original certification.

Before the candidate material, irrespective of its source, can be processed further an initial check on its quality at arrival (by the laboratory receiving the material) is mandatory. The criteria of this check depend on the internal quality system requirements of the laboratory as well as documentation supplied, and the conditions set by the supplier (e.g. transport under certain controlled conditions or time restrictions). If these criteria are not met, the material maybe rejected (see also Deliverable D3.1 paragraph 5.10). Depending on the source of the material its use may be governed by conditions upon which it was obtained defined by e.g. Nagoya protocol requirements, material transfer agreements and other. These conditions should be met before it can be further processed.

In the actual SOP a description of the target organism should be included as well as a description of the final form and the intended test(s) it is to be used in.

4.2 Identity test

If the material intended for use as RM comes either from field material, a working collection or is based on reference material for which the necessary documentation is missing, it should be treated as not sufficiently characterized material and should go through proper identification steps. These should comprise of at least two independent tests (test 1 and 2, both preferably validated), preferably based on different physical characteristics (e.g. serological and genetic) to prove the identity of the pest in the material. If one or both tests fail to confirm its identity the material should be rejected. If both tests confirm the identity of the pest, the material can be regarded as candidate RM. If sufficient material is available, the candidate RM may go in the production phase.

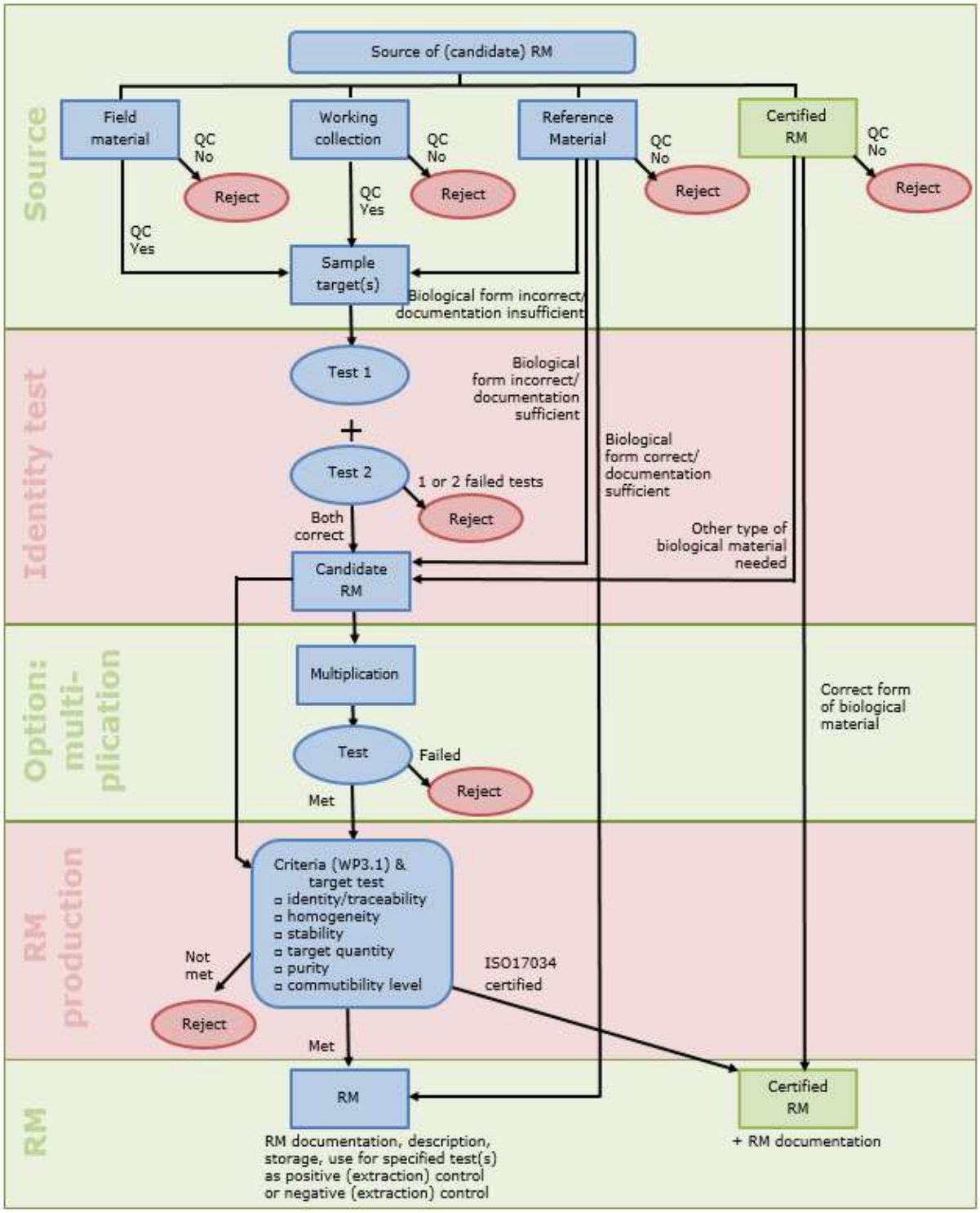

Figure 1: Graphical presentation of the general Standard Operating Procedure (SOP) for the production of reference material to be used in specified tests as either positive or negative samples. For the different blocks additional information can be found in paragraphs 4.1 to 4.5. For identity tests, two independent tests are recommended, but if not available, one will have to suffice.

4.3 Multiplication (optional)

If not enough material is available for future use, the candidate RM may need to go through one or more multiplication steps. Following this, the identity of the material needs re-confirmation using a preferably validated test, which may be one of the tests used in the identity test. If the identity of the RM cannot be confirmed the material should be rejected

4.4 Reference material (RM) production

The final step is the actual production and testing of the required minimal criteria of the reference material. Precise steps and procedures will depend on the actual physical properties and intended end-use (i.e. specific test) of the material. Following production, the material should meet all the general criteria for RM as defined in section 5 (Descriptors for reference material (RM) for validations and TPSs) in the report on Deliverable 3.1 of WP3. Table 1 lists the different criteria to be assessed in the production of RM, their possible values and the minimum criterion that should be reached. As discussed, and described in Deliverable 3.1, the minimum level identified is considered as a required criterion. However, the specific levels of each criterion required for a specific RM will depend on the scope of its use and purpose. As already stated in Deliverable 3.1 (paragraph 6) it is important to note '*that since criteria are inherently linked to the intended use of the reference materials and may be test-specific, the criteria may be different for different uses and should be defined by the producer*'.

The criteria considered for reference material are:

- Identity
- Material traceability
- Quantification of the material
- Homogeneity
- Stability
- Assigned Value
- Purity
- Commutability

Assigned values can be given with their uncertainties. These values can vary depending on the nature of the RM and its commutability. For instance as known concentration i.e. absolute quantification of the target pest and/or its components (e.g. DNA copy numbers), as level of concentration (high/medium/low) known (as determined through use of at least one semi-quantitative or quantitative test), as qualitative status known (positive/negative above the determined limit of detection using at least one test), as consensus values from participants in proficiency test or as originating from plants with known health statues with a recent test result (a given period of time depends on the plant-pest combination and previous experience). Rules for the definition of the values should be defined beforehand: statistical methods, outlier's effect (e.g. a virology interlaboratory comparison may assign the values this way). Also the uncertainty of assigned values should be defined.

Reports on an assigned/certified value of particular RM should include and reflect the method used to determine them. E.g. if turbidity measurements were used to determine bacterial cells concentrations this should be reported as 'the turbidity of XY which corresponds to xy cells/unit'. Similarly, if the concentration was determined through colony counts, the concentration should be reported as colony forming units/unit also stating the media and growth conditions used.

When all criteria for the candidate RM are met, it can be regarded as reference material.

4.5 Reference material

Passing all the tests, the final RM is ready for use, but it needs to be accompanied by suitable documentation regarding the defined minimal criteria set above (identity, material traceability, quantification, homogeneity, stability, purity and commutability level). The documentation should also include data on the intended use and for which tests the RM is suitable and advice on required storage conditions. Only ISO 17034:2007 certified laboratories can produce certified RM.

A useful tool for the description of the different criteria was produced as part of deliverable 3.1 and is reproduced here as Table 1. This table lists the different criteria for the production of reference material, the several levels available for each criterion and the minimum level required for each criterion required in the production of reference material.

Table 1: List of descriptors of criteria to be assessed in the production of reference material (RM). Where several levels are available for a descriptor, the lowest corresponds to the minimum criterion for the specific descriptor (Reproduced from Deliverable 3.1).

Descriptor	Value	Minimum criterion
Intended use	**should be defined (in this case it equals preparation of RM for the scope of the individual test or TPS)**	**yes**
Identity	**identified to the level of internationally recognized diagnostic protocols**	**yes**
Traceability	traceability to a specimen from a reference culture collection	no
	traceability to a specimen from a working culture collection	no
	traceability provided for the target pest and matrix used (the latter if relevant)	**yes**
Commutability level	naturally infested plant material	no
	artificially infested plant material	no
	spiked plant material	no
	purified organisms	no
	total nucleic acids from a sample (target organism in background)	no
	purified nucleic acids	no
	synthetic nucleic acids	**yes**
Homogeneity	**homogenous**	**yes**
Stability	**stable**	**yes**
	stability - short term	no
	stability - long term	no
Assigned value	absolute concentration known	no
	level of concentration known (high/medium/low)	no
	qualitative status known (above LOD level)	no
	originating from plants with known health statues with a recent test result (a given period of time depends on the plant-pest combination and previous experience)	**yes**
Purity	absence of non-targets	no
	absence of interfering non-targets	no
	known ratio of target vs. non-target interfering with the test - high	no
	known ratio of target vs. non-target interfering with the test - medium	no
	known ratio of target vs. non-target interfering with the test - low	**yes**

5 Final notes, limitations and further perspectives

The current deliverable describes the requirements and procedures for the production of reference material and necessary accompanying documentation by the RM producer, suitable for intra and interlaboratory studies (e.g. test performance studies, proficiency). These requirements and procedures were used to design a format for a general SOP for the production of reference material (Annex 1). An example of a specific SOP for the production of reference material of *Fusarium circinatum* using this format is listed in Annex 2.

This general SOP was designed for use within the VALITEST project and has been evaluated after VALITEST Test Performance study 1 (TPS1) to be used in VALITEST TPS2.

6 Acknowledgements

We thank all VALITEST collaborators for their help in putting together this SOP for RM and accompanying documents. This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°773139.

7 Sources of operating procedures

ANSES. Procédure spécifique: Gestion des différents matériaux de référence.

ANSES. Method for undertaking and interpreting homogeneity and stability studies.

EPPO (2018) PM 7/76 (5) Use of EPPO Diagnostic protocols. *Bulletin OEPP/EPPO Bulletin* 48 (3): 373–377.

EPPO (2018) PM 7/84 (2) Basic requirements for quality management in plant pest diagnosis laboratories. *Bulletin OEPP/EPPO Bulletin* 48 (3): 378–386.

EPPO (2018) PM 7/98 (3) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. *Bulletin OEPP/EPPO Bulletin* 48 (3): 387–404.

EPPO (2014) PM7/122 (1) Guidelines for the organization of interlaboratory comparisons by plant pest diagnostic laboratories. *Bulletin OEPP/EPPO Bulletin* 44 (3): 390-399

FERA (2016) The basic procedure of mechanical inoculation, including maintenance of Potato spindle tuber viroid (PSTVd) reference cultures. PLH/195 (5)

FERA (2011) The Restoration of Nematode Specimens Slide Mounted in Anhydrous Glycerol. PLH/849 (1)

ISO 13528:2015(en) Statistical methods for use in proficiency testing by interlaboratory comparison.

ISO 17034:2007 (2017) on the general requirements for the competence of reference material producers (RMPs).

ISO 31:2015 (2015) Reference materials- contents of certificates, labels and accompanying documentation.

ISO 35:2017 (2017) Reference materials- Guidance for characterization and assessment of homogeneity and stability.

NVWA (2016) Opname, beheer en uitgifte collectie- en referentiemateriaal MolBio. I-MOL-077 (3)

VALITEST (2019) Deliverable D3.1.

ANNEX 1

General Standard Operating Procedure (SOP) for the production of reference material (RM)

This general Standard Operating Procedure (SOP) for the production of reference material (RM) is meant as a stand-alone template for use in a laboratory for the production of RM.

Version	date	Relevant changes

1 Scope/intended use

This is a standard operating procedure (SOP) for the production of specify: particular organism as reference material as specify: particular form of the reference material is to be used in for use in specify: particular test in which the RM is to be used. It describes the different steps required in the production process, ranging from the different possible sources of the reference material, tests to confirm its identity, possibly required multiplication steps to the actual production process. For each step in the process, criteria and critical points are identified.

DISCLAIMER: Depending on the nature of the reference material, the exact criteria for its acceptance as RM need to be set. These will clearly differ when whole insects are the RM or when amplified nucleic acids solution are the RM. In this general SOP suggestions are made for the general procedure, steps and criteria; but no standard requirements can be set for all types of RM. This general SOP is meant as framework for the production of SOPs for RM for targeted use.

Only a ISO17034:2016 certified laboratory can produce certified reference materials (CRM). ISO 17034:2016 specifies the general requirements for the competence and consistent operation of reference material producers. It sets out the requirements in accordance with which reference materials are produced. In laboratories that are not ISO 17034:2017 certified, only reference material (RM) can produced and this SOP may provide a useful tool in their production of RM.

2 Definitions & Descriptions

Assigned Value:

EPPO Standard PM 7/122: Assigned quantity values correspond to the expected result of the test (pest present or absent, concentration of the pest, morphological characteristics of the specimen, etc.).

Certified reference material (CRM):

EPPO PM 7/76 (5): Reference material derived from a source that certifies the authenticity of the material. The material should preferably come from an internationally recognized source such as a national reference collection. The material should have a unique identification code allowing traceability and the name of the person who certified its authenticity. Details of how the material was authenticated should also be supplied. If appropriate, information about its activity (e.g. pathogenicity, antigenic properties) under specified conditions should also be supplied along with any related uncertainty at a stated level of confidence.

ISO 17034:2016: Reference material characterized by a metrological valid procedure (ISO Guide 35) for one or more specified properties, accompanied by a reference material certificates (ISO Guide 31) that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability .

For clarification: The ISO 9000 family of quality management systems standards is designed to help organizations ensure that they meet the needs of customers and other stakeholders while meeting statutory and regulatory requirements related to a product or service. ISO 9001 is the only standard in the ISO 9000 series to which organizations can certify. ISO 17034:2016 defines the qualifications for reference material producers. While ISO Guide 35 gives details on metrological valid procedures for properties of RM, ISO Guide 31 provides information for reference material certificates. Per definition only ISO 17034:2016 certified reference material producers can produce certified reference material according to the other mentioned guidelines.

Certified value:

ISO Guide 30: 2015, section 2.2.3 Value assigned to a property of a (certified) reference material that is accompanied by an uncertainty statement and a statement of metrological traceability, identified as such in the reference material certificate.

Commutability (RM):

Characteristic describing the extent to which the reference material resembles actual samples. A reference material is considered commutable when a test produces the same result as it does for an authentic sample that contained the same analyte concentration.

While naturally contaminated samples are often considered as the most appropriate material for reference material, this is not always available. We recognise seven classes of commutability, ranging from naturally infested plant material to completely artificial synthetic nucleic acids (see paragraph 4.1; sources of reference material).

Field material:

Material collected directly from the field or at import, usually plant parts. These may or may not show typical symptoms of the pest. The material may (or may not) contain the target pest and it may contain other organisms which may interfere in one or more of the steps later in the procedure. As working

collection material, most likely it will not meet all requirements for proper form and/or documentation and should therefore go through identification steps (and possibly multiplication steps).

Homogeneity (RM):

EPPO PM 7/122 section 3.5.1: the assessment of homogeneity should generally be performed after the samples have been packaged in the final form and before distribution to participants. Homogeneity can be demonstrated prior to packaging where no influence of packaging is reasonably expected. The EPPO guideline provides further information on the assessment of homogeneity for different types of material also stating: it recommends to test a minimum of 10 randomly chosen samples (for each pest/ matrix/infestation level, including negative samples) in duplicate.

ISO Guide 35:2017 gives further guidance on the assessment of within unit and between unit homogeneity and suggests a number of test samples between 10 and 30 when testing for homogeneity.

In cases where it is not technically possible -for example when limited material is available- less samples may be checked. The following numbers of samples for the homogeneity test samples are recommended, depending on the number of actually produced samples:

Number of samples prepared Per production lot	Number of samples to be tested
≤19	3
20-39	4
40-49	5
50-59	6
60-69	7
70-79	8
80-95	9
≥95	10

Depending on the type of material and intended use of the RM, properties and desired levels of the RM should be defined before the homogeneity testing (ANSES: Method for undertaking and interpreting homogeneity and stability studies).

Identity (RM):

The reference material should be clearly identified and characterized to ensure its correct identification. Following the recommendation this should be done if possible by at least two preferably validated and independent tests based on different physical characteristic of the RM. If the specimen used originates from and is available in several reference collections working according to commonly agree quality standards this provides additional guarantee for its identity. At minimum, the material should be thoroughly identified to the level of international accepted diagnostic protocols (when available) to ensure it is properly identified. The list of tests used for its identification should be clear from the RM documentation.

Material traceability (RM):

Material traceability of the RM covers the origin of the material including prior handling and multiplication (if applicable). If the material is derived from a collection, it should be traceable to a specific specimen in that collection including its history of maintenance and handling in that collection. If it is derived from

field material metadata should be documented; i.e. when and where the material was collected, from what plant species and part and whether or not it was showing which symptoms.

Multiplication (of RM):

If more or a different type of (biological) material of a certain candidate reference material is needed, the material may be used for multiplication/ amplification. This may for instance mean multiplication in or on plants or specific substrates, but also be an amplification of specific target genes.

NAC:

Negative amplification control (in molecular or other tests).

NC:

Negative control.

NIC:

Negative isolation control (in molecular or other tests).

PAC:

Positive amplification control (in molecular or other tests).

PC:

Positive control.

Pest (IPPC, 2017):

Any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products.

PIC:

Positive isolation control (in molecular or other tests).

Purity (RM):

Purity is defined as a ratio of target pest versus non-targets, particularly non-targets interfering with a test.

Presence of non-target material cannot always be avoided and does not need to be a problem however, this depends on the exclusivity specificity of the test used i.e. the performance of a test with regard to cross-reaction with a range of non-targets (e.g. closely related organisms, contaminants) (EPPO PM7/76 (5).

Quality assurance:

Part of quality management, focussed on providing confidence that quality requirements will be fulfilled.

Dependent on the laboratories own quality management system, samples and materials may be rejected if basic quality requirements are not met. For example, samples clearly contaminated with non-target organisms or (spoiled) samples transported under sub-optimal conditions may be discarded.

Reference collection:

Q-Collect: A collection of individuals maintained for the purpose of study and authentication. Reference collections are generally large undertakings maintained by institutions; instead of having a single representative of each species, they will typically have multiples, so as to illustrate variations and, be able to provide samples externally for comparisons and research. Reference collections are an important source of information about variations of populations within a species. They are also the repository of type strains or holotypes used as the official definition of a particular species.

Examples of reference collections are microbial culture collections focussing on acquisition, authentication, production, cataloguing and distribution on microbial cultures.

Reference material (RM):

EPPO PM 7/76 (5) Material appropriate to the test and diagnosis being performed such as live cultures, infested plant material, DNA/RNA preparations, images of a diagnostic quality or mounted specimens. The reference material used should be documented and appropriate for the test and diagnosis being performed. It should be ensured that it has the features for which it was selected, for example expressing a desired antigen for use in serological diagnosis or display specific physical features (e.g. sporulation) if used for morphological diagnosis ().

Reference material document (RM document):

ISO17034:2016 Document containing all the information that is essential for using any reference material.

The RM document covers both the product information sheet and reference material certificate. It should contain certified values: values assigned to a property of a reference material (*e.g.* homogeneity or stability) that is accompanied by an uncertainty statement and a statement of metrological traceability, identified as such in the reference material certificate. For more information ISO Guide 31:2015 gives more data on the contents, labels and accompanying documentation needed for reference materials.

Stability (RM):

Samples should be demonstrated to be sufficiently stable to ensure that they will not undergo any significant change, including storage and transport conditions. The number of aliquots to be tested for stability depends on the quantity of the reference material produced (recommended minimum 3 random chosen samples). ISO Guide 35:2017 gives further guidance on the assessment of stability and the management of the risks associated with possible stability issues related to the properties of interest. The ISO Guidelines state that RM should be sufficiently stable for its intended use, defining stability further in stability under long-term storage conditions, transport conditions and where applicable storage conditions at the RM user's laboratory. It can include considerations of stability after opening of RM, if re-use is permitted.

EPPO PM 7/122(1) recommends testing a minimum of 3 randomly chosen samples in duplicate. However, as this may not always be feasible, testing may be reduced if suitable data are available from previous stability testing or according to the expertise of the organizer. The choice of the number of tested samples should be documented (EPPO 7/122 (1)/ ISO13528). Further information regarding the assessment of stability for different type of material is provided in EPPO PM 7/122(1). ISO Guide 35:2017 allows less experimental stability studies if the RM producer has prior information on stability from previous materials held for an extended period under the same planned storage conditions and which were characterised for the same properties and share the same matrix composition.

Test:

EPPO PM 7/76 (5): the application of a method to a specific pest and a specific matrix.

Test performance study (also referred to as ring tests or collaborative trials):

EPPO PM 7/76 (5): Evaluation of the performance of one or more tests by two or more laboratories using defined samples.

Traceability (RM):

Traceability can be considered as an aspect of both identity and origin of the material in the sense that it may provide some additional guarantees to the correct identity of the materials used to prepare the reference material and its future availability. To ensure traceability it is important to properly identify and describe the matrix as well and ensure its proper identification. Provide any relevant meta-data available for the RM.-see material traceability.

Traceability can also refer to how certain values of the reference material were determined, the actual determined values and uncertainties in them.

Working collection:

Q-collect: Collections, usually of individuals belonging to a single organism or group of related organisms, maintained for the purpose of scientific investigation by experts. Working collections are usually maintained by individual researchers or research groups with recognized knowledge of the organism(s) in question. They do not usually provide samples externally other than to deposit individuals of interest into one or more reference collections for safe keeping and/or protection of intellectual property.

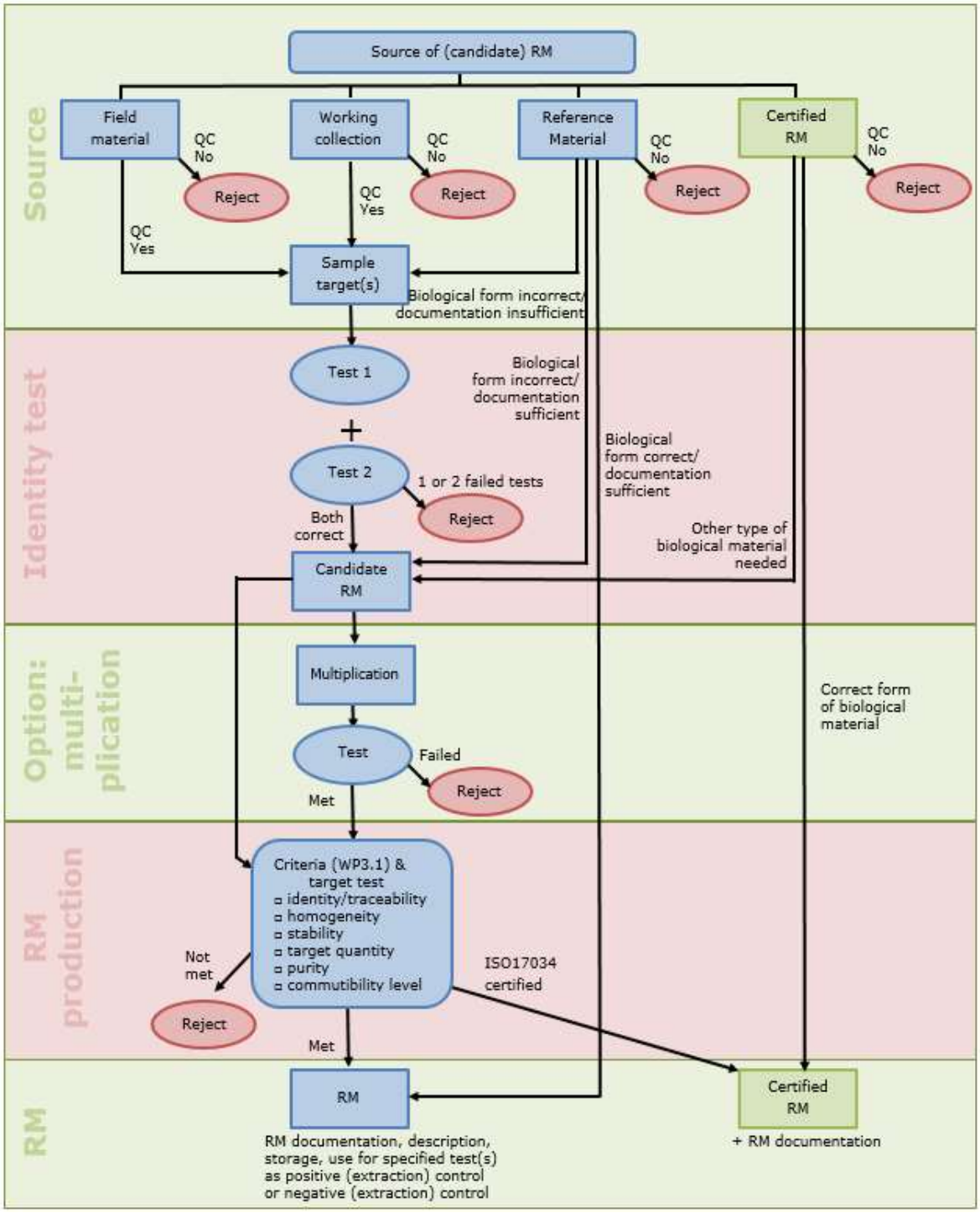

Figure 1: Graphical presentation of the general Standard Operating Procedure (SOP) for the production of reference material to be used in specified tests as either positive or negative samples. For the different blocks additional information can be found in paragraphs 4.1 to 4.5. For identity tests, two independent tests are recommended, but if not available, one will have to suffice.

3 Source of (candidate) reference material

Add description of the target material. The source of the material should be identified i.e. field collected material/working collection material/reference material/certified reference material), including all available metadata.

Both the biological nature as well as the source of the material intended as positive of negative RM can be quite diverse and may differ from the biological form needed for the test(s) the RM is intended for. The majority of pests are not available in pure or cultured form. The biological type of reference material should be defined. Add type of candidate material and needed form for RM:

- naturally infested plant material
- artificially infested plant material
- spiked plant material
- purified or isolated organisms
- total nucleic acids from a sample (target organism in background)
- purified nucleic acids
- synthetic nucleic acids

Provide: documentation on the handling protocol of target organism and sampling and on sample registration.

Initial check: Before material, irrespective of its source, can be processed an initial check at arrival on its quality by the laboratory receiving the material is mandatory. The criteria of this check depend on the internal quality system requirements of the laboratory as well as documentation supplied, and the conditions set by the supplier (e.g. transport under certain controlled conditions or time restrictions). If these criteria are not met, the material should be rejected.

Additional requirements/restrictions: Depending on the source of the material its use may be governed by conditions upon which it was obtained defined by e.g. Nagoya protocol requirements, material transfer agreements and other. These conditions should be met before it can be further processed.

4 Identification of candidate reference material

If the material intended for use as RM comes either from field material, or a working collection, reference material but either is in the incorrect form or necessary documentation is missing, it should be treated as not sufficiently characterized material and should go through proper identification steps. In the case of certified reference material we assume the reference material has been correctly identified and this step is not necessary. However, when in doubt of the correctness for instance due to suboptimal packaging /problems in transport, treat it as RM and perform additional identification tests.

In principle two independent tests (preferably based on different physical characteristics (e.g. serological and genetic) should be performed for the target organism(s). In the case of destructive sampling in *e.g*. insects or nematodes this may not be possible.

Give: Depending on type of material, a list/selection of recommended tests for the identification of target species. Standard operation procedures for these test(s) can be given or it should be documented where to find them. The list contains preferably validated tests (accepted diagnostic protocols) but may contain other tests if these are not available *e.g.* in the case of new, emerging species.

If one or both tests fail to confirm its identity the material should be rejected. If both tests confirm the identity of the pest, the material can be regarded as candidate RM. If sufficient material is available, the candidate RM may go in the production phase. If not enough material is available for later use as reference material, a multiplication step may be optional.

5 Multiplication of material

If not enough material is available for future use, the candidate RM may need to go through one or more multiplication steps. Following this, the identity of the material needs re-confirmation using a preferably validated test, which may be one of the tests used in the identity test. If the identity of the RM cannot be confirmed the material should be rejected.

Describe: which method is used for the multiplication of the material. Describe how quality and quantity will be checked.

6 Criteria for reference materials

Here the 7 different criteria are listed and described that are to be assessed during the production of reference material. This can be done by describing them in text as below in sections 6.1-6.7 or for instance in Table 1. This table 1 summarizes these and lists possible values of these criteria. It also indicates which minimum value of each criterion is to be met. It is important to note, that since criteria are inherently linked to the intended use of the reference materials and may be test-specific, the criteria may be different for different uses and should be defined by the producer. The **Intended Use** of the reference material should be defined prior to the production of the material.

6.1 Identity: The RM should be clearly identified and characterized preferably by two validated and independent tests (section 4). If the material originates from a reference collection operating under commonly agreed quality standards, this provides additional guarantee into its identity. At minimum, the material should be thoroughly identified to the level of international accepted diagnostic protocols (when available) to ensure it is properly identified.

6.2 Material Traceability: Traceability of the RM covers the origin of the material including prior handling and multiplication (if applicable). If the material is derived from a collection, it should be traceable to a specific specimen in that collection including its history of maintenance and handling in that collection. If it is derived from field material metadata should be documented;

i.e. when and where the material was collected, from what plant species and part and whether or not it was showing which symptoms. In case RM is mixed with matrix material, the matrix should be properly be identified and described.

6.3 Commutability: Describe the extent to which the RM is similar to actual samples.

6.4 Homogeneity: Homogeneity should generally be determined after the reference material has been packaged in its final form. EPPO guideline PM 7/122 recommends to test a minimum of 10 randomly chosen samples (for each pest/ matrix/infestation level, including negative samples) in duplicate.

6.5 Stability: RM should be tested to determine it is sufficiently stable for its intended purpose and will not undergo any significant changes for a required period of time, including transport and storage, which may influence the test result. Minimum period of time and the required conditions should be clearly indicated in the documentation accompanying each individual lot of RM. The number of aliquots to be tested for stability also depends on the quantity of the reference material produced.

6.6 Assigned value: Assigned quantity values correspond to the expected result of the test (pest present or absent, concentration of the pest, morphological characteristics of the specimen, etc.). Include the method used to determine the assigned value.

6.7 Purity: defined as a ratio of target pest versus non-targets, particularly non-targets interfering with a test. Different levels of purity can be defined ranging from an absence of any non-targets to a relative high amount of non-target in your reference material. Determining the presence of non-targets should preferably be done through unbiased methods e.g. high throughput sequencing.

7 Documentation

Prepare RM documentation: A reference material document should be prepared to accompany the produced RM. The document must contain all information that is essential regarding the determined minimal criteria (if possible in certified values), the targeted use(s) of the RM and suggestions for its storage. Table 1 could be incorporated in the RM documentation.

8 References

Include any relevant references.

Table 1: List of descriptors of criteria to be assessed in the production of reference material (RM).

Descriptor	Value	Minimum criterion	Description
Intended use	**should be defined (in this case it equals preparation of RM for the scope of the individual test or TPS)**	**yes**	(Describe)
Identity	**identified to the level of internationally recognized diagnostic protocols (mention tests and outcome)**	**yes**	(Describe)
Traceability	traceability to a specimen from a reference culture collection	no	(Describe)
	traceability to a specimen from a working culture collection	no	
	traceability provided for the target pest and matrix used (the latter if relevant)	**yes**	
Commutability level	☐ naturally infested plant material	no	(tick the right level)
	☐ artificially infested plant material	no	
	☐ spiked plant material	no	
	☐ purified organisms	no	
	☐ total nucleic acids from a sample (target organism in background)	no	
	☐ purified nucleic acids	no	
	☐ **synthetic nucleic acids**	**yes**	
Homogeneity	**homogenous Provide test & test results**	**yes**	(Describe)
Stability	**stable**	**yes**	(Describe)
	stability - short term	no	
	stability - long term	no	
Assigned value	absolute concentration known	no	(Describe)
	level of concentration known (high/medium/low)	no	
	qualitative status known (above LOD level)	no	
	originating from plants with known health statues with a recent test result (a given period of time depends on the plant-pest combination and previous experience)	**yes**	
Purity	absence of non-targets	no	(Describe)
	absence of interfering non-targets	no	
	known ratio of target vs. non-target interfering with the test - high	no	
	known ratio of target vs. non-target interfering with the test - medium	no	
	known ratio of target vs. non-target interfering with the test - low	**yes**	

ANNEX 2

Standard Operating Procedure (SOP) for the production of Reference Material (RM) of *Fusarium circinatum*

This general Standard Operating Procedure (SOP) for the production of reference material (RM) of reference material is meant as example how a SOP for a specific type of RM -*Fusarium circinatum* samples for (Real-Time) PCR tests – may look like. It includes definitions and standard text regarding RM production because of the intended use as stand-alone RM procedure.

This example does not describe actual produced RM and no rights can be derived from any of the descriptions.

title	**SOP Production of Reference Material of *Fusarium circinatum***			
code		version 1	date 01-03-2019	page x of y

Version	date	explanation

1 Scope/intended use

This is a standard operating procedure (SOP) for the production of ***Fusarium circinatum*** as reference material **as purified organism/purified nucleic acid for use in PCR and Real-time PCR tests**. It describes the different steps required in the production process, ranging from the different possible sources of the reference material, tests to confirm its identity, possibly required multiplication steps to the actual production process. For each step in the process, criteria and critical points are identified.

For traceability of materials and future adjustments of use, registration of all steps and tests in the production of a given reference material is important and are registered in E-lab under the VALITEST directory.

IMPORTANT: *Fusarium circinatum* has a Q-status; follow Q-protocols.

2 Definitions

Assigned Value:

EPPO Standard PM 7/122: Assigned quantity values correspond to the expected result of the test (pest present or absent, concentration of the pest, morphological characteristics of the specimen, etc.).

Certified reference material (CRM):

EPPO PM 7/76 (5): Reference material derived from a source that certifies the authenticity of the material. The material should preferably come from an internationally recognized source such as a national reference collection. The material should have a unique identification code allowing traceability and the name of the person who certified its authenticity. Details of how the material was authenticated should also be supplied. If appropriate, information about its activity (e.g. pathogenicity, antigenic properties) under specified conditions should also be supplied along with any related uncertainty at a stated level of confidence.

ISO 17034:2016: Reference material characterized by a metrological valid procedure (ISO Guide 35) for one or more specified properties, accompanied by a reference material certificates (ISO Guide 31) that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability .

For clarification: The ISO 9000 family of quality management systems standards is designed to help organizations ensure that they meet the needs of customers and other stakeholders while meeting statutory and regulatory requirements related to a product or service. ISO 9001 is the only standard in the ISO 9000 series to which organizations can certify. ISO 17034:2016 defines the qualifications for reference material producers. While ISO Guide 35 gives details on metrological valid procedures for properties of RM, ISO Guide 31 provides information for reference material certificates. Per definition only ISO 17034:2016 certified reference material producers can produce certified reference material according to the other mentioned guidelines.

Certified value:

ISO Guide 30: 2015, section 2.2.3 Value assigned to a property of a (certified) reference material that is accompanied by an uncertainty statement and a statement of metrological traceability, identified as such in the reference material certificate.

Commutability (RM):

Characteristic describing the extent to which the reference material resembles actual samples. A reference material is considered commutable when a test produces the same result as it does for an authentic sample that contained the same analyte concentration.

While naturally contaminated samples are often considered as the most appropriate material for reference material, this is not always available. We recognise seven classes of commutability, ranging from naturally infested plant material to completely artificial synthetic nucleic acids (see paragraph 4.1; sources of reference material).

Field material:

Material collected directly from the field or at import, usually plant parts. These may or may not show typical symptoms of the pest. The material may (or may not) contain the target pest and it may contain other organisms which may interfere in one or more of the steps later in the procedure. As working collection material, most likely it will not meet all requirements for proper form and/or documentation and should therefore go through identification steps (and possibly multiplication steps).

Homogeneity (RM):

EPPO PM 7/122 section 3.5.1: the assessment of homogeneity should generally be performed after the samples have been packaged in the final form and before distribution to participants. Homogeneity can be demonstrated prior to packaging where no influence of packaging is reasonably expected. The EPPO guideline provides further information on the assessment of homogeneity for different types of material also stating: it recommends to test a minimum of 10 randomly chosen samples (for each pest/ matrix/infestation level, including negative samples) in duplicate.

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Number of samples prepared Per production lot	Number of samples to be tested
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≥95	10

Depending on the type of material and intended use of the RM, properties and desired levels of the RM should be defined before the homogeneity testing (ANSES: Method for undertaking and interpreting homogeneity and stability studies).

Identity (RM):

The reference material should be clearly identified and characterized to ensure its correct identification. Following the recommendation this should be done if possible by at least two preferably validated and independent tests based on different physical characteristic of the RM. If the specimen used originates from and is available in several reference collections working according to commonly agree quality standards this provides additional guarantee for its identity. At minimum, the material should be thoroughly identified to the level of international accepted diagnostic protocols (when available) to ensure it is properly identified. The list of tests used for its identification should be clear from the RM documentation.

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Material traceability of the RM covers the origin of the material including prior handling and multiplication (if applicable). If the material is derived from a collection, it should be traceable to a specific specimen in that collection including its history of maintenance and handling in that collection. If it is derived from field material metadata should be documented; i.e. when and where the material was collected, from what plant species and part and whether or not it was showing which symptoms.

Multiplication (of RM):

If more or a different type of (biological) material of a certain candidate reference material is needed, the material may be used for multiplication/ amplification. This may for instance mean multiplication in or on plants or specific substrates, but also be an amplification of specific target genes.

NAC:

Negative amplification control (in molecular or other tests).

NC:

Negative control.

NIC:

Negative isolation control (in molecular or other tests).

PAC:

Positive amplification control (in molecular or other tests).

PC:

Positive control.

Pest (IPPC, 2017):

Any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products.

PIC:

Positive isolation control (in molecular or other tests).

Purity (RM):

Purity is defined as a ratio of target pest versus non-targets, particularly non-targets interfering with a test.

Presence of non-target material cannot always be avoided and does not need to be a problem however, this depends on the exclusivity specificity of the test used i.e. the performance of a test with regard to cross-reaction with a range of non-targets (e.g. closely related organisms, contaminants) (EPPO PM7/76 (5).

Quality assurance:

Part of quality management, focussed on providing confidence that quality requirements will be fulfilled.

Dependent on the laboratories own quality management system, samples and materials may be rejected if basic quality requirements are not met. For example, samples clearly contaminated with non-target organisms or (spoiled) samples transported under sub-optimal conditions may be discarded.

Reference collection:

Q-Collect: A collection of individuals maintained for the purpose of study and authentication. Reference collections are generally large undertakings maintained by institutions; instead of having a single representative of each species, they will typically have multiples, so as to illustrate variations and, be able to provide samples externally for comparisons and research. Reference collections are an important source of information about variations of populations within a species. They are also the repository of type strains or holotypes used as the official definition of a particular species.

Examples of reference collections are microbial culture collections focussing on acquisition, authentication, production, cataloguing and distribution on microbial cultures.

Reference material (RM):

EPPO PM 7/76 (5) Material appropriate to the test and diagnosis being performed such as live cultures, infested plant material, DNA/RNA preparations, images of a diagnostic quality or mounted specimens. The reference material used should be documented and appropriate for the test and diagnosis being performed. It should be ensured that it has the features for which it was selected, for example expressing a desired antigen for use in serological diagnosis or display specific physical features (e.g. sporulation) if used for morphological diagnosis ().

Reference material document (RM document):

ISO17034:2016 Document containing all the information that is essential for using any reference material.

The RM document covers both the product information sheet and reference material certificate. It should contain certified values: values assigned to a property of a reference material (*e.g.* homogeneity or stability) that is accompanied by an uncertainty statement and a statement of metrological traceability, identified as such in the reference material certificate. For more information ISO Guide 31:2015 gives more data on the contents, labels and accompanying documentation needed for reference materials.

Stability (RM):

Samples should be demonstrated to be sufficiently stable to ensure that they will not undergo any significant change, including storage and transport conditions. The number of aliquots to be tested for stability depends on the quantity of the reference material produced (recommended minimum 3 random chosen samples). ISO Guide 35:2017 gives further guidance on the assessment of stability and the management of the risks associated with possible stability issues related to the properties of interest. The ISO Guidelines state that RM should be sufficiently stable for its intended use, defining stability further in stability under long-term storage conditions, transport conditions and where applicable storage conditions at the RM user's laboratory. It can include considerations of stability after opening of RM, if re-use is permitted.

EPPO PM 7/122(1) recommends testing a minimum of 3 randomly chosen samples in duplicate. However, as this may not always be feasible, testing may be reduced if suitable data are available from previous stability testing or according to the expertise of the organizer. The choice of the number of tested samples should be documented (EPPO 7/122 (1)/ ISO13528). Further information regarding the assessment of stability for different type of material is provided in EPPO PM 7/122(1). ISO Guide 35:2017 allows less experimental stability studies if the RM producer has prior information on stability from previous materials held for an extended period under the same planned storage conditions and which were characterised for the same properties and share the same matrix composition.

Test:

EPPO PM 7/76 (5): the application of a method to a specific pest and a specific matrix.

Test performance study (also referred to as ring tests or collaborative trials):

EPPO PM 7/76 (5): Evaluation of the performance of one or more tests by two or more laboratories using defined samples.

Traceability (RM):

Traceability can be considered as an aspect of both identity and origin of the material in the sense that it may provide some additional guarantees to the correct identity of the materials used to prepare the reference material and its future availability. To ensure traceability it is important to properly identify and describe the matrix as well and ensure its proper identification. Provide any relevant meta-data available for the RM.-see material traceability.

Traceability can also refer to how certain values of the reference material were determined, the actual determined values and uncertainties in them.

Working collection:

Q-collect: Collections, usually of individuals belonging to a single organism or group of related organisms, maintained for the purpose of scientific investigation by experts. Working collections are usually maintained by individual researchers or research groups with recognized knowledge of the organism(s) in question. They do not usually provide samples externally other than to deposit individuals of interest into one or more reference collections for safe keeping and/or protection of intellectual property.

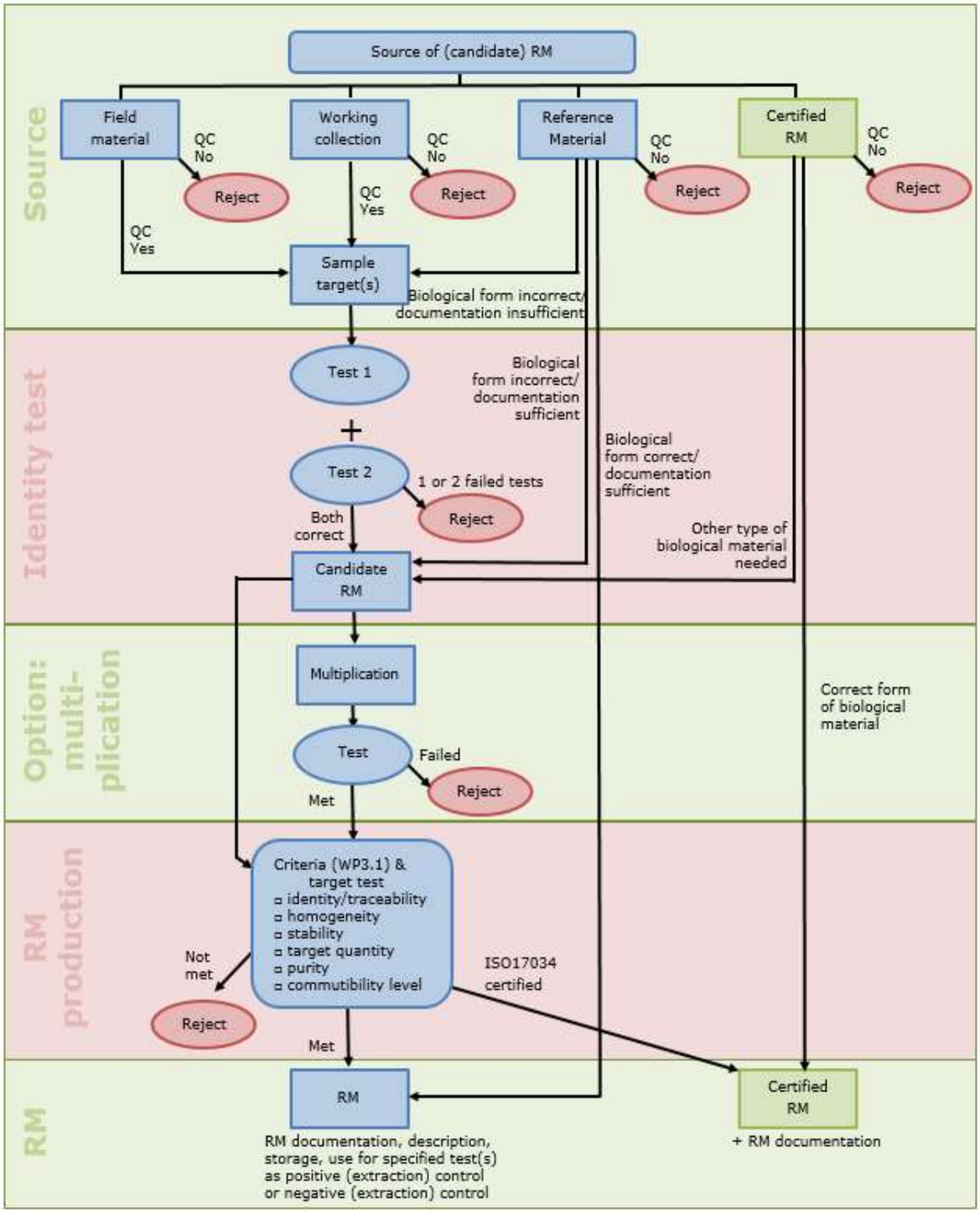

Figure 1: Graphical presentation of the general Standard Operating Procedure (SOP) for the production of reference material to be used in specified tests as either positive or negative samples. For the different blocks additional information can be found in paragraphs 4.1 to 4.5. For identity tests, two independent tests are recommended, but if not available, one will have to suffice.

3 Source of (candidate) reference material

The fungus *F. circinatum* is the causal organisms of pitch canker in pine (pitch because of its often spectacular resin exudates), a destructive disease of *Pinus* spp. and Douglas-fir (*Pseudotsuga menziesii*). The disease results in extensive tree mortality, reduced growth and timber quality. Multiple branch infections may cause severe crown dieback and eventually lead to the death of the tree, while it can also cause root rot. This aggressive fungus may also cryptically infect the *Pinus* seeds and may cause damping-off in seedlings. Conifer seeds can be colonized by *F. circinatum* internally (where it can remain dormant until seed germination) and externally (Storer et al., 1998).

F. circinatum is predominantly a wound pathogen and enters the host tree through mechanical wounds or feeding holes caused by woodboring insects. The taxon is listed as a quarantine fungus in Europe and several other regional plant protection organizations throughout the world. Whereas long-distance spread of the disease is made possible through the trade of infected pine seeds, local spread is caused by aerial dispersion or insect transportation of the fungal conidia.

The fungus is officially reported in the USA, Mexico, Haiti, South Africa, Japan, Chile (OEPP⁄EPPO, 2005) and has been officially reported in the EPPO region only recently: Spain (Landeras et al., 2005; under eradication), Italy (Carlucci et al., 2007 eradicated), France (OEPP⁄EPPO, 2008 under eradication). In most instances of introduction into new areas the pest was first found in nurseries.

Culture collection = *Reference material*

As *F. circinatum* is not known in our country, the **type strain of *F. circinatum* (CBS 405.97) was obtained as a lyophilized culture from the Westerdijk Fungal Biodiversity Institute**, Utrecht (NL). Strain material comes under Material Transfer Agreement (document 195 in e-lab). The material is intended to be used in the form of as purified organism/purified nucleic acid.

4 Identification of candidate reference material

A list of recommended tests for validation of target species is given below. In principle two independent tests (on different characters) should be performed for the target organism(s).

(1) Diagnostic method A: culture and morphology

Cultures are observed on Potato dextrose agar (PDA) and Spezieller-Nährstoffarmer Agar (SNA) plates for morphological identification. The correct morphological identification of *F. circinatum* in pure culture requires experience and a molecular confirmation should be carried out in case of uncertainty (PM7/91).

(2) Diagnostic Method B: Molecular tests

Direct detection in planta using molecular techniques (plant tissue, including seeds) include conventional PCR, SyBr green real-time PCR or dual-labelled probe real-time PCR; Besides IGS

amplicon sequencing or WGS can be applied to confirm identity. The molecular tests rely on a previous DNA extraction.

(3) DNA extraction from pure culture

Fungal DNA should be extracted using an appropriate standard method for DNA extraction from fungi e.g. regular commercial plant DNA extraction kits (or other methods reviewed in Irlinger et al., 2008) and analysed following any of the tests.

(4) Molecular methods

There are several molecular methods currently available to confirm the identity of *F. circinatum* isolated in pure culture or to detect and identify directly *F. circinatum* in planta.

- A PCR-RFLP (Restriction Fragment Length Polymorphism) test, with primers and RFLP pattern developed by Steenkamp et al. (1999) and is appropriate for identification of the anamorphic stage of F. circinatum in pure culture only as contaminants or host material may affect the quality and numbers of PCR amplicons.
- SyBr green real-time PCR or conventional PCR tests with primers designed by Schweigkofler et al. (2004) can be useful for identification of the fungus in pure culture, as well as for direct detection of the pathogen in seeds. However, when carried out on plant samples DNA, verification of the nature of the PCR amplicon should be carried out by sequencing for conventional PCR, or by melting analysis for SyBr green real-time PCR cross-reaction might occur with phylogenetically close Fusarium sp., especially with high amounts of Fusarium template DNA.
- Method for real-time PCR with primers and a dual-labelled probe designed by Ioos et al. (2009) can be useful for identification of the fungus in pure culture, as well as for direct detection of the pathogen in plant tissue, including seeds. This method proved to be more sensitive than the conventional PCR (diagnostic sensitivities of 79.1% and 58.6%, respectively; Ioos et al., 2009) and its specificity is strengthened thanks to the combination of specific primers and probe.
- Sequencing: Regions of the IGS rDNA, such as that amplified by the CIRC1A/CIRC4A primers (Schweigkofler et al., 2004), or the region of the translation elongation factor 1-alpha (EF-1alpha) gene amplified by the EF1/EF2 primers (O'Donnell et al., 1998), must be sequenced and used for species identification. The CIRC1A/CIRC4A PCR product may be generated from DNA extracted from a pure fungal culture or from plant tissue or seeds, whereas the EF1/EF2 PCR product may be generated only from DNA extracted from a pure fungal culture. The EF-1alpha sequence is sufficient to assign the identity of a Fusarium strain to *F. circinatum* (O'Donnell et al., 1998) but other markers may also be useful (e.g. largest RNA polymerase II B-subunit (RPB1), second largest RNA polymerase II B-subunit (RPB2), beta-tubulin, IGS) (Steenkamp et al., 2002; O'Donnell et al., 2010). The universal barcode ITS, while very useful for fungi in general, should not be used for the Fusarium genus as it is not sufficiently polymorphic for several closely related species, including *F. circinatum*.

5 Multiplication of material

Cultures are best grown on potato dextrose agar (PDA), potato dextrose broth (PDB) and Spezieller-Nährstoffarmer Agar (SNA) for growth and spore production. Transfer under aseptic conditions under the required safety conditions. Optimal growth conditions 25-27 °C, in dark or UV light conditions. Isolations of nucleic acids as described above.

6 Criteria for reference materials

Target tests of this reference material are the (real-time) PCR tests described in the section above. All RM criteria and their assessed values are listed in Table 1.

Table 1: List of descriptors of criteria assessed in the production of *Fusarium circinatum* as reference material (RM).

Descriptor	Value	Minimum criterion	Description
Intended use	**should be defined (in this case it equals preparation of RM for the scope of the individual test or TPS)**	**yes**	(real-time) PCR tests
Identity	**identified to the level of internationally recognized diagnostic protocols (mention tests and outcome)**	**yes**	**Westerdijk Fungal Biodiversity** Institute: strain CBS 405.97 **identified** based on morphology and multi-locus sequencing as *F. circinatum* (26S; 18S; ITS and EF1) **in-house** sequencing EF-1α as confirmation
Traceability	**traceability to a specimen from a reference culture collection**	**yes**	**Type strain CBS 405.97**
	traceability to a specimen from a working culture collection	no	
	traceability provided for the target pest and matrix used (the latter if relevant)	no	
Commutability level	☐ naturally infested plant material	no	
	☐ artificially infested plant material	no	
	☐ spiked plant material	no	
	☐ purified organisms	**yes**	**X**

	☐ total nucleic acids from a sample (target organism in background)	no	
	X purified nucleic acids	**yes**	**X**
	☐ synthetic nucleic acids	no	
Homogeneity	**homogenous** **Provide test & test results**	**yes**	tested: 10 samples checked in duplicate in RT-PCR; all positive; Ct value of 23±0.4)
Stability	**stable**	**yes**	(3 random samples)
	stability - short term	yes	*5 °C/RT at least month*
	stability - long term	yes	stability - long term; *20 °C at least 1 year* *-80 °C at least 2 years*
Assigned value	**absolute concentration known**	**yes**	Genomic DNA extract in extraction buffer at a concentration of 10 ng/µl (3 ml); in aliquots of 5 µl
	level of concentration known (high/medium/low)	no	
	qualitative status known (above LOD level)	no	
	originating from plants with known health statues with a recent test result (a given period of time depends on the plant-pest combination and previous experience)	yes	
Purity	**absence of non-targets**	**yes**	yes
	absence of interfering non-targets	no	
	known ratio of target vs. non-target interfering with the test - high	no	
	known ratio of target vs. non-target interfering with the test - medium	no	
	known ratio of target vs. non-target interfering with the test - low	yes	

7 Documentation

A reference material document should be prepared to accompany the produced RM. The document must contain all information (see for instance Table above) that is essential regarding the determined minimal criteria (if possible in certified values), the targeted use(s) of the RM and suggestions for its storage. Chosen format is up to the RM producer.

8 References

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