

Technical Working Party for Ornamental Plants and Forest Trees
 Fifty-Seventh Session
 Roelofarendsveen, Kingdom of the Netherlands, March 31 to April 3, 2025

TWP/9/4

Original: English
Date: February 24, 2025

Technical Working Party on Testing Methods and Techniques
 Third Session
 Beijing, China, April 28 to May 1, 2025

Technical Working Party for Vegetables
 Fifty-Ninth Session
 Virtual meeting, May 5 to 8, 2025

Technical Working Party for Agricultural Crops
 Fifty-Fourth Session
 Arusha, United Republic of Tanzania, May 19 to 22, 2025

Technical Working Party for Fruit Crops
 Fifty-Sixth Session
 Bursa, Türkiye, June 23 to 26, 2025

**GUIDELINES FOR THE VALIDATION OF A NEW CHARACTERISTIC-SPECIFIC MOLECULAR MARKER
 PROTOCOL AS AN ALTERNATIVE METHOD FOR OBSERVATION**

Document prepared by experts from France, Italy and the Kingdom of the Netherlands

Disclaimer: this document does not represent UPOV policies or guidance

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ASSOCIATED DOCUMENTS

- TG/1/3: General Introduction to the Examination of Distinctness, Uniformity and Stability and the Development of Harmonized Descriptions of new Varieties of Plants
- TG/44: Guidelines for the conduct of tests for distinctness, uniformity and stability for Tomato
- TGP/9: Examining distinctness
- TGP/10: Examining uniformity
- TGP/12: Guidance on Certain Physiological Characteristics
- TGP/15: Guidance on the Use of Biochemical and Molecular Markers in the Examination of Distinctness, Uniformity and Stability (DUS)
- UPOV/INF/17 Guidelines for DNA-Profiling: Molecular Marker Selection and Database Construction
- UPOV/INF/18 Possible use of Molecular Markers in the Examination of Distinctness, Uniformity and Stability (DUS)
- TWV/54/7 + Add Use of molecular techniques in DUS examination

I. OBJECTIVES OF THESE GUIDELINES

1. The purpose of these guidelines is to elaborate the principles contained in the General Introduction (document TG/1/3), and its associated TGP documents, into detailed practical guidance for the harmonized validation of a new method based on characteristic-specific molecular marker before its use as an alternative test. Performance criteria required for the validation are described and guidance on their assessment is given. These guidelines also describe a standard protocol with mandatory and optional chapters. Survey after acceptance is also described.
2. If a different technique is used, the laboratory must validate its method in comparison to the reference method (to show that the alternative technique gives the same results).

II. SCOPE OF THESE GUIDELINES

All crops

Characteristic-Specific Molecular Markers

For the examination of Distinctness, Uniformity, and Stability (DUS).

III. PERFORMANCE CRITERIA FOR A NEW MOLECULAR MARKER BASED PROTOCOL

Specificity

Definition

3. Correlation between the genotype and the phenotype, *i.e.* reliability of the link between the marker and the characteristic.

Requirement

4. In principle 100% of correlation between the genotype and the phenotype. If the correlation is less than 100% a follow-up test(s) should be performed to ensure the reliability of the results. A decision rule can be used in that case. Less than 100% correlation can be caused by other genetics.

How to evaluate it?

5. Number of varieties: To start the marker selection process an appropriate number of varieties (development set) is needed to reflect at the most the diversity observed within the group/crop/species/type for which the markers are intended to be discriminative.
6. Varieties should represent the different states of expression (if known varieties with heterozygous and homozygous state), coming from different plant breeders, with different genetic background of the characteristic and different types. Well phenotypically characterized varieties for the trait of interest should be used when available.

7. Number of plants per variety: At least one plant per variety if available varieties are phenotypically well characterized. If not, the number of plants should be the same as for the morphological observation described in the UPOV Test Guidelines.

8. The specificity can be assessed within one laboratory.

Sensitivity and limit of detection

Definition

9. The limit of detection is defined as the minimal quantity of the target that can be reliably detected.

10. In case of analyses performed on bulk samples (e.g. pool of different plants of the same variety) the sensitivity is critical and must be assessed. For the use on individual plants, the quantity of the target is not critical and this performance criterium is optional.

Requirement

11. In the case of the pool, the requirement would be to detect at least one off-type in the pool.

How to evaluate it?

12. To use artificial samples by mixing one off-type to a pool to check the sensitivity of the detection.

Repeatability

Definition (based on ISO 16 577:2016; reference to UPOV/INF/17)

13. *“Repeatability; where identical test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.”*

14. For qualitative methods, accordance is equivalent to the repeatability of quantitative methods (Langton *et al.*, 2002).

Requirement

15. Ideally 100%, a performance $\geq 90\%$ is generally accepted. If the repeatability of the reference method is published the repeatability of the alternative method should be at least equivalent.

How to evaluate it?

16. The repeatability can be evaluated within one laboratory.

17. At least three technical replicates drawn from a same plant (three independent DNA extractions). To include at least all expected types of genotype.

Reproducibility

Definition (based on ISO 16 577:2016; reference to UPOV/INF/17)

18. *“Reproducibility; where test results are obtained with the same method, on identical test items, within the same laboratory or between different laboratories, with different operators, using different equipment” at different times.*

19. For qualitative methods, concordance is equivalent to the reproducibility of quantitative methods (Langton *et al.*, 2002).

Requirement

20. Ideally 100%, a performance $\geq 90\%$ is generally accepted. If the reproducibility of the reference method is published the reproducibility of the alternative method should be at least equivalent.

How to evaluate it?

21. Reproducibility should be assessed between different laboratories by an interlaboratory validation study (Ring-test) with coded samples of known genotypes. All expected types of genotype should be included.
22. The ring-test should involve at least, three different laboratories including at least two different examination offices (e.g. in INVITE EU-project 817970 DOI [10.3030/817970](https://doi.org/10.3030/817970), 3 examination offices were involved in the validation test). If possible, experienced laboratories familiar with the species and the technique should be involved. If not, a training can be organized ahead of the ring-test with un-coded samples. Laboratories can participate in a ring-test on voluntary basis. In case there are no volunteers, then an intra-laboratory reproducibility assesment will be possible with different operators.
23. All laboratories must follow the protocol to be validated. In the protocol compulsory and optional parts can be defined by the validation team.
24. Number of varieties: To include at least all expected types of genotype.
25. Guidelines/Norms on interlaboratory studies can be followed: ISO 13495 *Foodstuffs - Principles of selection and criteria of validation for varietal identification methods u sing specific nucleic acid*, ISO 17043 *Conformity assessment - General requirements for proficiency testing*, EPPO pm7-122-2 *Guidelines for the organization of interlaboratory comparisons by plant pest diagnostic laboratories*, ISTA TCOM-P-10-*Validation of seed health methods and organization and analysis of interlaboratory comparative tests (CT)*... The validation team can cite the followed guidelines in its report.

Robustness

Definition (based on ISO 16 577:2016; reference to UPOV/INF/17)

26. “Robustness; a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure parameters and provides an indication of its reliability during normal usage” (e.g. change of DNA extraction method or change of real time machine).

Requirement

27. Ideally 100%, if less that means that the method is not robust to a change of one parameter and this should be indicated in the protocol as a mandatory step (e.g. a change of a mastermix that would be critical).

How to evaluate it?

28. It is optional to assess, and robustness is evaluated partially during the ring test (reproducibility), (different laboratories, equipment, machinery, persons, etc.).

IV. VALIDATION REPORT

29. The validation report and results must be peer-reviewed by two (preferably 3 if the reproducibility was done within one laboratory) of the responsible bodies. Reviewing is on voluntary basis but preferably perform review by laboratory familiar with the species and the method.

30. During the reviewing process, the reviewers can require extra validation data in concertation with the validation team.

Content of the validation report

- Raw data generated during the different steps of the validation process
- Detail protocol with optional and compulsory steps defined
- Performance criteria assessment
- Conclusion

Publicity

31. The validation report should be available upon request. In the new protocol the validation process should be mentioned with the contact examination office. In some particular cases, e.g. a “trade secret protocol”

(cytoplasmic male sterility in cabbage), the protocol and the validation report could not be shared outside of the examination offices.

V. STANDARD PROTOCOL FOR CHARACTERISTIC-SPECIFIC MOLECULAR MARKER PROTOCOL

32. Compulsory elements are indicated in the column “essential information”, the other elements may be used depending on the characteristic test protocol. If a laboratory wants to adapt/modify/change a mandatory chapter or element of a mandatory chapter it must validate its method in comparison to the reference method (to show that you obtain the same results as the published method).

Table 1: Standard characteristic-specific molecular marker protocol (see document TWV/54/7 “Use of molecular techniques in DUS examination”. Modifications are highlighted in grey)

Chapter	Elements in a Standard characteristic-specific molecular marker protocol	Example	Essential information for harmonization	Remark
1	characteristic	Resistance to Tomato mosaic virus (ToMV) <i>See TG/44/11/rev3 – Ad 51: ii DNA marker test</i>	YES	
2	Genes and alleles	<i>See TG/44/11/rev3 – Ad 51: ii DNA marker test add 2</i>	YES	Need to avoid dominant marker or presence/absence marker otherwise the robustness should be assessed
2.1	Targeted gene(s)	Resistance Gene Tm2 Arens, P. et al (2010)	YES	a) file(s) containing the DNA sequence information (order of nucleotides) b) reference to DNA information in public databases (like GeneBank) c) reference to (scientific) publications in which the DNA sequence information of the states of expression of the characteristic is revealed. d) reference to a particular position on the published reference genome version.
2.2	Allele corresponding to expression state 1	tm2 Arens, P. et al (2010)	YES	a) file(s) containing the DNA sequence information (order of nucleotides) b) reference to DNA information in public databases (like GeneBank) c) reference to (scientific) publications in which the DNA sequence information of the states of expression of the characteristic is revealed. d) reference to a particular position on the published reference genome version in combination with the SNP or INDEL that is responsible for the state of expression.
2.3	Allele corresponding to expression state n	Tm2 and Tm2 ² Arens, P. et al (2010)	YES	a) file(s) containing the DNA sequence information (order of nucleotides) b) reference to DNA information in public databases (like GeneBank) c) reference to (scientific) publications in which the DNA sequence information of the states of expression of the characteristic is revealed. d) reference to a particular position on the published reference genome version in combination with the SNP or INDEL that is responsible for the state of expression.
3	Primers (and probes)	<i>See TG/44/11/rev3 – Ad 51: ii DNA marker test add 3, 3.1 and 3.2</i>	YES	Primer and probe sequences, reference to accessions and sequences in public databases (Genebank numbers), literature

Chapter	Elements in a Standard characteristic-specific molecular marker protocol	Example	Essential information for harmonization	Remark
3.1	Primers (and probes) to detect allele '9'		YES	Primer Sequences corresponding to allele(s) for expression '9' (resistance)
3.2	Primers (and probes) to detect allele '1'		YES	Primer Sequences corresponding to allele(s) for expression '1' (susceptibility)
3.3	Primers (and probes) to detect allele 'x'		YES	Primer Sequences corresponding to allele(s) for expression 'x'
4	Format of the test			
4.1	Number of plants per genotype	≥20	YES	A minimal number of individual plants required: the test for the marker is conducted on the same number of individual plants, with the same criteria for distinctness, uniformity and stability as for the examination of the characteristic by an observation assay (documents TGP/9 and TGP/10)
4.2	Control varieties	See TG/44/11/rev3 – Ad 51: ii DNA marker test add 4.2	YES	Control varieties (same as in observation assay) as standards representing all relevant combination of alleles. For example homozygous for Allele corresponding to expression state 9 (present), homozygous for allele corresponding to expression state 1 (absent) and heterozygous (both alleles are present in a diploid) corresponding to either resistant, susceptibility or intermediate resistance of the variety (depending on gene function; dominant - recessive). DNA controls can be directly used.
4.3	Process controls	e.g. buffer used for extraction; a marker targeting the cytochrome oxidase gene as an internal amplification marker	YES	<ul style="list-style-type: none"> a) Negative process control(s) b) Positive DNA control(s) that can be the control varieties c) Internal amplification control in case of a presence/absence marker
5	Preparations	e.g. Sampling of seedlings 4 days old followed by DNA extraction using CTAB method	NO	Depending on the method used. Not in the Test Guideline. Detailed protocol(s) can be provided as an example in annex or available on request from the organization that developed the marker
6	Technique of the method	e.g. conventional PCR, TETRA-ARMS, qPCR, KASP, amplicon sequencing See TG/44/11/rev3 – Ad 51: ii DNA marker test add 6	YES	.
6.1	Particular conditions	e.g. PCR protocol describing primer, enzyme, dNTP concentrations, PCR cycle scheme	NO	Depending on the method used. Not in the Test Guidelines. Detailed protocol(s) can be provided as an example in annex or available on request from the institute that developed the marker
6.2	Particular hardware or infrastructure	e.g. machines, commercial kits, manufactures of components, lot numbers of chemicals	NO	Depending on the method used. Not in the Test Guidelines. Detailed protocol(s) can be provided as an example in annex or available on request from the institute that developed the marker

Chapter	Elements in a Standard characteristic-specific molecular marker protocol	Example	Essential information for harmonization	Remark
7	Observations	e.g. Bands on agarose gel (conventional PCR), Ct values (qPCR) Variant call based on sequencing reads	NO	Depending on the method used. Not in the Test Guidelines. Detailed protocol(s) can be provided as an example in annex or available on request from the institute that developed the marker
7.1	Validity of the results	e.g. for qPCR, Check for typical exponential amplification curves. Check if the controls are as expected (negative controls = no signal; positive controls = shows expected signals for all fluorophores).	YES	Depending on the method used.
8	Interpretation of the test results	See TG/44/11/rev3 – Ad 51: ii DNA marker test add 8	YES	Relation between alleles and expressions (with its notes) In case the DNA marker test result does not confirm the declaration in the Technical Questionnaire, a field trial or bio-assay should be performed to assess the correctness of the declaration in the Technical Questionnaire.
9	Validation of the method.	This protocol was validated by a ring-test with different laboratories	YES	(e.g. interlaboratory Comparative Test INVITE 2024 817970 DOI 10.3030/817970)
9.1	Contact Examination Office	e.g. Naktuinbouw	YES	Contact of the institute that developed this protocol, Name of the service.

VI. FOLLOW-UP SURVEY AFTER APPROVAL

33. Validation of the marker is not fixed as new genetics can arise from the market. This is a continuous evaluation process. Specificity should be re-assessed after validation acceptance using parallel testing (marker test and bioassay) at least during the first year with observation method.

34. After the first year of acceptance of the protocol, morphological checks on about 10% of the new varieties must be performed.

VII. LITERATURE

Arens P., Mansilla C., Deinum D., Cavellini L., Moretti A., Rolland S., van der Schoot H., Calvache D., Ponz F., Collonnier C., Mathis R., Smilde D., Caranta C.; Vosman B., 2010: Development and evaluation of robust molecular markers linked to disease resistance in tomato for distinctness, uniformity and stability testing. *Theoretical and applied genetics* 120(3). pp. 655-64

Langton, S.D., Chevennement, R., Nagelkerke, N. and Lombard, B. (2002). Analysing collaborative trials for qualitative microbiological methods: concordance and concordance. *International Journal of Food Microbiology*, 79, 175-181

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