
**Foodstuffs — Principles of selection
and criteria of validation for varietal
identification methods using specific
nucleic acid**

*Produits alimentaires — Principes de sélection et critères de
validation des méthodes d'identification variétale utilisant des acides
nucléiques spécifiques*

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2. www.iso.org/directives

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The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

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Introduction

This International Standard outlines guidelines designed to support decision-making and validation on the protocols used to produce high-quality molecular data for varietal identification.

Varietal identification testing requires high-quality markers, which are able to provide reproducible data using a variety of equipment, chemistries and reagents. Accordingly, this International Standard only addresses specific amplification methods.

The aims of this International Standard are to ensure that the methods of analysis are compatible with customer requests, to list the different steps towards method validation, and to define acceptance criteria. It also guarantees that the general principles employed in performing these analyses will be the same across all laboratories (reference material, sample size, laboratory sample, test portion, extraction, results analysis and interpretation, certificate of analysis).

Finally, this International Standard plays a role in standardizing the results obtained by different laboratories.

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Foodstuffs — Principles of selection and criteria of validation for varietal identification methods using specific nucleic acid

1 Scope

This International Standard specifies molecular tools for generating molecular profiles of varieties of plant species, enabling varietal identification, i.e. confirmation of identity in relation to one or more references.

This International Standard is applicable to various matrices, seeds, leaves, roots, industrial products composed of only one variety. Matrices presented in the form of mixtures of varieties (such as purees, compotes, flours) are excluded from the scope of this document.

This International Standard does not deal with genetic purity.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable to its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/IEC 17025:2005, *General requirements for the competence of testing and calibration laboratories*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1 Terms related to variety

3.1.1 cultivar

group of cultivated plants which may be clearly defined by morphological, physical, cytological, chemical or other characteristics and which, after sexual or asexual reproduction, keeps its distinct character

[SOURCE: ISO 7563:1998, definition 1.12]

Note 1 to entry: The concept of “cultivar” is essentially different from the concept of the botanical variety “varietas”, in that

- “cultivar” is an infraspecific division resulting from controlled selection, even if empirical;
- “varietas” is an infraspecific division resulting from natural selection.

The terms “cultivar” and “variety” (in the sense of cultivated variety) are equivalent. In translations or adaptations of botanical nomenclature for particular uses, the terms “cultivar” or “variety” (or their equivalents in other languages) may be used in text.

Note 2 to entry: The names of botanical varieties and species are always in Latin form and are governed by botanical nomenclature.

3.1.2 species

group of organisms that have a high level of genetic (DNA) similarity and are capable of interbreeding; often containing subspecies, varieties or races

Note 1 to entry: A species is designated in italics by the genus name followed by the specific name, e.g. *Ananas comosus*.

3.1.3

variety

unique and uniform member of a species of plant (except for hybrid species) that retains its characteristics from generation to generation through its natural mode of reproduction

[SOURCE: ISO 5527:—, definition 2.1.7, modified — the other preferred term “cultivar” has been deleted.]

3.2 Terms related to DNA/RNA extraction and purification

3.2.1

nucleic acid extraction

sample treatment for the liberation of target nucleic acid

Note 1 to entry: The nucleic acid extraction procedure is used for isolating nucleic acids from other cellular components, such as protein, lipids, carbohydrates and other impurities in a test sample.

[SOURCE: ISO 22174:2005, definition 3.2.1, modified — Note 1 has been added.]

3.2.2

nucleic acid purification

method resulting in a more purified DNA

Note 1 to entry: A procedure or process involving sequential steps used to separate DNA and/or RNA from other components in a sample. A highly purified DNA or RNA sample contains negligible observable or measurable effects attributable to inhibitors of the polymerase chain reaction.

Note 2 to entry: In this context, purity refers to the reduction of observable and measurable effects of PCR inhibitors.

[SOURCE: ISO 22174:2005, definition 3.2.2, modified — Note 1 has been added; Note 2 has been modified.]

3.3 Terms related to PCR amplification of nucleic acids

3.3.1

amplicon

specific DNA fragment produced by a DNA-amplification technology, such as the polymerase chain reaction (PCR)

3.3.2

hybridization

specific binding of complementary nucleic acid sequences under suitable reaction conditions

[SOURCE: ISO 22174:2005, definition 3.6.3]

3.3.3

multiplex PCR

PCR that uses multiple pairs of primers in different loci combined within a single reaction mixture to produce multiple amplicons simultaneously

[SOURCE: ISO 22174:2005, definition 3.4.11, modified — the phrase following “primers” has been added.]

3.3.4

polymerase chain reaction

PCR

enzymatic procedure which allows *in vitro* amplification of DNA

[SOURCE: ISO 22174:2005, definition 3.4.1]

3.3.5**primer**

oligonucleotide of defined length and sequence complementary to a segment of an analytically relevant DNA sequence

[SOURCE: ISO 22174:2005, definition 3.4.12]

3.3.6**probe**

labelled nucleic acid molecule with a defined sequence used to detect target DNA by hybridization

[SOURCE: ISO 22174:2005, definition 3.6.1, modified — the term was originally “DNA probe”.]

3.3.7**specificity**

property of a method to respond exclusively to the characteristic or analyte under investigation

Note 1 to entry: It describes the ability to specifically recognize the nucleic acid sequence to be detected by distinguishing it from other nucleic acid sequences, and the tendency for a primer or probe to hybridize with its intended target and not hybridize with other non-target sequences.

[SOURCE: ISO 24276:2006, definition 3.1.4, modified — Note 1 has been added.]

3.3.8**thermocycler**

automated laboratory apparatus used to repeatedly raise and lower the temperature of a sample by cycling through a series of discrete, pre-programmed steps

Note 1 to entry: This cycling of temperatures drives the PCR process.

3.4 Terms related to detection**3.4.1****electrophoresis**

method of separating electrically-charged particles by their differential migration under an electric field

Note 1 to entry: PCR products can be separated by various types of electrophoresis.

3.5 Terms related to controls**3.5.1****reference sample
reference material**

material or substance, one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials

Note 1 to entry: The reference material may be either provided by the customer, internal to the laboratory, or an officially-designated reference.

[SOURCE: ISO Guide 30]

[SOURCE: ISO 24276:2006, definition 3.5.1, modified — Note 1 has been added.]

3.5.2**test control**

one or more samples that have undergone all or part of the analytical process designed for the target samples and which can reveal known alleles of the markers used, thereby signalling any process errors and providing reference alleles which can facilitate the reading of results

3.6 Terms related to markers

3.6.1

allele competition

preferential amplification of one allele over another in a heterozygote or a mixture

3.6.2

allele frequency

measure of how common an allele is in a population; the proportion or percentage of all of the occurrences of a locus that is occupied by a given allele

3.6.3

marker

genetic marker that typically applies to DNA fragments matching a given locus that gives information on the genotype of the carrier or on the genotype of neighbouring loci

3.6.4

null allele

<context of PCR> sequence variant that precludes PCR amplification of a particular target, resulting in the absence of detectable PCR product

3.6.5

repeat region

genomic region in which a particular DNA or RNA sequence occurs as multiple copies

3.6.6

simple sequence repeat

SSR

region of DNA consisting of a short (1 bp to 6 bp) sequence (repeat unit) that is tandemly repeated many (typically five to 50) times

Note 1 to entry: SSRs are commonly known as microsatellites.

Note 2 to entry: The number of repeat units present at a specified SSR, and thus the overall length of the SSR, often varies among individuals.

3.6.7

single nucleotide polymorphism

SNP

single nucleotide variation in a genetic sequence that occurs at appreciable frequency in the population

Note 1 to entry: SNP is often pronounced "snip".

[SOURCE: ISO 25720:2009, definition 4.23, modified — Note 1 has been added.]

4 Quality assurance on the test results

The requirements and guidelines set out in [Table 1](#) are coded as follows:

- C: compulsory;
- R: Recommended.

Table 1 — Requirements and guidelines on the test results

STAFF		
All staff carrying out specific tasks shall be qualified based on an appropriate level of education, training, experience and/or proven competency, according to the task skills required.		
	Criteria	Requirement (C or R)
	Training in use of the equipment	C
	Handling chemicals	C
	Staff responsible for issuing opinions and interpretations shall be fully conversant with the various genetic structures and seed production systems involved, in order to provide customers with advice and/or suggest ways to interpret the results.	C
EQUIPMENT		
Maintenance operations shall be run on all apparatus and equipment following the manufacturer's instructions. Suitable apparatus calibration systems shall be made available.		
Maintenance on key equipment	The equipment used for preparing the sample	C
	Example: mixer mills	
	Pipetting equipment	C
	The autoclaves used for sterilizing the laboratory equipment and the various buffers and solutions used	C
	Storage freezer(s) designed for conserving extracts, products, mixes, etc.	C
	Refrigerator/walk-in cooler for storing extract samples, solutions and chemicals	C
	Equipment used to perform DNA amplification cycles (thermocycler, water bath, etc.)	C
	Amplicon imaging and recording system (photocopier, scanners, etc.)	C
	Electrophoresis system, genetic analyser	R
	Precision scales	C
	pH meter	R
	Robots	R
FACILITIES		
	All necessary occupational health and environmental protection measures have been taken	R
	Manual or automatically-controlled pipetting equipment specifically assigned to each workspace	C
	Two separate workspace areas	
	Pre- and post-PCR	C
REAGENTS		
	The products used shall not represent a potential source of DNA degradation or contamination. All reagents shall be conserved and used according to the manufacturer's guidelines.	C

5 Selection of methods

The methodology and the markers used for varietal identification shall be reliable and robust, and shall enable each laboratory to annotate the marker profiles simply and with the utmost confidence.

This requires the implementation of quality criteria, such as in the choice of the reference alleles and the allele designation/annotation system; the detection system shall be appropriate for the size of PCR products.

The methods employed should be selected according to the markers available for the species studied.

The following is a list of the most important criteria governing the selection of a methodology:

- a) availability;
- b) reproducibility of the data generated between laboratories and detection platform (equipment types employed);
- c) repeatability;
- d) discriminatory power.

6 Markers selection

6.1 General criteria

The general criteria governing the selection of a set of markers are:

- a) the level of discrimination against the reference varieties;
- b) marker set repeatability and reproducibility intralaboratory variance (see [8.2.1](#) and [8.2.2](#));
- c) going as far as possible to avoid markers presenting null alleles;
- d) convenience for scoring.

6.2 Marker set selection

In order to confirm an identity in comparison to one or more references (parental lines, synthetic hybrids, clones), the laboratory shall use a set of markers generating a sufficiently discriminatory number of alleles to meet the customer's request. The laboratory shall state the rationale for the marker set selected.

The markers should be distributed across the entire genome when the linkage map is available. Although this is not a mandatory point, it is nevertheless very useful guidance to avoid opting for potentially related markers.

6.3 SSR markers

Efficient and effective SSR analysis hinges on choosing top-quality markers. Marker choice should thus be guided by the following listed points:

- a) preference shall be given to markers that display few or no stutter bands;
- b) the selection of markers shall take into consideration the separation and visualization systems;
- c) preference shall be given to markers that do not present allele competition, and also an attempt should be made to eliminate any competition between markers in multiplex PCR runs.

6.4 Single nucleotide polymorphism (SNP)

Repeat regions should be avoided.

NOTE Repeat regions can lead to a dominant SNP allele if simultaneous detection of a homologous region occurs.

The very nature of SNPs means they generally have two allele states. It is strongly recommended not to choose SNPs with more than two allele states.

SNPs can be annotated reliably and without complication. It is also recommended to analyse a large number of markers, either independently or through multiplexing, in order to achieve sufficient discriminatory power to distinguish among varieties.

7 Laboratory samples

7.1 Sample

The sample size and pre-analysis storage conditions shall be appropriate for the analysis requested.

Special focus shall be given to identifying samples, subsamples and test portions, as stipulated in ISO/IEC 17025:2005, 5.8.2, in order to guarantee traceability.

7.2 Sample size

For analyses of reference material, sufficient numbers of individuals should be included to allow characterization of intravarietal polymorphism, taking into account reproductive characteristics (see 7.3) and other knowledge of crop- and/or variety-specific factors. For test samples, analysis of several single or pooled individuals is also recommended when possible.

7.3 Reference material

It is mandatory to use a reference material (see 3.5.1), which shall be processed in the same way as the test sample. If the reference material is a DNA template, the only identical steps will be those following DNA extraction.

The molecular profile pre-acquired under the same conditions can nevertheless be employed as a reference molecular profile.

The marker profiles of the reference material can be used to build an in-house database internal to each laboratory, and that can then be used for routine analyses.

- **Vegetatively-reproducing varieties:** In principle, analyses on vegetatively-reproducing varieties can be run using just one individual, given that all the individuals will be identical.
- **Autogamous or primarily autogamous varieties:** Autogamous or primarily autogamous varieties will not necessarily be monomorphic at all loci, including SSR or SNP loci. It is generally recommended to analyse a number of single seeds or plants to allow the characterization of polymorphism within a variety. However, a bulk sample of a number of individuals may also be used to represent the variety.
- **Varieties in a population:** It is recommended to analyse seeds or plants individually in order to determine the allele frequency profiles characterizing each variety in a population. A representative sample size shall be used, as well as statistical approaches to determine confidence level of the results expressed in accordance with 9.4. In this case, a reference material shall be used for each variety in population.
- **Fixed-parent (F1) simple hybrids:** In principle, the analyses can, in this case, be run using just one individual, given that all the individuals will be identical. There may be heterogeneity stemming for example from a residual heterozygosity of at least one of the parent lines, cross-pollination, or

a physical mix. This is why the guideline is to analyse several individuals in order to represent the DNA profile of a given variety.

- **3-way hybrids:** The genetic structure of this kind of hybrid dictates that the analyses should be run on a mix of an appropriate number of individuals (at least 20).
- **Outcrossing or primarily outcrossing varieties:** It is generally recommended to analyse a number of single seeds or plants to allow the characterization of polymorphism that might be present within an outcrossing or primarily outcrossing variety. However, a bulk sample of a number of individuals may also be used to represent the variety.

8 Laboratory validation

8.1 General

Each laboratory shall use a method validated by an interlaboratory study, or failing this, an intralaboratory study.

In the field of molecular marker-mediated varietal identification, method validation equates to validating a set of markers.

The method is validated marker-by-marker for a given species. Interpretation and validation of the results are performed as set out in [Clause 9](#).

The set of markers shall provide sufficient discriminatory power to meet customer requests. The method is validated across the entire analytical process (on each matrix of each species).

8.2 Intralaboratory validation criteria

Validation covers a marker set selected by the laboratory according to the criteria set out in [Clause 6](#).

Validations shall include a set of an appropriate number of different varieties (at least five, if possible).

For species able to present hybrid varieties, it is recommended to screen for allele competition using heterozygotes (hybrid plant or mix of parent DNA) and, if possible, their parent lines.

8.2.1 Repeatability

Every marker used shall be repeatable. The aim is to test precision under the same conditions.

Each marker shall be tested for repeatability on one nucleic acid extraction per variety and three PCRs per extraction.

The marker is considered repeatable if all three results are identical for each of the varieties tested.

8.2.2 Reproducibility conditions (or intermediate precision)

Every marker used shall be reproducible. The aim is to test precision under different conditions.

Each marker is tested for reproducibility by running three, non-simultaneous tests per variety, with one PCR per extracted DNA. Each test should include sufficient numbers of single or pooled individuals per variety to allow recognition of intravarietal polymorphism. Varieties known to have uncommon variants should be excluded from use in reproducibility tests.

Non simultaneous means that each test shall be carried out using different conditions (personnel, equipment, time, reagent, etc.).

The marker is considered reproducible if it yields the same results for each variety across all three tests.

8.2.3 Discrimination

Use of a validated set of markers is to be limited to the varieties among which it can distinguish.

It is recognized that there can be instances where varieties derived through procedures, such as the selection of a natural or induced mutant or repeated backcrossing, might not be distinguished from the initial variety using a single all-purpose marker set. Specific supplementary markers or other additional means may be required for unequivocal identification of varieties in such cases. Known limitations of a marker set shall be clearly acknowledged.

8.2.4 Validation when changing equipment or plant matrix

NOTE Equipment includes thermocycler, automated pipetting platform, and separation and detection system.

In the event that there is a change to genotyping equipment, the set of markers already validated shall be re-characterized using DNA from the varieties previously used for the validations.

Each marker is tested via 1 PCR on 1 DNA on at least five different varieties, if possible.

Although there can be differences in parameters, such as inferred allele size when determined by different sets of equipment, the conclusions drawn with respect to variety identity shall not vary.

8.3 Interlaboratory validation criteria

8.3.1 General

Validation concerns the result only. Each participating laboratory is free to choose its own analytical processes (DNA extraction, nucleic acid purification, PCR protocol, equipment, etc.) during interlaboratory validation testing. This freedom does not extend to the choice of markers used.

8.3.2 Validation by interlaboratory testing

8.3.2.1 General

This interlaboratory validation shall further refine the process of selecting the most robust marker set possible from among the markers validated during the previous intralaboratory validation process (see 8.2).

8.3.2.2 Minimum number of participating laboratories

The national-level validation shall be led by at least four laboratories (if possible) or at least eight for international validation.

8.3.2.3 Biological material

The test shall be conducted on an appropriate number of varieties (at least five, if possible) presenting well-known national (or international) variability. The number of single or pooled individuals per variety examined by each participating laboratory should be sufficient to allow recognition of intravarietal polymorphism. Varieties known to have uncommon variants should be excluded from use in interlaboratory validation trials.

NOTE Biological material can be seeds, plant tissues, flour. If flour is used, it is intended to be derived from a single variety.

8.3.2.4 Working protocol

The working protocol, including basic requirements for PCR, such as primer and probe sequences, including any fluorochromes, quenchers, minor groove binders and/or other specificity-promoting moieties, as well as recommended PCR amplification conditions, shall be provided.

The participating laboratories may adapt these conditions to suit the equipment used. Addition or removal of 5' primer sequence extensions or modification of fluorochromes is allowed; however, the portion of the primer sequence that is specific to the target sequence shall remain unchanged.

8.3.2.5 Annotation of the results

See [9.2](#).

8.3.2.6 Individual marker validation

A marker is validated when all of the results are identical among laboratories for all varieties tested.

For microsatellite markers, relative sizes of alleles for a given set of varieties shall be consistent among laboratories.

8.3.2.7 Laboratory method validation

The method is validated once a sufficient number of reproducible markers have been obtained. The laboratory shall determine the number of markers that is sufficient. The number depends on the type of markers used, the species and the intended application.

It is important to validate a method with the detection system used in each of the participating laboratories.

8.3.3 Methodology transfer

If a laboratory wishes to use a set of markers, which has been validated by another laboratory, it shall engage in an intralaboratory validation procedure (see [8.2](#)).

8.3.4 Matrix change

Any prospective matrix changes shall require intralaboratory validation only.

NOTE Matrix change means change of any part of tissue at any stage.

9 Interpretation and expression of the results

9.1 General

The use of test controls to aid in detection of experimental or reading errors is recommended.

9.2 Data annotation system

When working with size-based markers such as SSRs, use of an appropriate DNA ladder (molecular weight standard) is recommended to facilitate annotation of data. The DNA ladder shall not be used as a substitute for a test control (see [3.5.2](#)).

Visual checks should be run if the data are read by an automated system.

Annotation can preferably be in locus-allele format.

The alleles can be annotated using a common coding for SSRs, e.g. A, B, C, D and so on, where A is the smallest allele and so on.

For size-based markers, such as SSRs, results reported shall include an estimated size for each marker.

When working with SNPs, the results should be expressed in three formats:

— homozygous 1 (fluorescence 1 or band 1), written as A;