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Microbiology of the food chain — Method validation —

Part 7: Protocol for the validation of identification methods of microorganisms

Microbiologie de la chaîne alimentaire — Validation des méthodes —

Partie 7: Protocole pour la validation de méthodes d'identification des micro-organismes

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Foreword

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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 document should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 463, *Microbiology of the food chain*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

A list of all parts in the ISO 16140 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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Introduction

0.1 The ISO 16140 series

The ISO 16140 series has been expanded in response to the need for various ways to validate or verify test methods. It is the successor to ISO 16140:2003. The ISO 16140 series consists of several parts with the general title, *Microbiology of the food chain — Method validation:*

— *Part 1: Vocabulary*;

— *Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method*;

— *Part 3: Protocol for the verification of reference methods and validated alternative methods in a single laboratory*;

— *Part 4: Protocol for method validation in a single laboratory*;

— *Part 5: Protocol for factorial interlaboratory validation for non-proprietary methods*;

— *Part 6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures*;

— *Part 7: Protocol for the validation of identification methods of microorganisms.*

ISO 17468 is a closely linked International Standard, which establishes technical rules for the development and validation of standardized methods.

In general, two stages are needed before a method can be used in a laboratory.

— The first stage is the validation of the method. Validation is conducted using a study in a single laboratory followed by an interlaboratory study (see ISO 16140-2, ISO 16140-5, ISO 16140-6 and as described in this document). In the case when a method is validated within one laboratory (see ISO 16140-4), no interlaboratory study is conducted.

— The second stage is method verification, where a laboratory demonstrates that it can satisfactorily perform a validated method. This is described in ISO 16140-3. Verification is only applicable to methods that have been validated using an interlaboratory study.

In general, two types of methods are distinguished: reference methods and alternative methods.

A reference method is defined in ISO 16140-1:2016, 2.59, as an “internationally recognized and widely accepted method”. The note to entry clarifies that “these are ISO standards and standards jointly published by ISO and CEN or other regional/national standards of equivalent standing”.

In the ISO 16140 series, reference methods include standardized reference (ISO and CEN) methods as defined in ISO 17468:2023, 3.7, as a “reference method described in a standard”.

An alternative method (method submitted for validation) is defined in ISO 16140-1:2016, 2.4, as a “method of analysis that detects or quantifies, for a given category of products, the same analyte as is detected or quantified using the corresponding reference method”. The note to entry clarifies that: “The method can be proprietary. The term ‘alternative’ is used to refer to the entire ‘test procedure and reaction system’. This term includes all ingredients, whether material or otherwise, required for implementing the method”.

ISO 16140-4 addresses validation within a single laboratory. The results are therefore only valid for the laboratory that conducted the study. In this case, verification (as described in ISO 16140-3) is not applicable. ISO 16140-5 describes protocols for non-proprietary methods where a more rapid validation is required or when the method to be validated is highly specialized and the number of participating laboratories required by ISO 16140-2 cannot be reached. ISO 16140-4 and ISO 16140-5 can be used for validation against a reference method. ISO 16140-4 (regarding qualitative and quantitative methods) and ISO 16140-5 (regarding quantitative methods only) can also be used for validation without a reference method.

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The flow chart in Figure 1 gives an overview of the links between the different parts mentioned above. It also guides the user in selecting the right part of the ISO 16140 series, taking into account the purpose of the study and the remarks given above.

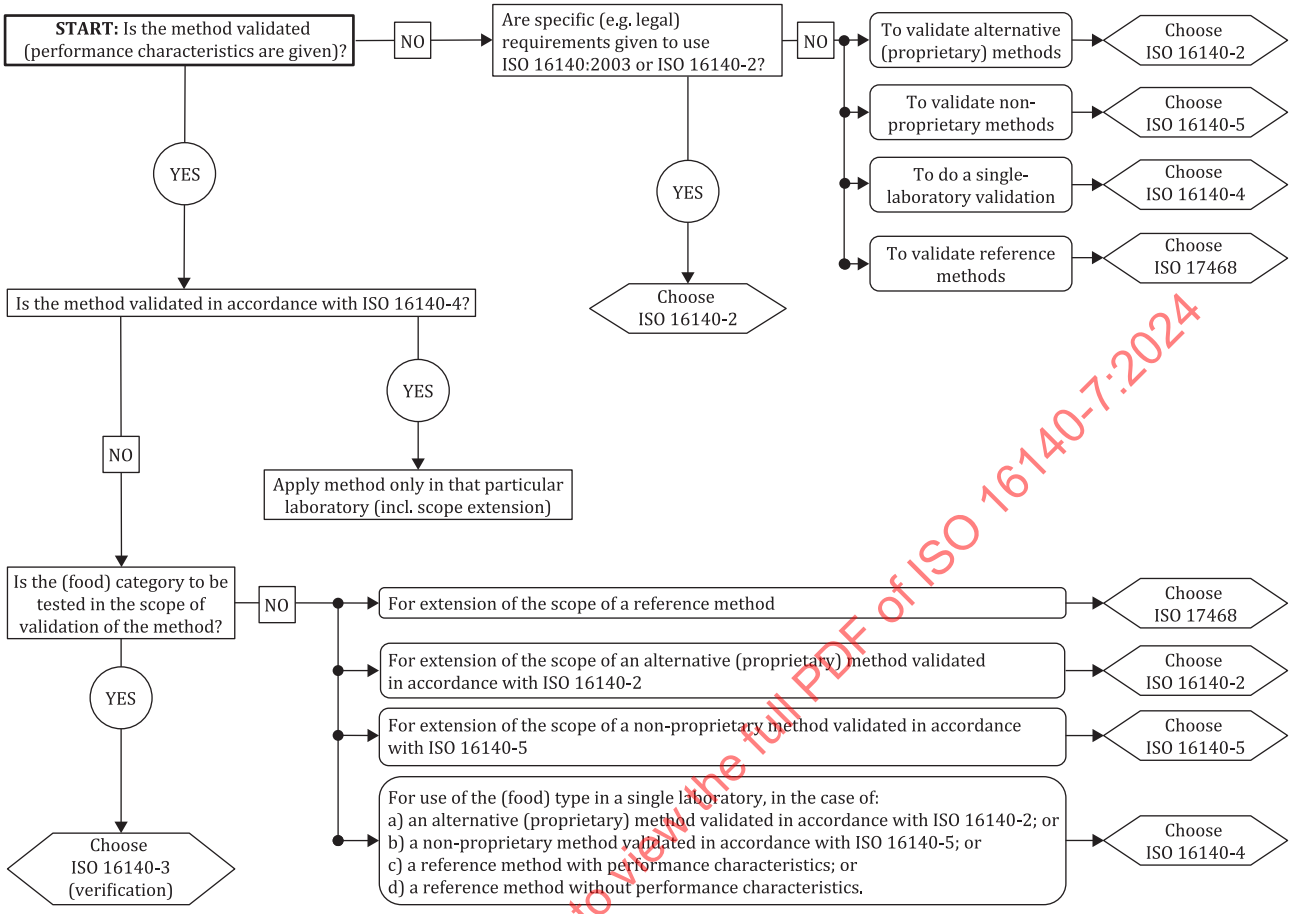

Figure 1 — Flow chart for application of ISO 16140-2 to ISO 16140-5

NOTE 1 In this document, the words "category", "type" and/or "item" are sometimes combined with "(food)" to improve readability. However, the word "(food)" is interchangeable with "(feed)" and other areas of the food chain as mentioned in Clause 1.

ISO 16140-6 and this document (ISO 16140-7) are somewhat different from the other parts in the ISO 16140 series in that they relate to very specific situations.

ISO 16140-6 is restricted to the confirmation procedure of a method to be validated [e.g. the biochemical confirmation of *Enterobacteriaceae* (see ISO 21528-2)]. The confirmation procedure advances a suspected (presumptive) result to a confirmed positive result. The validation of alternative typing techniques (e.g. serotyping of *Salmonella*) is also covered by ISO 16140-6. The validation study in ISO 16140-6 clearly specifies the selective agar(s) from which strains can be confirmed using the alternative confirmation method. If successfully validated, the alternative confirmation method can only be used if strains are recovered on an agar that was used and was shown to be acceptable within the validation study. Figure 2 shows the possibilities where an alternative confirmation method validated in accordance with ISO 16140-6 can be applied (see text in the boxes).

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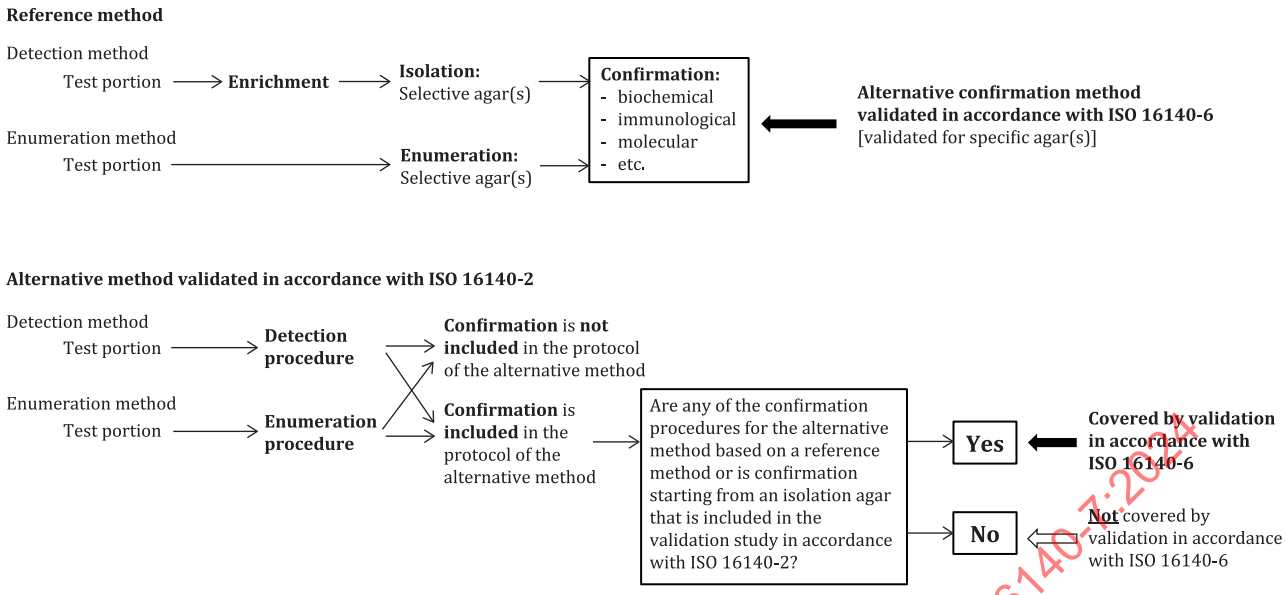

Figure 2 — Use of validated alternative confirmation methods (see ISO 16140-6)

EXAMPLE 1 An example application of a validated alternative confirmation method is as follows.

An alternative confirmation method based on ELISA has been validated to replace the biochemical confirmation for *Salmonella* as described in ISO 6579-1. In the validation study, XLD (mandatory agar in accordance with ISO 6579-1) plus BGA and a specified chromogenic agar (two optional agars for second plating in accordance with ISO 6579-1) were used as the agars to start the confirmation. The validated confirmation method can be used to replace the biochemical confirmation under the following conditions:

— by laboratories using ISO 6579-1; or

— by laboratories using an ISO 16140-2 validated alternative method that refers to ISO 6579-1 for confirmation; or

— by laboratories using an ISO 16140-2 validated alternative method that starts the confirmation from XLD and/or BGA agar and/or the specified chromogenic agar.

The validated confirmation method cannot be used under the following conditions:

— by laboratories using an ISO 16140-2 validated alternative method that refers only to agars other than those included in the validation to start the confirmation (e.g. Hektoen agar and SS agar only); or

— by laboratories using an ISO 16140-2 validated alternative method that refers only to a confirmation procedure that does not require isolation on agar.

This document (ISO 16140-7) addresses the validation of identification procedures (e.g. molecular identification using multiplex PCR or DNA sequencing or mass spectrometry). This document differs from the other parts in the ISO 16140 series, as it is intended for microbial identification for which there is no reference method and, therefore, it is not possible to run a method comparison study. The validation study in this document specifies the identification method principle, the identification database and algorithm when appropriate, and the agar(s) from which strains can be identified. If properly characterized and successfully validated, the identification method can only be validly used on strains recovered on the agars covered and shown to have been acceptable within the validation study.

NOTE 2 Whole-genome sequencing (WGS) in accordance with ISO 23418 will eventually be a reference method for all microorganisms, but the implementation of this technique is still at an early stage. Therefore, the use of WGS cannot currently be requested as a reference method for a large panel of strains.

Figure 3 shows the possibilities where an alternative confirmation method validated in accordance with ISO 16140-6 and an alternative identification method validated in accordance with this document can be applied within a reference method or an ISO 16140-2 validated detection or enumeration method. The result provided by the ISO 16140-7 validated method can be considered as additional information on the identity of the tested colony(ies); this result cannot be taken as a confirmation result. When there is a discrepancy

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between the results of the ISO 16140-6 validated method and the ISO 16140-7 validated method, a root cause analysis is conducted. An ISO 16140-7 validated method can also be used to identify colonies within methods that do not require a confirmation step.

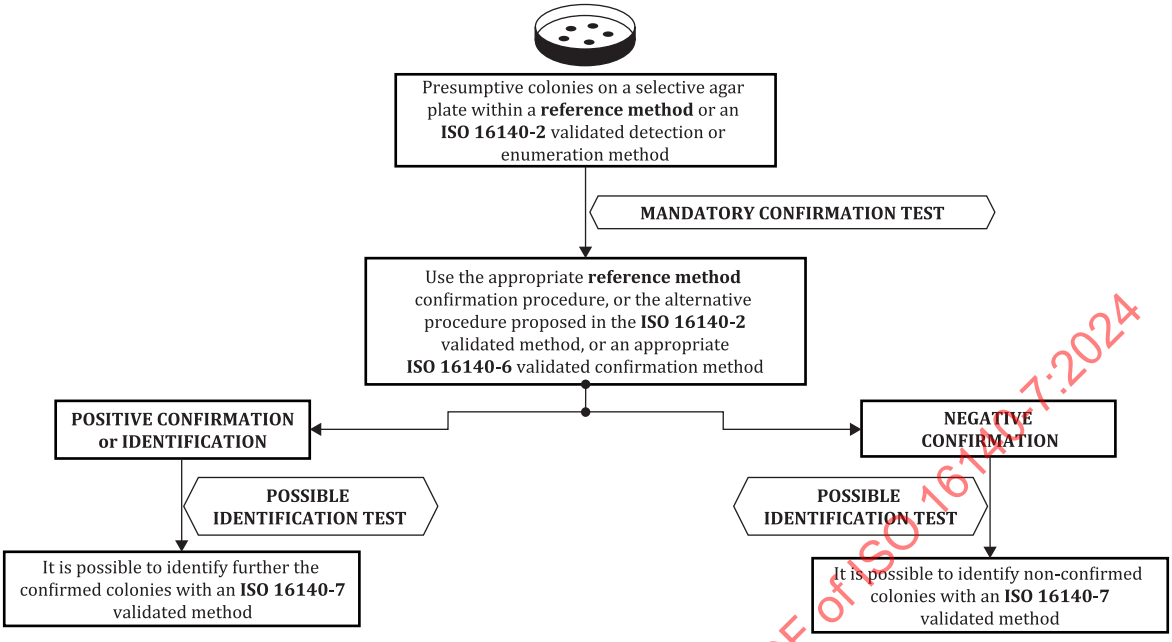

Figure 3 — Flow chart for the application of ISO 16140-6 and this document for the confirmation and identification of colonies within a reference method or an ISO 16140-2 validated detection or enumeration method

If the identification method is also validated in accordance with ISO 16140-6, the same method can be used for both confirmation and identification.

When a confirmation method is used, it is possible to apply an identification method validated in accordance with this document for further identification.

EXAMPLE 2 An alternative confirmation method of *Campylobacter* genus can be validated in accordance with ISO 16140-6 and compared to the mandatory confirmation procedure at the genus level described in ISO 10272-1. The identification at the *Campylobacter* species level is optional in ISO 10272-1 and ISO 10272-2 and is therefore not mandatory. In this instance, an identification method at the *Campylobacter* species level can be validated in accordance with this document. If the method is validated by ISO 16140-6 and this document, it can be used for both confirmation and identification purposes.

0.2 Validation and verification of identification methods of microorganisms

The procedure described in this document is intended for validation of identification methods of microorganisms. This procedure comprises two parts: a performance characteristics study and an interlaboratory study.

The procedure for validation of identification methods of microorganisms in a single laboratory is described in ISO 16140-4. The procedure for verification of identification methods of microorganisms in a single laboratory is described in ISO 16140-3.

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Microbiology of the food chain — Method validation —

Part 7: Protocol for the validation of identification methods of microorganisms

1 Scope

This document specifies the general principle and the technical protocol for the validation of identification methods of microorganisms for microbiology in the food chain. As there is no reference method, no method comparison study can be run. Therefore, this document provides a protocol to evaluate the performance characteristics and validate the method workflow using well-defined strains. When required, an additional identification method can be used.

This document is applicable to the validation of identification methods of microorganisms that are used for the analysis of isolated colonies from:

— products intended for human consumption;

— products for feeding animals;

— environmental samples in the area of food and feed production and handling;

— samples from the primary production stage.

Identification methods only validated in accordance with this document cannot be used instead of confirmation described in:

— the reference method;

— an alternative method validated in accordance with ISO 16140-2;

— an alternative method validated in accordance with ISO 16140-6.

In these instances, the identification method is validated in accordance with ISO 16140-6 method that is used as a confirmation method.

This document is applicable to bacteria and fungi. Some clauses can be applicable to other (micro)organisms, which can be determined on a case-by-case basis.

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2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 16140-1, *Microbiology of the food chain — Method validation — Part 1: Vocabulary*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 16140-1 and the following apply.

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ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
acceptability limit
AL
maximum acceptable proportion of deviations between the reference identities (or if not known, the accepted reference values) of the strains or specimens and the corresponding identification results obtained when applying the operating procedure of the candidate identification method

Note 1 to entry: In the context of this document, the reference value can be the assigned identity of the strain.

3.2
agreement
identification agreement
method under validation study provides the same identification result as the assigned, i.e. original, identification of the tested strain

3.3
assigned identity
result of the microorganism identification displaying generally accepted molecular and/or biochemical characteristics

EXAMPLE Bergey's Manual of Systematics of Archaea and Bacteria[13].

3.4
comparison algorithm
defined calculation rules used to compare the profile of the analysed strain to the database

3.5
confirmation procedure
confirmation test
procedure or test which is carried out to verify a presumptive result

Note 1 to entry: Not all methods have a confirmation procedure

Note 2 to entry: A confirmation test can provide a positive or negative result, without yielding the identity of the analyte.

[SOURCE: ISO 16140-1:2016, 2.17, modified — Note 2 has been included.]

3.6
database
library
collection of data categories and concept entry structure of an identification database

Note 1 to entry: An identification database usually gathers the phenotypic or molecular data information of several strains from the same species or genus.

Note 2 to entry: Some identification methods can have a restricted scope and do not imply a database (e.g. multiplex PCR assay).

3.7
deviation
identification deviation
method under validation study does not provide the same identification result as the assigned, i.e. original, identification of the tested strain

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3.8
group
group of microbial ecosystems
specimens processed in a similar way, with similar intrinsic characteristics and a similar microbial ecology

EXAMPLE Enrichment broths.

3.9
homology
score
identity between the profile of the analysed strain and the entry(ies) in the database

Note 1 to entry: This is normally measured as % or with score value(s).

Note 2 to entry: For select identification methods (e.g. microarray), a homology score may not be obtained.

3.10
identification method
method submitted for validation
method of analysis that provides the name (identity) of the microorganism (e.g. species or higher taxonomy ranking level)

Note 1 to entry: The method can be non-proprietary or proprietary.

Note 2 to entry: The methods can be based on various principles (e.g. phenotypic and molecular principles).

Note 3 to entry: The identification of microorganisms can help for example in determining whether it is a safety or spoilage concern, or is a specific technological or probiotic strain, or is likely to be resistant to an inactivation treatment.

3.11
identification procedure
identification test
procedure or test yielding the identity of the analyte (e.g. species or higher taxonomy ranking level)

3.12
level of detection
LOD_x
relative concentration of the measured analyte in proportion to the total count of the specimen, obtained by a given measurement procedure, for which the probability of detection is x

EXAMPLE LOD_{50} is the level of detection for which 50 % of tests give a positive result.

[SOURCE: ISO 16140-1:2016, 2.35, modified — In the definition, “relative” and “in proportion to the total count of the specimen” have been added and the Note 1 to entry has been deleted.]

3.13
profile
set of characteristics that identify or are used to identify a strain

Note 1 to entry: The profile can be phenotypic (e.g. biochemical or serological) and/or molecular (e.g. DNA fingerprint, DNA sequence or mass spectra).

3.14
reliability
identification reliability
closeness of agreement between an identification result and the assigned identity of the tested strain

Note 1 to entry: The concept “identification reliability” is related to the identity of the analyte, i.e. genus or/and species names(s).

Note 2 to entry: “Identification reliability” is sometimes understood as closeness of agreement with the identification result that are being attributed to the identity of the strain given by another identification method.

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3.15
risk of non-identification
ratio of the probability of getting no identification result within a specified set of strains included in the scope of validation

Note 1 to entry: This does not apply to misidentification.

3.16
scope of validation
database content and version, culture media or group of microbial ecosystems and comparison algorithm for which a validated method for the identification of microorganisms can be used satisfactorily

[SOURCE: ISO 16140-1:2016, 2.70 modified — In the definition, “analytes, matrices, and concentrations” has been replaced by “database content and version, culture media or group of microbial ecosystems and comparison algorithm”.]

4 General principles for the validation of identification methods of microorganisms

The validation protocol comprises two phases:

— a performance characteristics study;

— an interlaboratory study.

NOTE 1 For proprietary methods, the validation study is conducted by an organizing laboratory (see ISO 16140-1:2016, 2.45).

NOTE 2 It is possible, if relevant, to include inclusivity or exclusivity data obtained in an ISO 16140-2 or an ISO 16140-6 validation study into a study related to this document.

NOTE 3 As there is no reference identification method, there is no method comparison study.

The technical rules for performing the method performance characteristics study and the interlaboratory study are given in Clauses 6 and 7. An extended set of strains from a non-selective agar or a selective agar when appropriate (e.g. yeasts and moulds) will be tested for both parts.

Where appropriate, the validation protocol shall also specify the selective media from which strains can be identified using the identification method. A specified number of strains shall be tested.

NOTE 4 The term “agar” is often referred to as “solid culture medium”.

NOTE 5 Other applications of identification methods are possible (e.g. identification of microorganisms in an ecosystem), see Annex A for further information.

NOTE 6 The identification procedure can be ensured by the collaboration of multiple laboratories. For instance, a first laboratory can isolate the strains and run the sample preparation such as DNA extraction and sequencing; the generated data can be analysed by comparison to the database held and managed by a second laboratory. In such a case, during the interlaboratory study, multiple collaborators will be involved in the sample preparation and will send the results for analysis to the laboratory managing the identification data interpretation.

5 Strains

The pure strains used for determining the identification reliability of the method shall be well-characterized in line with the purpose of the validation study. When necessary, the identification information provided by a second identification is used to evaluate the discrepancies between the results of the tested identification method and the assigned identity of the strain.

NOTE 1 National, regional or international reference laboratories can be contacted during such investigations.

NOTE 2 Well-characterized strains are strains that can be obtained from national/international culture collections, or ones that have been obtained locally and have been previously identified and given the same identity using usually two or more identification methods that are based on dissimilar principles.

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NOTE 3 When appropriate, select strains with available WGS data in accordance with ISO 23418.

6 Performance characteristics of an identification method

6.1 General

The performance characteristics study is the part of the validation that is performed in one laboratory. It consists of a description of the concept and limitation(s) of the identification method, and an identification reliability study of the method. The results are then compared to those of the assigned identities of the tested set of strains.

6.2 Description of the concept and limitation(s) of the identification method

The method characteristics described in Table 1 shall be carefully described in the method protocol (e.g. standard operation procedure, instructions for use) to enable proper use of the identification method by the end-user.

Table 1 — Essential components of the performance characteristics study

Characteristics	Detailed information
Scope of validation	Database description including name, version and content Culture media Microorganism name or type (e.g. bacteria, yeast, moulds, lactic acid bacteria, *Listeriaceae*) Comparison algorithm version
Identification method principle	Description of the strain profile/sequence and instrument signal acquisition used for identification of microorganisms
Database content	List of the genera and species in the database version used in the validation study
Concept of the database	Description of the types of signal profiles in the database, e.g. one unique profile selected as a representative for various strains of the same species or several signal profiles of various strains of the same species
Strains per species and genera in database	Number of strains per species and genera A minimum of 5 strains is required to claim a species in the database content
Limitations	List of the closely related species that are not differentiated by the identification method, size of the database
Comparison algorithm	Calculation of the result reporting, e.g. comparison of the instrument signal profile of the analysed strain to each single profile in the database or comparison of the profile of the analysed strain to unique average profiles for each species of the database.
Result reporting	Description of the homology concept used to assess the identification results with high confidence or low confidence
Strain preparation and troubleshooting	Description of the strain preparation and method workflow, particularly when obtaining homology result of low confidence or when no identification result is generated

In some very specific cases, the minimum number of strains per species in the database can be lower than the required 5 strains due to the lack of available strains. There should be a disclaimer in the database content for strains that do not meet the minimum requirement.

If the identification method does not imply a database (e.g. multiplex PCR assay), the description of the concept and limitation(s) of the identification method should be restricted to the scope of validation, the identification method principle, the limitations, the result reporting, the strain preparation and troubleshooting.

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6.3 Identification reliability of the identification method

6.3.1 Number of strains to be tested

The set of strains to be tested depends on the database content and the scope of the validation. Select, as much as possible, one strain per species to ensure that at least 25 % of genera and 10 % of species contained in the database are tested. However, a minimum and a maximum number of strains to be tested has been specified at, respectively, 250 strains and 1 000 strains. For restricted database content (e.g. 25 genera and 200 species), test all the genera and species.

Figure 4 provides examples of how to determine the appropriate number of strains to be tested.

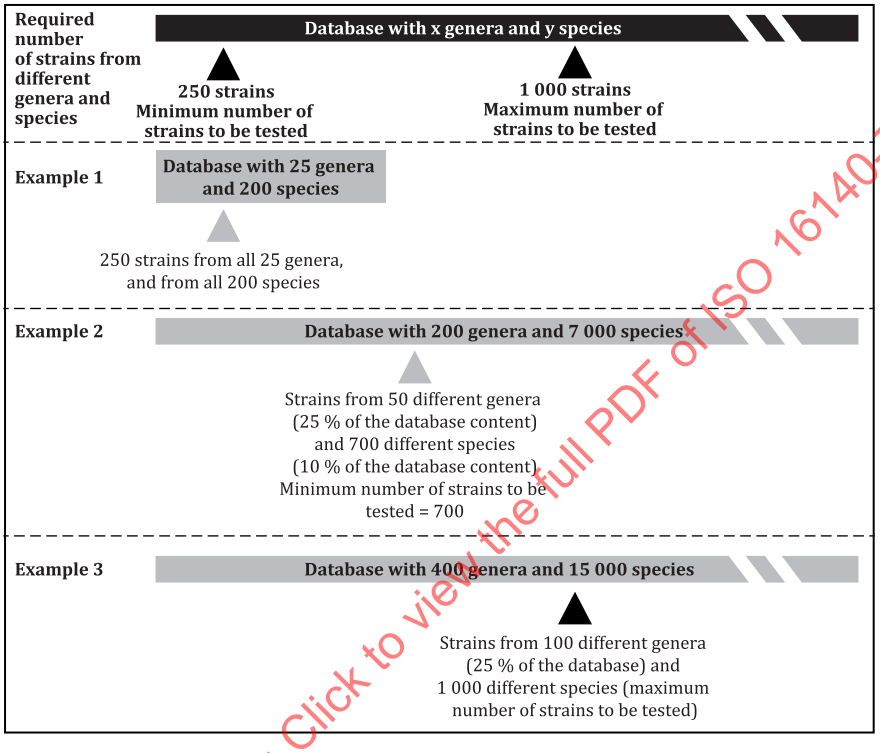

Figure 4 — Examples on how to determine the number of strains to be tested

Pure cultures from a non-selective agar of all selected strains shall be tested by the identification method.

NOTE 1 Non-selective agars (e.g. TSA) enable the recovery of a broad range of microorganisms and can favour the recovery of some specified groups of microorganisms. For microorganisms, for which non-selective agars are not known or are not supported by corresponding standardized reference methods for the isolation of the respective microorganism, only selective agars can be used instead.

If the scope of the validation study also includes selective agars, refer to Table 2 for the number of relevant genera and species to be tested for each selective agar included in the scope.

NOTE 2 Selective agars can enable the recovery of microorganisms from specific clades, (i) family, (ii) genus or group of species (e.g. *Bacillus cereus* group). In this document, “group of species ” is substituted with “genus” to improve readability. However, the word “genus” is interchangeable with “group of species”.

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Table 2 — Number of strains to be tested for each of the selective agars included in the scope

Taxonomy level of the strains recovered on the tested selective agars	Number of strains to be tested for each of the selective agars included in the scope
Family	100 strains, from as many different genera and species as possible, including strains from multiple families that can grow on the selective agar
Genus	50 strains, from as many different species as possible, including strains from multiple genera that can grow on the selective agar
Species	25 strains that can grow on the selective agar
Sub-species, types	25 strains that can grow on the selective agar

For a method with a restricted scope (e.g. multiplex PCR assay), it is recommended to test a relevant panel of strains that should not produce identification results (negative control strains) as expected by the scope of the method (see the example in Clause C.4).

6.3.2 Selection of the strains

A broad range of strains shall be used. Criteria used for selecting test strains shall be in accordance with Annex B. The strains selected should take into account the measurement principle (e.g. phenotypic or molecular based) and the database content of the identification method. Different measurement principles can require the use of panels of different test strains, representing the diversity of the studied microorganism(s). As much as possible, strains used in the development of the database should not be used in the validation of the identification method unless availability of strains is limited. In this case, disclosure shall be included in the validation study report. The rationale for the choice of the strains and their characteristics shall be included in the validation study report (e.g. coverage of the database, closely related species, recent changes in taxonomy).

Select strains from species able to be recovered on the test agar(s). For selective agars, select test strains from multiple families or genera.

Each strain shall be characterized in sufficient detail for its assigned identity to be known using relevant phenotypic or molecular tests. The method or the method principle used for the identification shall be known.

Strains should preferably have been isolated from foods, feed, the food-processing environment or from primary production; depending on the scope of the validation. However, clinical strains can also be used. The original source of all strains should be known, and they should be held in a local (e.g. expert laboratory), national or international culture collection to enable them to be used in future testing if required. See ISO 11133 for guidance on the local maintenance of stock cultures.

Results generated by a specialized reference laboratory, using an appropriate identification method, can be used if the laboratory performing the validation study is not able to perform the identification of rare strains.

6.3.3 Testing of the strains

Pure cultures of all selected strains shall be tested with the identification method from a non-selective agar and the selected selective agars (if any) included in the scope of validation.

In case of discrepancies between the assigned identity of a tested strain and the result provided by the identification method evaluated, run a root cause analysis:

— Repeat the identification of the tested strain with both, the method, or the method principle, used to assign the identification of the strain and the identification method evaluated during the validation study. If the discrepancy is confirmed, identify the strain with a third method.

— The third appropriate method shall preferably use different characteristics than the method used to initially assign the identity of the strain and the identification method being validated.

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6.3.4 Expression and interpretation of results

Tabulate the results from strains grown on non-selective (and selective agars) as in Table 3 and include the final interpretation for the identification deviations in accordance with Table 4. The outcome, preferably tabulated, with an explanation, shall be included in the validation study report.

A deviation at a higher taxonomy ranking level is also regarded as a deviation at a lower taxonomy ranking level. For instance, since the genus is a higher taxonomy ranking level than species, a deviation at the genus level is also regarded as a deviation at the species level.

If the third method used for the root cause analysis matches the result of the assigned identity and confirms the identification deviation, then report the final interpretation as an identification reliability deviation (*IRD*) in Table 4.

The identification reliability deviation (*IRD*) should be adapted to the scope of the method. For instance, IRD_{genus} or $IRD_{species}$ specify if the *IRD* is at the genus or species levels and are given as examples. If the third method used for the root cause analysis matches the identification result provided by the method evaluated and does not confirm therefore the identification deviation, then report the final interpretation as an identification agreement (*IA*) in Table 4.

If the identification results differ with the three methods, it is most likely that the tested strain cannot be identified with the currently available method principles. This result should be reported but not included in the data interpretation.

NOTE 1 Detailed examples are given in Annex C (expression, interpretation and evaluation of results).

NOTE 2 In some specific cases, the method under validation is able to identify strains at a lower taxonomy level than the method(s) used for comparison. The reliability will be first assessed regarding the taxonomy level shared by the methods used in the validation study. Additional information will be considered to evaluate the reliability of the identification at a lower taxonomy level given by the method under validation, e.g. whole genome sequencing as described in ISO 23418 or any relevant fingerprinting or sequences. These additional data will be interpreted as described in Table 4.

Table 3 — Results of the identification reliability study

Tested strain	Agar(s) used to recover the strain	Identity of the strain		Identity of the strain by the method under validation[a]	Interpretation of results ($IA/ID/N_0$)
		Assigned identity	Identification method, or identification method principle		
1					
2					
3					
Etc.					

Key

IA: identification agreement, *ID*: identification deviation, N_0: no identification.

[a] Include results with no identification.

NOTE Assigned identity of the individual strains are, as a minimum: the name of the strain, (culture) collection number and origin of the strain. Other available characteristics can be added as well.

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Table 4 — Interpretation of the identification deviations of the identification reliability study

Tested strain	Assigned identity	Identity of the strain by the method under validation[a]	Identity of the strain by a third method using a different principle		Interpretation of results (*IA*/ IRD_{genus} or $IRD_{species}$)
			Method principle	Identity of the strain	
1					
2					
3					
Etc.					

Key

IA: Identification agreement, IRD_{genus}: identification reliability deviation at the genus level, $IRD_{species}$: identification reliability deviation at the species level.

[a] Include results with no identification.

NOTE Assigned identity of the individual strains are as a minimum: the name of the strain, (culture) collection number and origin of the strain. Other available characteristics can be added as well.

6.4 Evaluation

Summarize the results of the identification reliability study for each of the tested agars, as in Table 5 (in total numbers of N_0, *IA* and *IRD* found), based on the interpretations in accordance with Table 3 (results of the identification reliability study) and Table 4 (interpretation for the identification deviations of the identification reliability study).

Table 5 — Summary of the results in the identification reliability study

Tested agar	Number of				
	Strains (N)	*IA*	N_0	IRD_{genus}	$IRD_{species}$
x					
Etc.					

Key

N: number of strains, *IA*: identification agreement, N_0: number of non-identified strains, IRD_{genus}: identification reliability deviation at the genus level, $IRD_{species}$: identification reliability deviation at the species level

Calculate the percent of risk of non-identification (R_{ni}) and 95 % confidence intervals for each of the tested agars in the specified scope of validation in accordance with Formula (1), and round to the first decimal place:

$$R_{ni} = \frac{N_0}{N} \times 100 \quad (1)$$

Calculate the percent identification reliability (*IRC*) and 95 % confidence intervals at the genus and species levels for each of the tested agars in accordance with Formula (2) and Formula (3), and round to the first decimal place:

$$IRC_{genus} = \frac{N - \left(N_0 + IRD_{genus}\right)}{N - N_0} \times 100 \quad (2)$$

$$IRC_{species} = \frac{N - \left(N_0 + IRD_{species}\right)}{N - N_0} \times 100 \quad (3)$$

Record the results.

For each of the tested agars, use Table 6 to evaluate the results of the identification reliability (*IRC*) at the genus and species levels in accordance with the given acceptability limits (*ALs*). The *AL* is met when 100 – IRC_{genus}, 100 – $IRC_{species}$ are less than or equal to the *AL* values listed in Table 6.

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The acceptability limits specified are for each non-selective or selective agar plate evaluated in the validation study.

An investigation shall be conducted (e.g. root cause analysis) in order to provide an interpretation for the observed discrepant results. The fitness for purpose of the identification method is then determined, based on the *AL* and the expert laboratory's interpretation (e.g. risk analysis) from the investigation. The reasons for acceptance of the identification method shall be stated in the validation study report.

NOTE Detailed examples are given in Annex C (expression, interpretation and evaluation of results).

Table 6 — Evaluation of the identification reliability study results

Tested agars	Identification reliability at genus level (%)			Identification reliability at species level (%)		
	100 – IRC_{genus}	*AL*	Evaluation[a]	100 – $IRC_{species}$	*AL*	Evaluation[a]
Non-selective agar for the validation of a broad range of microorganisms (e.g. bacteria, yeasts, moulds)		3			6	
Appropriate agar for the validation of a restricted range of microorganisms (e.g. lactic acid bacteria)		3			6	
Selective agar at the family level		3			4	
Selective agar at the genus level		3			4	
Selective agar at the species, sub-species and type levels		0			4	

Key

IRC_{genus}: identification reliability at the genus level, $IRC_{species}$: identification reliability at the species level, *AL*: acceptability limit.

[a] Evaluation: Results reported as Accepted or Not accepted.

7 Interlaboratory study

7.1 General

The aim of the interlaboratory study is to determine the variability of the results obtained by different collaborators using the same strains (reproducibility conditions). Whenever possible, the study conditions should reflect the normal variation between laboratories.

7.2 Data sets to be obtained

The interlaboratory study shall produce at least 10 valid data sets from at least 10 collaborators. The collaborators shall come from a minimum of 5, but preferably 10, different organizations, excluding the organizing laboratory. Technicians, involved in the preparation of the strains used in the interlaboratory study, shall not take part in the testing of those strains within the interlaboratory study.

NOTE Laboratories in different locations, but belonging to one company or institute, are accepted as different organizations.

— A total of 24 strains shall be tested per collaborator and all strains shall be tested with the identification method from a non-selective agar plate. The 24 selected strains shall represent the range of genera/species that are within the scope of the identification method and shall be as diverse as possible.

— If selective agars are included in the scope of validation, a total of 8 strains able to be recovered on the tested selective agars shall be additionally tested. The choice of selective agar tested should be determined based on their respective principle. These 8 strains may be selected from the 24 total strains tested, if applicable.

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— The interlaboratory study shall use strains that were correctly identified by all identification methods used during the identification reliability study.

7.3 Protocol

Strains shall be prepared by the organizing laboratory to ensure homogeneity between shipments (originating from the one purified colony per test strain).

The organizing laboratory shall blind code the strains prior to shipment.

Depending on the characteristics of the strains to be sent, shipment of strains can take place on, for example, agar transport swabs, agar tubes or non-selective agar plates.

Shipment of strains shall take into account the applicable transportation safety requirements.

The organizing laboratory shall provide the participants with adequate information on safe handling of the strains upon arrival and storage, and protocol(s) for the testing and reporting of results.

The analysis of the strains will start from the isolation of the strains on the tested agars prior to performing the identification method.

Plates and/or strains shall be retained for a certain period to be able to confirm results obtained by a collaborator, if needed.

The analysis of strains shall be performed by each collaborator within a stipulated time frame.

A minimum of 240 results from a non-selective agar and 80 from each selective agar used are required for the interpretation (10 complete data sets from each collaborator).

When the interlaboratory study is completed, all the information on data sheets and the results shall be submitted to the organizing laboratory and examined. Data shall be disregarded from collaborators:

— who received strains/test kits, etc. that were damaged during transportation;

— who deviated from the method protocol;

— if the reported technical results suggest that the laboratory has deviated from either the testing protocol(s) or any critical operating conditions.

7.4 Expression of results

Tabulate the results for each of the tested strains for the interlaboratory study as given in Table 7.

Any result discrepancies shall be included in the validation study report, accompanied by an explanation.

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Table 7 — Results for the interlaboratory study

Collaborators	Identification from non-selective agar		Identification from selective agar[a]	
	Number of incorrectly identified strains (N_{ic})	Number of correctly identified strains (IA)	Number of incorrectly identified strains (N_{ic})	Number of correctly identified strains (IA)
Collaborator 1	/24	/24	/8	/8
Collaborator 2	/24	/24	/8	/8
Etc.	/24	/24	/8	/8
Collaborator 10[b]	/24	/24	/8	/8
Total	**/240**	**/240**	**/80**	**/80**

Key

(N_{ic}): includes the number of non-identified strains (N_0) and the identification reliability deviation (IRD); IA: identification agreement.

[a] A representative of each type of agar tested in the performance characteristics study is evaluated in the interlaboratory study.

[b] At least 10 collaborators are required, but more may be included if available.

7.5 Interpretation and evaluation

Summarize the results of the interlaboratory study for each of the tested agars as in Table 8 (in total numbers of N_0, IA and IRD found), based on the interpretations in accordance with Table 7 (Results for the interlaboratory study). The identification reliability deviation (IRD) should be adapted to the scope of the method. For instance, IRD_{genus} or $IRD_{species}$ specify if the IRD is at the genus or species levels and are given as examples.

Table 8 — Summary of the results in the interlaboratory study

Tested agar	Number of				
	Strains (N)	IA	N_0	IRD_{genus}	$IRD_{species}$
x					
Etc.					

Key

N: number of strains, IA: identification agreement, N_0: number of non-identified strains, IRD_{genus}: identification reliability deviation at the genus level, $IRD_{species}$: identification reliability deviation at the species level.

Use the data from all collaborators to calculate the risk of non-identification (R_{ni}) in the specified scope of validation in accordance with Formula (1), and round to the first decimal place.

Calculate the identification reliability (IRC) at the genus and species levels for each of the tested agars in accordance with Formula (2) and Formula (3), and round to the first decimal place.

Record the results.

For each of the tested agars, use Table 9 to evaluate the results of the identification reliability (IRC) at the genus and species levels in accordance with the given acceptability limits (ALs).

The AL is met when 100 – IRC_{genus}, 100 – $IRC_{species}$ are below or equal to the AL values listed in Table 9.

When the AL is not met, investigations should be conducted (e.g. root cause analysis) in order to provide an explanation for the observed results. The fitness for purpose of the identification method is then determined, based on the AL and the additional information from the investigation. The reasons for acceptance of the identification method, when the AL is not met, shall be stated in the validation study report.

NOTE Detailed examples are given in Annex C (expression, interpretation and evaluation of results).

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Table 9 — Evaluation of the interlaboratory study results

Tested agars	Identification reliability at genus level (%)			Identification reliability at species level (%)		
	100 – IRC_{genus}	*AL*	**Evaluation**[a]	100 – $IRC_{species}$	*AL*	**Evaluation**[a]
Non-selective agar for the validation of a broad range of microorganisms (e.g. bacteria, yeasts, moulds)		4			6	
Appropriate agar for the validation of a restricted range of microorganisms (e.g. lactic acid bacteria)		3			5	
Selective agar at the family level		3			3	
Selective agar at the genus level		2			3	
Selective agar at the species, sub-species and type levels		0			0	

Key

IRC_{genus}: identification reliability at the genus level, $IRC_{species}$: identification reliability at the species level, *AL:* acceptability limit.

[a] Evaluation: Results reported as Accepted or Not accepted.

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Annex A
(informative)

Guidelines for the validation of methods for the identification of microorganisms in ecosystems

A.1 General

This annex provides guidelines on how to validate identification methods for the analysis of complex microbial ecosystems (e.g. enrichment broths, environment or food samples). This can be conducted only if the validation study using isolated strains on relevant agar(s) has been successfully conducted. Additional performance characteristics should be determined for the analysis of complex microbial ecosystems, and the results are only valid for the laboratory where the performance characteristics study was performed. An interlaboratory study is proposed where a network of laboratories is using the developed method.

Currently, no reference identification method can be specified. Therefore, the method will be validated on its own.

The validation protocol comprises two phases:

— a performance characteristics study;

— an interlaboratory study.

The validation protocol shall specify the group(s) of microbial ecosystems from which strains can be identified using the identification method. A specified number of microbial ecosystems should be tested.

Illustrations are provided in Annex D.

A.2 Performance characteristics of an identification method

A.2.1 General

The performance characteristics study is the part of the validation that is performed in one laboratory. The study consists of two parts:

— determining the level of detection (LOD_{50});

— determining the risk of non-identification study.

A.2.2 Description of the concept and limitation(s) of the identification method

In addition to the essential components of the performance characteristics study described in Table 1 (see 6.2), the group of microbial ecosystems for the identification of microorganisms should be carefully described, e.g. enrichment broths, as well as the claimed level of detection, e.g. 2 log cfu/g below the Total Viable Count (TVC).

NOTE TVC can be obtained with colony count at 30 °C in accordance with ISO 4833-1. However, other methods and techniques can be used if relevant, e.g. enumeration of citrate-fermenting lactic acid bacteria in accordance with ISO 17792. The selected inoculated species and related strains are able to grow in the group of microbial ecosystems being examined in order to provided satisfactory results in the identification reliability of the method. The determination of the LOD of a method based on TVC or culture-based results can be misleading for some advanced molecular methods. More suitable techniques can be considered (e.g. quantitative PCR).

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A.2.3 Level of detection (LOD_{50}) of the identification method

A.2.3.1 General

In the study, replicates of microbial ecosystems are used at three or more levels of contamination of the target microorganism(s). The evaluation is based on the:

a) calculation of the level of detection of a given strain relative to the main population (i.e. TVC);

b) selection of a number of microbial ecosystems;

c) replicates tested.

LOD_{50} is the level of detection for which 50 % of tests give a positive result. The LOD_{50} assesses the ability to detect and identify a target and tested species in a complex ecosystem that comprises microbial organisms at a higher level than the target and tested species.

A.2.3.2 Microbial ecosystem selection and preparation

For each group of microbial ecosystems to be studied, select three relevant media or samples. Use sterile media (e.g. three culture broths such as BPW, BLEB, TSB) or samples (e.g. UHT milk, UHT soy milk, UHT fruit juice). Check the sterility of the selected media or samples using a cultural method. Artificially inoculate each sterile medium or sample with a set of at least 5 different species from different relevant genera. These genera should be inoculated at a high level in order to mimic naturally encountered TVC (e.g. 6 to 8 log cfu/ml for culture broths). Each medium or sample should be preferably inoculated with a set of several different species. For the readability of the document, the set of 5 different species is referred to as the species cocktail. In addition, a non-culture identification method, e.g. based on DNA, should be used to determine if there is any interference caused by residual analytes (for example, residual free DNA).

A.2.3.3 Inoculation of microbial ecosystems

One analyst shall prepare, randomize, and blind code the ecosystem test portions and a second analyst should perform the identification method on the blind coded test portions.

Inoculate each prepared microbial ecosystem with a microbial species that is not included in the species cocktail.

A minimum of three levels per microbial ecosystem will be prepared consisting of at least a negative control level (L_0), a low level (L_1), and a higher level (L_2). Ideally, the low level (L_1) should be the theoretical detection level (e.g. 2 log cfu below the TVC per test portion) and the higher level (L_2) above the theoretical detection level (e.g. 1 log cfu below or at the TVC per test portion). An estimate for the levels of contamination (except for the negative control) should be made. At least 5 replicates will be prepared for the control level (L_0), 20 replicates for the low level (L_1), and 5 replicates for the higher level (L_2).

Perform the identification method on the test portions.

The species cocktail should be identified at all levels.

The negative control level should not produce identification results for the added strains. When positive results are obtained for a negative control level with the added strains, a root cause analysis should be performed. Depending on the outcomes of the root cause analysis, the experiments should be repeated for all levels.

The low level (L_1) should have fractional recovery by the identification method (fractional recovery at the low level should be between 25 % and 75 % of the number of test portions tested) for the added strains.

The high level (L_2) should have fractional recovery or only positive results for the additional species.

NOTE In order to have a better assurance that fractional recovery will be obtained, more levels of contamination can be produced and tested.

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Tabulate the study design as presented in Table A.1.

Table A.1 — Summary of LOD_{50} study details for one group of microbial ecosystems

Microbial ecosystem identification number[a]	Species cocktail[a]	TVC[a] (cfu/g or cfu/ml)	Inoculated strain below TVC level, i.e. levels L_1 and L_2[a]	Theoretical inoculation level to achieve fractional recovery (cfu/g or cfu/ml)
1				
2				
3				

[a] Provide the characteristics of the individual strains inoculated for each microbial ecosystem in a separate table such as the name of the strain, (culture) collection number and origin of the strain; other available characteristics can be added.

A.2.3.4 Calculation and interpretation of the LOD_{50}

It is expected that the strains of the species cocktail are all correctly identified, and no deviation is considered acceptable.

For each microbial ecosystem, tabulate the results with the additional species inoculated at levels L_1 and L_2 as in Table A.2.

Table A.2 — Results of the LOD_{50} study for one group of microbial ecosystems

Microbial ecosystem identification number	Blank level (L_0)		Inoculation level providing fractional recovery (L_1) ideally at the theoretical detection level (e.g. 2 log cfu below the TVC per test portion)		High inoculation level (L_2) ideally at the theoretical detection level (e.g. 1 log cfu below the TVC per test portion)		Total number of replicates
	Inoculation level (cfu/g or ml)	Number of positive replicates out of 5 replicates	Inoculation level (cfu/g or ml)	Number of positive replicates out of 20 replicates	Inoculation level (cfu/g or ml)	Number of positive replicates out of 5 replicates	
1	0	/5		/20		/5	30
2	0	/5		/20		/5	30
3	0	/5		/20		/5	30

For each microbial ecosystem, the LOD_{50} should be estimated by fitting the *POD-LOD* model[14] to the combined absence/presence data of the identification method.

Summarize the results of the LOD_{50} study for each of the tested microbial ecosystem, as in Table A.3. Calculate the ratio between the determined TVC and LOD_{50} and express the data in $\log_{10}$. Calculate the difference between the highest TVC/LOD_{50} value and the lowest TVC/LOD_{50} value. Consistency is expected between the observed TVC/LOD_{50} ratio, and no more than 0,5 $\log_{10}$ should be obtained between highest TVC/LOD_{50} and lowest TVC/LOD_{50} values.

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Table A.3 — Calculation and interpretation of the LOD_{50} study for one group of microbial ecosystems

Microbial ecosystem identification number	TVC (cfu/g or ml)	LOD_{50} (cfu/g or ml)	TVC/LOD_{50}		$\log_{10}$ (highest TVC/LOD_{50} value) – $\log_{10}$ (lowest TVC/LOD_{50} value)	Acceptability limit (AL)
			normal scale value	$\log_{10}$ value	$\log_{10}$	
1						0,5
2						
3						

The (high TVC/LOD_{50} value) – (low TVC/LOD_{50} value) should be below or equal to the AL. In case of discrepancies, a root cause analysis should be performed.

A.2.4 Risk of non-species-identification study

A.2.4.1 General

The evaluation of the risk of non-identification is based on the ability of the method to identify targeted analytes in a set of usually encountered microbial ecosystems.

A.2.4.2 Selection of number of microbial ecosystems and replicates tested

For each group of microbial ecosystems, a minimum of 30 microbial ecosystems (N) that are ordinarily encountered by the originating laboratory or prospective end-users of the method should be selected; 30 valid data sets (N) should be produced. The TVC of the microbial ecosystems should be determined, and an analysis with the identification method should be run prior to artificial inoculations of the microbial ecosystems. For each microbial ecosystem, inoculate one species that was not previously identified with the method; the inoculation level should be below the TVC and higher than the theoretical LOD_{50}, preferably $10 \times LOD_{50}$.

The microbial ecosystems should be stored in appropriate conditions (e.g. 5 °C ± 3 °C) to obtain the TVC results prior to inoculation with non-identified strains.

The storage should not exceed 72 h after incubation to minimize the potential growth of species.

Tabulate the details of the design as presented in Table A.4.

Table A.4 — Details of the study into the identification risk of non-species-identification for one group of microbial ecosystems

Microbial ecosystem identification number	TVC (cfu/g or ml)	LOD_{50} (cfu/g or ml)	Selected species for artificial inoculation				Inoculation level (cfu/g or ml)
			Reporting of the analysis with the identification method on the non-inoculated microbial ecosystem[a]	Species	Strain	Origin	
1							
…							
30							

[a] Only absence of the inoculated strain is expected for each microbial ecosystem.

A.2.4.3 Calculation of the risk of non-identification study

For each microbial ecosystem, tabulate the results of the tested inoculated microbial ecosystems as in Table A.5. Report if the inoculated strain is identified as expected for each tested microbial ecosystem, and the number of non-identified strains.

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Table A.5 — Results of the risk of non-species-identification for one group of microbial ecosystems

Microbial ecosystem identification number	**Selected species for artificial inoculation**	**Inoculation level** (cfu/g or ml)	**Identification of the inoculated strain** (yes/no)
1			
…			
30			

The total number of non-identified strains (N_0) should be reported as presented in Table A.6.

Table A.6 — Calculation of the risk of non-species-identification study for one group of microbial ecosystems

Microbial ecosystem identification number	**Microbial ecosystem description**	**TVC** (cfu/g or ml)	**Inoculated strain**		**Total number of non-identified strains** (N_0)
			Species	**Inoculation level** (cfu/g or ml)	
…					
…					
…					

Calculate the risk of non-identification (R_{ni}) for the tested group of microbial ecosystems according to Formula (A.1).

A.3 Interlaboratory study

A.3.1 General

The aim of the interlaboratory study is to determine the variability of the results obtained by different collaborators using the same strains (reproducibility conditions). Whenever possible, the study conditions should reflect the normal variation between laboratories.

The interlaboratory study should be run with one microbial ecosystem of one of the groups of microbial ecosystems tested of Clause A.2.

A.3.2 Protocol

The interlaboratory study shall produce 10 valid data sets from a minimum of 10 collaborators. The collaborators should come from a minimum of 5, but preferably 10, different organizations, excluding the organizing laboratory. Technicians, involved in the preparation of the strains used in the interlaboratory study, shall not take part in the testing of those strains within the interlaboratory study.

NOTE Laboratories in different locations, but belonging to one company or institute, are accepted as different organizations.

The protocol is as follows:

— A relevant microbial ecosystem should be produced by the organizing laboratory by artificially inoculating a set of 5 different species (species cocktail) at a high level to mimic naturally encountered TVC. Select a microbial ecosystem already tested in Clause A.2 and inoculate with the same set of strains. The TVC should be determined by the organizing laboratory. However, each collaborator should perform a TVC for quality control.

— An additional species will be added at lower inoculation levels by the organizing laboratory. A minimum of three levels per microbial ecosystem will be prepared consisting of a negative control level (L_0), a low level (L_1), and a higher level (L_2). Ideally, the low level (L_1) should be the theoretical detection level (e.g. 2 log cfu below the TVC per microbial ecosystem) and the higher level (L_2) above the theoretical

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detection level (e.g. 1 log cfu below or at the TVC per microbial ecosystem). At least the low level (L_1) should have fractional recovery by the identification method (fractional recovery at the low level should be between 25 % and 75 % of the number of microbial ecosystems tested). An estimate for the level of contamination (except for the negative control) should be made. Eight replicate microbial ecosystems should be prepared for each contamination level: the negative control (L_0) and the two inoculation levels (L_1 and L_2), achieving 24 data sets.

— The microbial ecosystems should be retained for a certain period of time to be able to confirm results obtained by a collaborator if needed.

— The analysis of microbial ecosystems should be performed by each collaborator at the stipulated date.

A minimum of 1 200 results are required for the calculation of the risk of non-identification, and a minimum of 240 results are required for the calculation of LOD_{50} (10 complete data sets from each collaborator).

When the interlaboratory study is completed, all the information on data sheets and the results shall be submitted to the organizing laboratory and examined. Data shall be disregarded from collaborators:

— who received microbial ecosystems/test kits, etc. that were damaged during transportation;

— who used reagents that are not in accordance with the method;

— if the reported technical results suggest that the laboratory has deviated from either the testing protocol(s) or any critical operating conditions.

A.3.3 Expression and interpretation of the results

A.3.3.1 General

Any result discrepancies shall be included in the validation study report, accompanied by an explanation.

A.3.3.2 Calculate the risk of non-identification

Tabulate the results in Table A.7. Record the non-identified strains (N_0) and the total number of data (N) as presented in Table A.8.

Twenty-four (24) data sets should be recorded per collaborator and species. The total number of data set (N) is 5 species × number of collaborators × 24 microbial ecosystems, i.e. minimum 1 200 data points.

Table A.7 — Results for the species cocktail inoculated at high levels

Collaborators	Number of identifications of the species cocktail				
	Species 1	Species 2	Species 3	Species 4	Species 5
Collaborator 1	/24	/24	/24	/24	/24
Collaborator 2	/24	/24	/24	/24	/24
Etc…	/24	/24	/24	/24	/24
Collaborator 10	/24	/24	/24	/24	/24
Non-identified strains	**/240**	**/240**	**/240**	**/240**	**/240**

Table A.8 — Results to calculate the risk on non-identification

Total number of data (N)	
Total number of non-identified strains (N_0)	

Calculate the risk of non-identification (R_{ni}) for the interlaboratory study in accordance with Formula (A.1):

$$R_{ni}\,(\%) = \frac{N_0}{N} \times 100 \qquad \text{(A.1)}$$

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A.3.3.3 Calculate the LOD_{50}

Tabulate the results of the identification data of the species inoculated at levels L_0, L_1 and L_2 as given in Table A.9.

Table A.9 — Results for the inoculated strain at levels L_0, L_1 *a*nd L_2

Collaborators	**Inoculation levels**		
	L_0	L_1	L_2
Collaborator 1	/8	/8	/8
Collaborator 2	/8	/8	/8
Etc…	/8	/8	/8
Collaborator 10	/8	/8	/8
Total	**/80**	**/80**	**/80**

The LOD_{50} should be estimated by fitting the *POD-LOD* model for the interlaboratory study to the combined absence/presence data of the identification method.

Summarize the results of the LOD_{50}, as in Table A.10. Calculate the ratio of the TVC by the organizing laboratory and the LOD_{50}. Express the data in $\log_{10}$.

Calculate the difference between the TVC/LOD_{50} value of the interlaboratory study and the TVC/LOD_{50} value of the performance characteristics study. Express the obtained result in absolute value. Consistency is expected between the TVC/LOD_{50} value of the interlaboratory study and the TVC/LOD_{50} value of the performance characteristics study. Perform a root cause analysis when the difference between the TVC/LOD_{50} values of the interlaboratory study and the performance characteristics study exceed the *AL*.

Table A.10 — Results for LOD_{50} of the interlaboratory study

Microbial ecosystem	**TVC** (cfu/g or ml)	LOD_{50} (cfu/g or ml)	**TVC/LOD_{50}**		| $\log_{10}$ **(TVC/LOD_{50} value) of the interlaboratory study** – $\log_{10}$ **(low TVC/LOD_{50} value) of the performance characteristic study** |	**Acceptability limit** (*AL*)
			Value	$\log_{10}$ **value**		
…						0,5

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Annex B
(normative)

Points to be considered when selecting strains for an identification reliability study

B.1 General

This annex outlines the minimum test requirements for general use. In the selection of test strains, the majority shall originate from foods, feed, the food-processing environment or the primary production, and should cover the recognized range of the target analyte with respect to the following: diversity in identification characteristics (e.g. phenotype or genotype), geographical distribution, incidence, and any other claims made by the producers of the identification method. In addition, (food-borne disease related) clinical, environmental and culture collection strains can also be used.

B.2 Identification group categories

a) Undefined group, e.g. bacteria, yeast, moulds.

b) Defined group, e.g. phylum, lactic acid bacteria.

c) Family, e.g. *Listeriaceae, Lactobacillaceae*.

d) Genus, e.g. *Listeria, Lactobacillus*.

e) Species, e.g. *Listeria monocytogenes, Lactobacillus delbrueckii.*

f) Sub-species, e.g. *Lactobacillus delbrueckii* subsp. *bulgaricus.*

g) Type, e.g. *Lactobacillus delbrueckii* subsp. *bulgaricus* fingerprint type 1.

B.3 Group selection

a) For undefined and defined groups included in the scope of validation: use strains selected from species included in the database content of the identification method.

b) For defined groups: use strains from a range of families and genera. If possible, include a representative member of all families and genera.

c) For family: use a range of species from that family and test as many species as possible; whenever possible, use additional strains from other families that are able to be recovered on the tested selective agar.

d) For genus or group of species (e.g. *Bacillus cereus* group): use a range of species from that genus and test as many species as possible; whenever possible, use additional strains from other genera that are able to be recovered on the tested selective agar.

e) Where appropriate, the number of strains tested can be rationally limited by selection of strains with biologically relevant differences.

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Annex C
(informative)

Expression, interpretation and evaluation of results

C.1 General

This annex shows three examples of validation studies:

a) Example of the validation of an identification method to an undefined group of microorganisms from non-selective agars, i.e. total microflora, and a selective *Bacillus cereus* group (see Clause C.2);

b) Example of the validation of an identification method to a defined group of microorganisms from specified agars suitable for the targeted group microorganisms, i.e. lactic acid bacteria on De Man, Rogosa and Sharpe agar (MRS) or M17 (see Clause C.3);

c) Example of the validation of an identification method to a defined and restricted group of microorganisms from a specified agar suitable for the targeted group microorganisms, i.e. *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (see Clause C.4).

C.2 Example of the validation of an identification method to an undefined group of microorganisms from non-selective agars and a selective agar

The scope of validation is the following:

a) groups of microorganisms: total microflora, and in addition the differentiation of the species within the *Bacillus cereus* group;

b) database name, version and content: company A, version 5, 7 000 species and 200 genera;

c) culture media: non-selective agars [e.g. tryptone soya agar (TSA) or tryptone soya yeast extract agar (TSYEA)], and Mossel mannitol-egg yolk-polymyxin (MYP) selective agar;

d) microorganism type: bacteria;

e) comparison algorithm: version 3.5.

The identification method principle is MALDI-TOF mass spectrometry.

The reliability study includes strains from species included in the database content as follows:

— 700 strains from minimum 50 different genera (and 700 different species) recovered on some non-selective agars, e.g. TSA or TSYEA;

— 45 strains from species of the *Bacillus cereus* group usually encountered in food microbiology, with as much as possible the same number of strains per species, and 5 strains from other *Bacillus* species recovered on MYP.

The organizing laboratory tested the 750 strains with the identification method.

The interlaboratory study includes:

— 24 strains from various families, genera and species recovered on some non-selective agars, e.g. TSA or TSYEA;

— 6 strains from species of the *Bacillus cereus* group and 2 strains from other *Bacillus* species recovered on MYP.

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All 14 participants to the interlaboratory study tested the 32 strains with the identification method.

The identification method was successfully validated based on the results presented in Tables C.1 to C.7.

Table C.1 — Results of the identification reliability study

Tested strain	Agar(s) used to recover the strain	Identity of the strain		Identity of the strain by the method under validation[a]	Interpretation of results ($IA/ID/N_0$)
		Assigned identity	Identification method, or identification method principle		
1	TSYEA	*Bacillus licheniformis*	Conventional test with biochemical gallery	*Bacillus licheniformis*	*IA*
2	TSYEA	*Listeria ivanovii*	Conventional test with biochemical gallery	*Listeria monocytogenes*	*ID*
3	TSYEA	*Escherichia coli*	Conventional test with biochemical gallery	*Shigella* spp.	*ID*
Etc.					

Key

IA: identification agreement, *ID*: identification deviation, N_0: no identification.

[a] Include results with no identification.

NOTE Characteristics of the individual strains are as a minimum: the name of the strain, (culture) collection number and origin of the strain. Other available characteristics can be added as well.

Table C.2 — Interpretation of the identification deviations of the identification reliability study

Tested strain	Assigned identity	Identity of the strain by the method under validation[a]	Identity of the strain by a third method using a different principle		Interpretation of results (IA/IRD_{genus} or $IRD_{species}$)
			Method principle	Identity of the strain	
2	*Listeria ivanovii*	*Listeria monocytogenes*	16S rDNA sequencing	*Listeria monocytogenes*	*IA*
3	*Escherichia coli*	*Shigella* spp.	16S rDNA sequencing	*Escherichia coli*	IRD_{genus}
Etc.					

Key

IA: identification agreement, IRD_{genus}: identification reliability deviation at the genus level, $IRD_{species}$: identification reliability deviation at the species level.

[a] Include results with no identification.

NOTE Characteristics of the individual strains are as a minimum: the name of the strain, (culture) collection number and origin of the strain. Other available characteristics can be added as well.

Table C.3 — Summary of the results in the identification reliability study

Tested agar	Number of				
	Strains (N)	*IA*	N_0	IRD_{genus}	$IRD_{species}$
TSYEA or TSA	700	683	2	5	15
MYP	50	48	1	0	1

Key

N: number of strains, *IA*: identification agreement, N_0: number of non-identified strains, IRD_{genus}: identification reliability deviation at the genus level, $IRD_{species}$: identification reliability deviation at the species level.

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In the identification reliability study, risk of non-identification (R_{ni}), IRC_{genus} and $IRC_{species}$ in the defined scope of validation are the following:

— From non-selective agar, e.g. TSA or TSYEA:

R_{ni} = 0,3 %; IRC_{genus} = 99,3 %; $IRC_{species}$ = 97,9 %.

— From MYP:

R_{ni} = 2,0 %; IRC_{genus} = 100 %; $IRC_{species}$ = 98,0 %.

Table C.4 — Evaluation of the identification reliability study results

Tested agars	Identification reliability at genus level (%)			Identification reliability at species level (%)		
	100 – IRC_{genus}	*AL*	Evaluation[a]	100 – $IRC_{species}$	*AL*	Evaluation[a]
TSYEA or TSA	0,7	3	Accepted	2,1	6	Accepted
MYP	0	3	Accepted	2,0	4	Accepted

Key

IRC_{genus}: identification reliability at the genus level, $IRC_{species}$: identification reliability at the species level, *AL*: acceptability limit.

[a] Evaluation: Results reported as Accepted or Not accepted.

Table C.5 — Results for the interlaboratory study

Collaborators	Number of identified strains from non-selective agar (TSYEA or TSA)		Number of identified strains from selective agar (MYP)	
	Number of incorrectly identified strains (N_{ic})	Number of correctly identified strains (*IA*)	Number of incorrectly identified strains (N_{ic})	Number of correctly identified strains (*IA*)
Collaborator 1	1/24	23/24	0/8	8/8
Collaborator 2	0/24	24/24	0/8	7/8
Etc.	/24	/24	/8	/8
Collaborator 14	0/24	24/24	1/8	7/8
Total	**2/336**	**330/336**	**1/112**	**110/112**

Key

(N_{ic}): includes the number of non-identified strains (N_0) and the identification reliability deviation (*IRD*); *IA*: identification agreement.

Table C.6 — Summary of the results in the interlaboratory study

Tested agar	Number of				
	Strains (*N*)	*IA*	N_0	IRD_{genus}	$IRD_{species}$
TSYEA or TSA	336	330	2	2	4
MYP	112	110	1	1	1

Key

N: number of strains, *IA*: identification agreement, N_0: number of non-identified strains, IRD_{genus}: identification reliability deviation at the genus level, $IRD_{species}$: identification reliability deviation at the species level.

In the identification reliability study, risk of non-identification (R_{ni}), IRC_{genus} and $IRC_{species}$ in the defined scope of validation are the following:

— From non-selective agar, e.g. TSA or TSYEA:

R_{ni} = 0,6 %; IRC_{genus} = 99,4 %; $IRC_{species}$ = 98,8 %.

— From MYP:

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R_{ni} = 0,9 %; IRC_{genus} = 99,1 %; $IRC_{species}$ = 99,1 %.

Table C.7 — Evaluation of the interlaboratory study results

Tested agars	Identification reliability at genus level (%)			Identification reliability at species level (%)		
	100 – IRC_{genus}	*AL*	Evaluation[a]	100 – $IRC_{species}$	*AL*	Evaluation[a]
TSYEA or TSA	0,6	4	Accepted	1,2	6	Accepted
MYP	0,9	3	Accepted	0,9	5	Accepted

Key

IRC_{genus}: identification reliability at the genus level, $IRC_{species}$: identification reliability at the species level, *AL*: acceptability limit.

[a] Evaluation: Results reported as Accepted or Not accepted.

C.3 Example of the validation of an identification method to a defined group of microorganisms from specified agars suitable for the targeted group microorganisms

The scope of validation is the following:

a) group of microorganisms: lactic acid bacteria;

b) database name, version and content: company B, version 1, 225 species;

c) culture media: agars for lactic acid bacteria (e.g. MRS or M17);

d) microorganism type: lactic acid bacteria;

e) comparison algorithm: version 2020/1.

The identification method principle is target DNA sequencing (*gyr*B)

The reliability study includes strains from species included in the database content as follows: 250 strains recovered on some agars suitable for lactic acid bacteria recovery, e.g. MRS or M17.

The organizing laboratory tested the 250 strains with the identification method. The strains were all able to grow on the tested agar plates used, i.e. MRS or M17. Most of the strains were gathered in the *Lactobacillales* order covering all the genera, but 3 strains were belonging to the *Sporolactobacillus* genus from the *Bacillales* order as they produce lactic acid.

The identification method was not successfully validated based on the results presented in Tables C.8 to C.11.

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Table C.8 — Results of the identification reliability study

Tested strain	Agar(s) used to re-cover the strain	Identity of the strain		Identity of the strain by the method under validation[a]	Interpretation of results ($IA/ID/N_0$)
		Assigned identity	Identification method or identification method principle		
1	MRS	*Lactobacillus brevis*	16S rDNA sequencing	*Lactobacillus hilgardii*	*ID*
2	MRS	*Lactococcus lactis*	16S rDNA sequencing	*Lactococcus lactis*	*IA*
3	MRS	*Leuconostoc mesenteroides*	16S rDNA sequencing	*Oenococcus oeni*	*ID*
Etc.					

Key

IA: identification agreement, *ID*: identification deviation, N_0: no identification.

[a] Include results with no identification.

NOTE Characteristics of the individual strains are as a minimum: the name of the strain, (culture) collection number and origin of the strain. Other available characteristics can be added as well.

Table C.9 — Interpretation for the identification deviations of the identification reliability

Tested strain	Assigned identity	Identity of the strain by the method under validation[a]	Identity of the strain by a third method using a different principle		Interpretation of results (IA/IRD_{genus} or $IRD_{species}$)
			Method principle	Identity of the strain	
1	*Lactobacillus brevis*	*Lactobacillus hilgardii*	Mass spectrometry	*Lactobacillus brevis*	$IRD_{species}$
3	*Leuconostoc mesenteroides*	*Oenococcus oeni*	MLST	*Leuconostoc mesenteroides*	IRD_{genus}
Etc.					

Key

IA: identification agreement, IRD_{genus}: identification reliability deviation at the genus level, $IRD_{species}$: identification reliability deviation at the species level.

[a] Include results with no identification.

NOTE Characteristics of the individual strains are as a minimum: the name of the strain, (culture) collection number and origin of the strain. Other available characteristics can be added as well.

Table C.10 — Summary of the results in the identification reliability study

Tested agar	Number of				
	Strains *(N)*	*IA*	N_0	IRD_{genus}	$IRD_{species}$
MRS or M17	250	228	2	10	20

Key

N: number of strains, *IA*: identification agreement, N_0: number of non-identified strains, IRD_{genus}: identification reliability deviation at the genus level, $IRD_{species}$: identification reliability deviation at the species level.

In the identification reliability study, risk of non-identification (R_{ni}), IRC_{genus} and $IRC_{species}$ in the defined scope of validation are the following:

R_{ni} = 0,8 %; IRC_{genus} = 96,0 %; $IRC_{species}$ = 92,0 %.

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Table C.11 — Evaluation of the identification reliability study results

Tested agars	Identification reliability at genus level (%)			Identification reliability at species level (%)		
	100 – IRC_{genus}	*AL*	**Evaluation**[a]	100 – $IRC_{species}$	*AL*	**Evaluation**[a]
MRS or M17	4,0	3	Not accepted	8,0	6	Not accepted
Key IRC_{genus}: identification reliability at the genus level, $IRC_{species}$: identification reliability at the species level, *AL*: acceptability limit. [a] Evaluation: Results reported as Accepted or Not accepted.						

The data and tables are not given for the interlaboratory study because the method did not pass the first part of the validation, i.e. the performance characteristics study.

C.4 Example of the validation of an identification method to a defined and restricted group of microorganisms from a specified agar suitable for the targeted group microorganisms

The scope of validation is the following:

a) group of microorganisms: *Lactobacillus delbrueckii* subsp. *lactis and Lactobacillus delbrueckii* subsp. *bulgaricus;*

b) no database as the identification is a multiplex PCR assay;

c) culture medium: MRS agar for lactic acid bacteria;

d) microorganism type: *Lactobacillus delbrueckii* subsp. *lactis and Lactobacillus delbrueckii* subsp. *bulgaricus;*

e) no database and therefore no algorithm.

The identification method principle is multiplex PCR run after a *Lactobacillus delbrueckii* species identification method.

The reliability study includes strains from sub-species *Lactobacillus delb*rueckii subsp. *lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus*: 25 strains of each sub-species recovered on MRS. In addition, 25 *Lactobacillus* strains from 25 different species are included in the tested panel.

The organizing laboratory tested the 75 strains with the identification method. The strains were all able to grow on the tested agar plates used, i.e. MRS.

The interlaboratory study includes:

— 12 strains from *Lactobacillus delbrueckii* subsp. *lactis* and 12 *Lactobacillus delbrueckii* subsp. *bulgaricus* recovered on some MRS;

— 8 strains from Lactobacillus different from *Lactobacillus delbrueckii* recovered on MRS.

All 12 participants to the interlaboratory study tested the 32 strains with the identification method.

The identification method was successfully validated based on the results presented in Tables C.12 to C.17.

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