International Standard

ISO 16140-7

First edition
2024-11

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

Reference number
ISO 16140-7:2024(en)

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Published in Switzerland

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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.

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.

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.

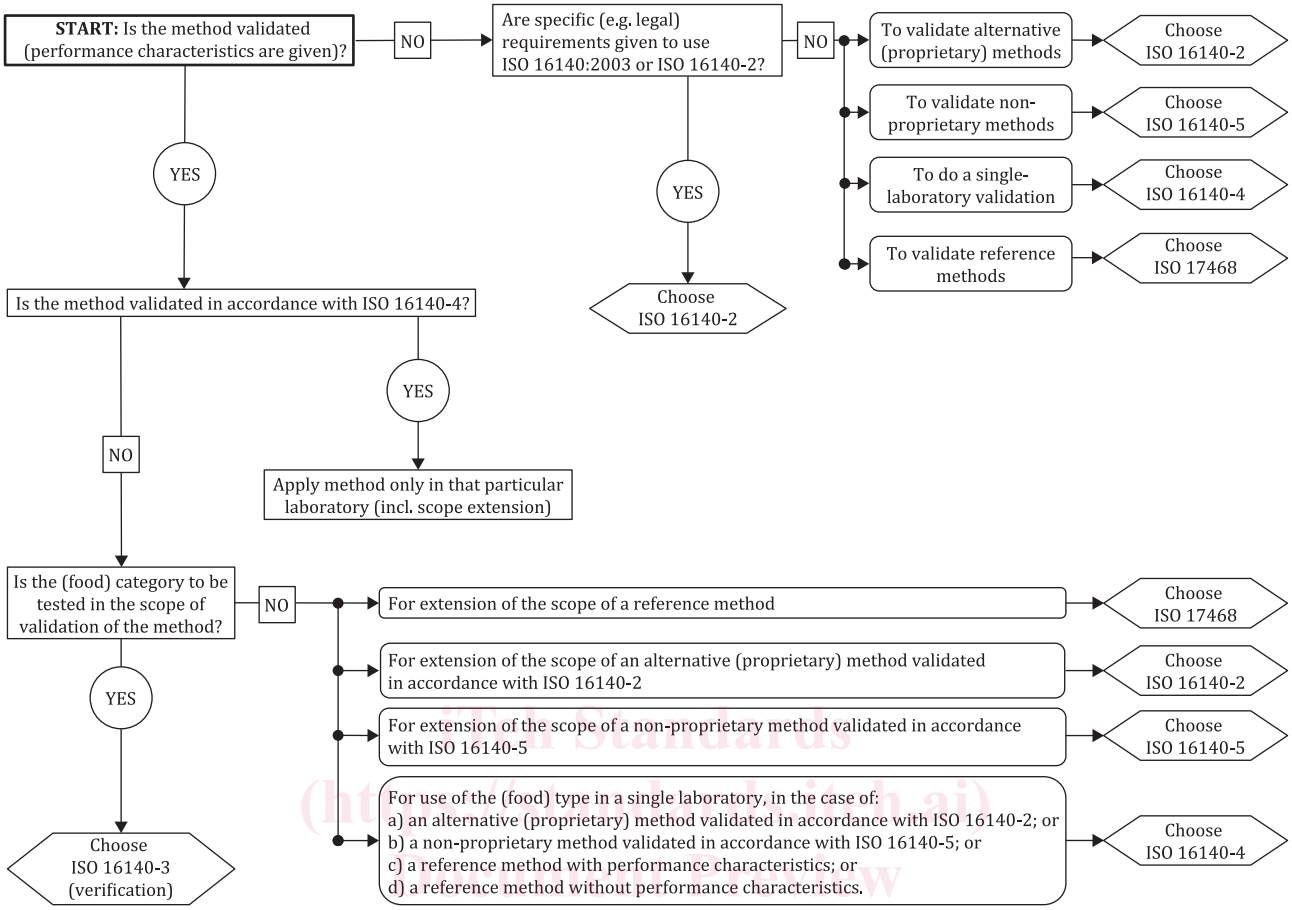

Figure 1 — Flow chart for application of ISO 16140-2 to ISO 16140-5

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).

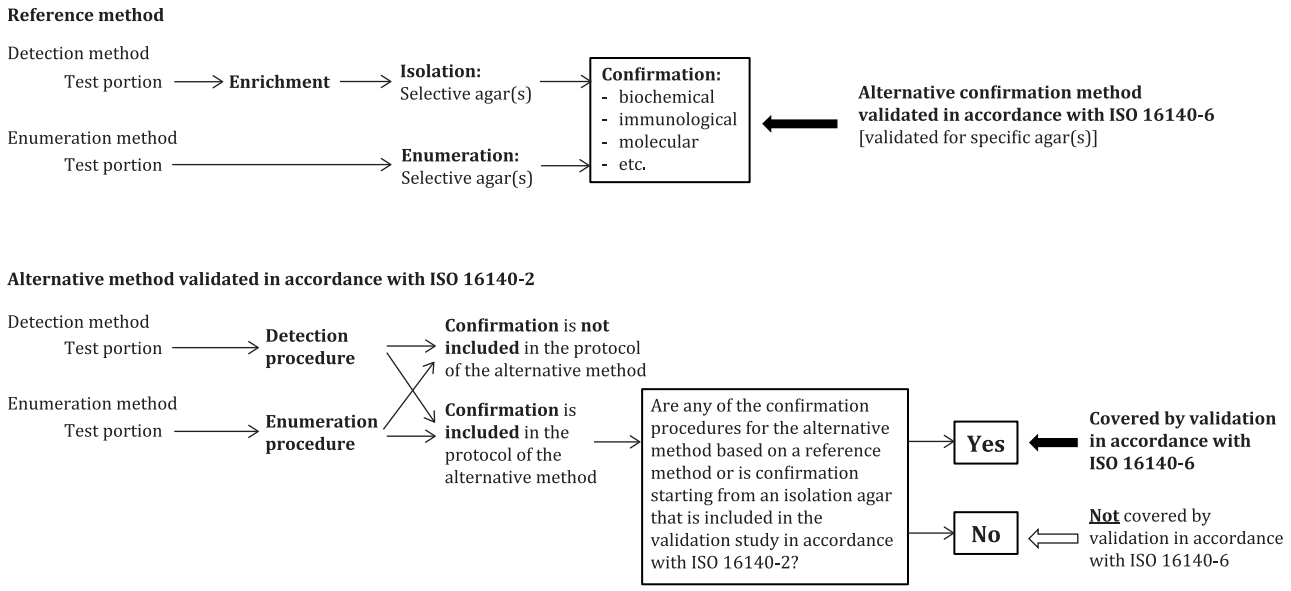

Figure 2 — Use of validated alternative confirmation methods (see ISO 16140-6)

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

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.

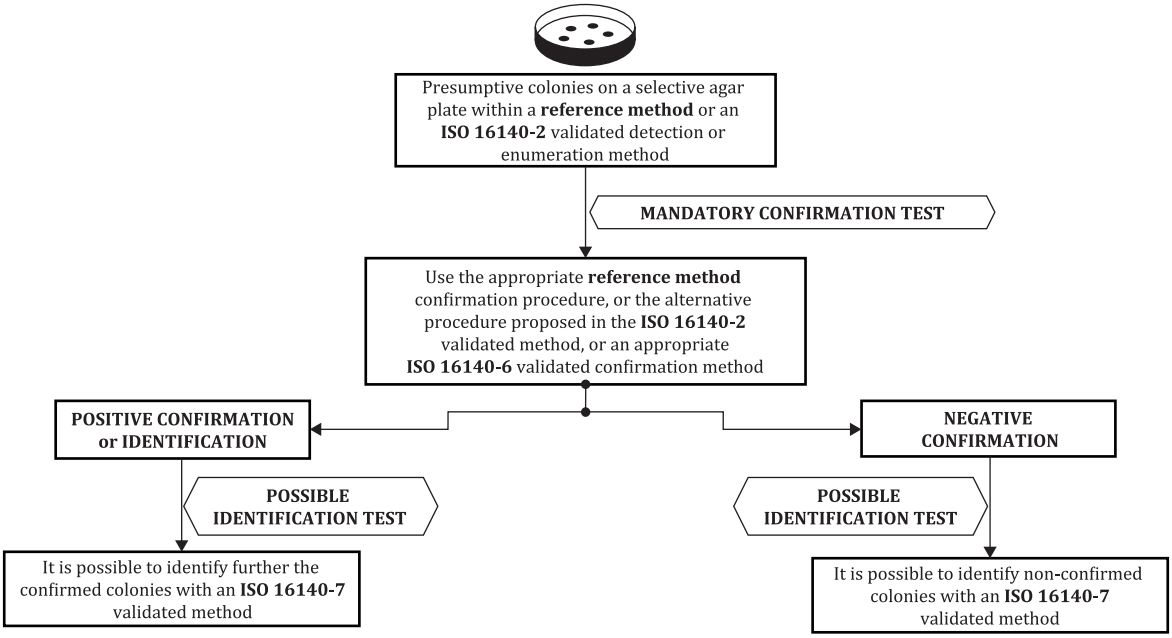

Figure 3 — Flow chart for the application of ISO 16140-6 and this document for the confirmation and identification of colonies within a reference method or an ISO 16140-2 validated detection or enumeration method

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.

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.

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.

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

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.

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.