



BSI Standards Publication

Microbiology of the food chain — Method validation

Part 4: Protocol for method validation in a single laboratory

National foreword

This British Standard is the UK implementation of EN ISO 16140-4:2020+A2:2025. It is identical to ISO 16140-4:2020, incorporating amendment 1:2024 and amendment 2:2025. It supersedes BS EN ISO 16140-4:2020+A1:2024, which is withdrawn.

The start and finish of text introduced or altered by amendment is indicated in the text by tags. Tags indicating changes to ISO text carry the number of the ISO amendment. For example, text altered by ISO amendment 1 is indicated by A1 A1.

The UK participation in its preparation was entrusted to Technical Committee AW/9, Microbiology.

A list of organizations represented on this committee can be obtained on request to its committee manager.

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Published by BSI Standards Limited 2025

ISBN 978 0 539 31743 5

ICS 07.100.30

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This British Standard was published under the authority of the Standards Policy and Strategy Committee on 30 September 2020.

Amendments/corrigenda issued since publication

Date	Text affected
30 September 2024	Implementation of ISO amendment 1:2024 with CEN endorsement A1:2024
31 August 2025	Implementation of ISO amendment 2:2025 with CEN endorsement A2:2025

English Version

**Microbiology of the food chain - Method validation - Part 4:
Protocol for method validation in a single laboratory (ISO
16140-4:2020)**

Microbiologie de la chaîne alimentaire - Validation des
méthodes - Partie 4: Protocole pour la validation de
méthodes dans un seul laboratoire (ISO 16140-4:2020)

Mikrobiologie der Lebensmittelkette -
Verfahrensvalidierung - Teil 4: Arbeitsvorschrift für
Einzel-Labor-Verfahrensvalidierung (ISO 16140-
4:2020)

This European Standard was approved by CEN on 25 May 2020.

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European foreword

This document (EN ISO 16140-4:2020) has been prepared by Technical Committee ISO/TC 34 "Food products" in collaboration with Technical Committee CEN/TC 463 "Microbiology of the food chain" the secretariat of which is held by AFNOR.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by February 2021, and conflicting national standards shall be withdrawn at the latest by February 2021.

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Endorsement notice

The text of ISO 16140-4:2020 has been approved by CEN as EN ISO 16140-4:2020 without any modification.

European foreword to Amendment 1

This document (EN ISO 16140-4:2020/A1:2024) has been prepared by Technical Committee ISO/TC 34 "Food products" in collaboration with Technical Committee CEN/TC 463 "Microbiology of the food chain" the secretariat of which is held by AFNOR.

This Amendment to the European Standard EN ISO 16140-4:2020 shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by March 2025, and conflicting national standards shall be withdrawn at the latest by March 2025.

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Endorsement notice

The text of ISO 16140-4:2020/Amd 1:2024 has been approved by CEN as EN ISO 16140-4:2020/A1:2024 without any modification.

European foreword to Amendment 2

This document (EN ISO 16140-4:2020/A2:2025) has been prepared by Technical Committee ISO/TC 34 "Food products" in collaboration with Technical Committee CEN/TC 463 "Microbiology of the food chain" the secretariat of which is held by AFNOR.

This Amendment to the European Standard EN ISO 16140-4:2020 shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by February 2026, and conflicting national standards shall be withdrawn at the latest by February 2026.

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Endorsement notice

The text of ISO 16140-4:2020/Amd 2:2025 has been approved by CEN as EN ISO 16140-4:2020/A2:2025 without any modification.

Contents

Page

Foreword	iv
Introduction	v
1 Scope	1
2 Normative references	1
3 Terms and definitions	2
4 General principles of the single-laboratory detection or quantification method validation	4
4.1 General.....	4
4.2 Principles of the factorial approach.....	5
4.3 Principles of the conventional approach.....	5
5 Technical protocol for validation — Factorial approach	7
5.1 Qualitative methods.....	7
5.1.1 Single-laboratory method validation study against a reference method.....	7
5.1.2 Single-laboratory method validation study without a reference method.....	13
5.2 Quantitative methods.....	15
5.2.1 Single-laboratory method validation study against a reference method.....	15
5.2.2 Single-laboratory method validation study without a reference method.....	18
6 Technical protocol for validation — Conventional approach	19
6.1 Qualitative methods.....	19
6.1.1 Single-laboratory method validation study against a reference method.....	19
6.1.2 Single-laboratory method validation study without a reference method.....	20
6.2 Quantitative methods.....	21
6.2.1 Single-laboratory method validation study against a reference method.....	21
6.2.2 Single-laboratory method validation study without a reference method.....	23
7 Summary of acceptability limits	26
Annex A (informative) List of factors and factor levels for factorial method validation	27
Annex B (informative) Calculation of in-house reproducibility for qualitative methods based on the LOD₅₀ study described in 6.1.2.3	29
Annex C (informative) Example of a factorial single-laboratory method validation study for a quantitative method against a reference method	30
Annex D (informative) Example of a factorial single-laboratory method validation study for a qualitative method against a reference method	36
Annex E (informative) Example of a factorial single-laboratory method validation study for the variability of the LOD₅₀ for a qualitative method without a reference method	40
Annex F (informative) Determination of precision if the artificially contaminated samples are unstable	43
Annex G (informative) Protocol for single-laboratory validation of alternative methods for microbiological confirmation and typing procedures	45
A₁ Annex H (normative) Validation of a larger test portion size for qualitative methods	46 A₁
A₂ Annex G (informative) Protocol for single-laboratory validation of identification methods of microorganisms	51 A₂
Bibliography	52

Foreword

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

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

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

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. ^{A2} The ISO 16140 series consists of several parts with the general title, *Microbiology of the food chain — Method validation*: ^{A2}

- *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;*

^{A2} — *Part 7: Protocol for the validation of identification methods of microorganisms.* ^{A2}

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 and ISO 16140-6). In the case when a method is validated within one laboratory (as described in this document), 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”.

^{A2} In the ISO 16140 series, reference methods include standardized reference (ISO and CEN) methods as defined in ISO 17468:2016, 3.7, as a “reference method described in a standard”. ^{A2}

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.”

This document, 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. This document and ISO 16140-5 can be used for validation against a reference method. This document (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.

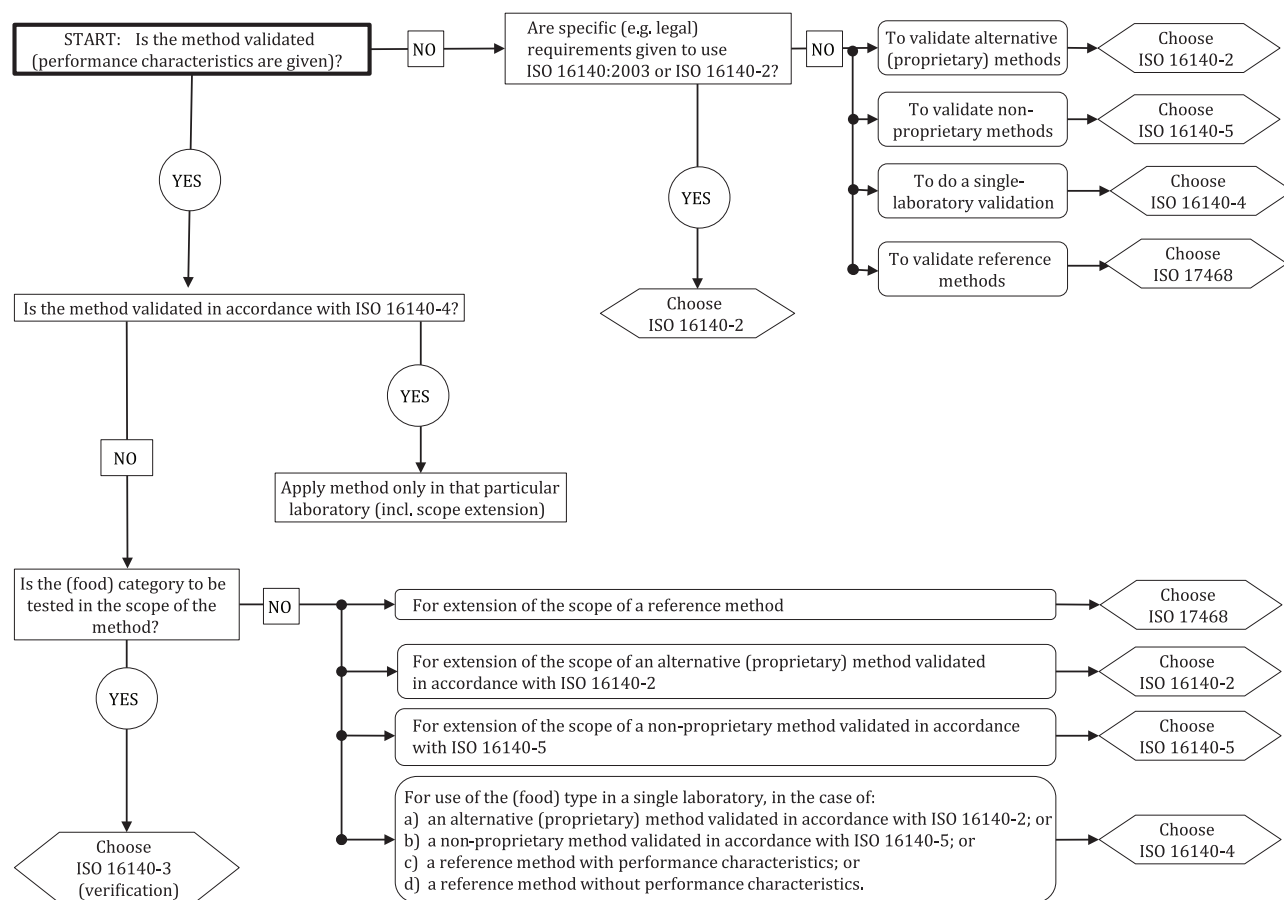


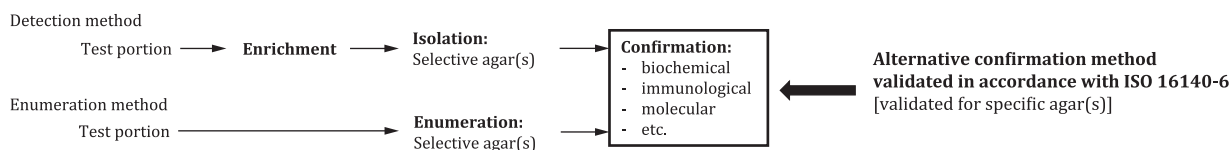
Figure 1 — Flow chart for application of the ISO 16140 series

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 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)] **A2**. 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 **A2** specifies **A2** 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 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).



Reference method



Alternative method validated in accordance with ISO 16140-2

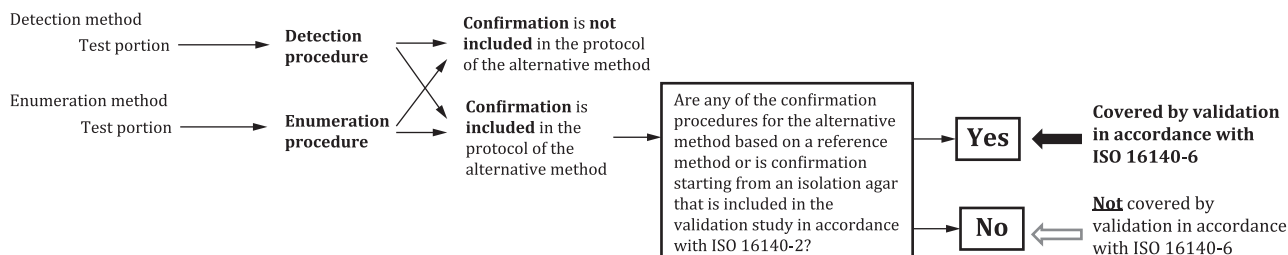


Figure 2 — Use of validated alternative confirmation methods (see ISO 16140-6)

EXAMPLE 1

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

An alternative confirmation method based on ELISA has been validated (in accordance with ISO 16140-6) 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 the 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.

ISO 16140-7 addresses the validation of identification procedures (e.g. molecular identification using multiplex PCR or DNA sequencing or mass spectrometry). ISO 16140-7 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 ISO 16140-7 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.

ISO 16140-7:2024, 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 ISO 16140-7 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.

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 ISO 16140-7 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 ISO 16140-7. If the method is validated by ISO 16140-6 and ISO 16140-7, it can be used for both confirmation and identification purposes. 

0.2 Validation protocols in the ISO 16140 series

An interlaboratory validation study, in accordance with ISO 16140-2, requires at least eight laboratories for quantitative methods and at least ten laboratories for qualitative methods. ISO 16140-5 is intended to be used for interlaboratory studies comprising four to seven laboratories for quantitative methods and four to nine laboratories for qualitative methods. ISO 16140-5 can only be used for non-proprietary methods. [Table 1](#) provides an overview of the different protocols.

Table 1 — Overview of different validation protocols described in the ISO 16140 series

Number of participating laboratories	With reference method	Without reference method
1	This document: — factorial (see 5.1.1 and 5.2.1), or — conventional (see 6.1.1 and 6.2.1)	This document: — factorial (see 5.1.2 and 5.2.2), or — conventional (see 6.1.2 and 6.2.2)
4 to 7 (quantitative method)/ 4 to 9 (qualitative method)	ISO 16140-5: for non-proprietary methods only	ISO 16140-5: for non-proprietary quantitative methods only
≥ 8 (quantitative method)/ ≥ 10 (qualitative method)	ISO 16140-2: for the interlaboratory study part	Not applicable

The aim of this document is to assess the performance of detection or quantification methods within a single laboratory, typically across a number of (food) categories and (food) types. Single-laboratory validation of alternative methods for microbiological confirmation and typing procedures can also be performed under certain conditions: the general principles are the same as those described in ISO 16140-6 for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures (except there is no interlaboratory study). Further information is given in [Annex G](#).

A2 Single-laboratory validation of identification methods of microorganisms can also be performed under certain conditions: the general principles are the same as those described in ISO 16140-7 for the validation of identification methods of microorganisms (except there is no interlaboratory study). Further information is given in Annex I. **A2**

The protocols in this document only validate the method for the particular laboratory. A generalization to other laboratories is not within the scope of these protocols. However, extension to other laboratories is possible if this document is used as the first phase of validation of a reference method, to be followed by an interlaboratory study as described in ISO 17468.

If a reference method is available, the validation of a method is conducted by comparing the alternative method to the reference method. This allows inclusion of naturally contaminated samples in the validation process and thus provides a more realistic picture of the performance of the method. If no reference method is available, the validation process is based on samples with known contamination levels only. This document provides protocols for both situations.

The general principles for single-laboratory validations of detection and quantification methods are the same as those described in ISO 16140-2 for the validation of alternative (proprietary) methods against a reference method. This document cannot be used without ISO 16140-1 or ISO 16140-2, as many definitions and procedures are given in these International Standards. In addition to the validation parameters described in ISO 16140-2, this document describes the calculation of in-house repeatability and in-house reproducibility. Calculation of these parameters is not required if an interlaboratory study is to be conducted after the single-laboratory validation (i.e. if the single-laboratory validation is only the first phase of validation). Reliability of performance parameters obtained with this document is comparable to ISO 16140-2. This also means that the workload associated with the technical protocols for the single laboratory is comparable with the method comparison study of ISO 16140-2.

This document provides two strategies for the single-laboratory method validation of detection and quantification methods. The first strategy is based on a factorial approach while the second strategy uses the conventional approach derived from the protocols of ISO 16140-2. In addition, protocols for the determination of the in-house reproducibility for quantitative methods are described.

The advantages of using a factorial approach, over the conventional approach, are that it takes into account specific conditions that the laboratory encounters during routine testing and provides more information on the factors (technicians, culture media, etc.) that vary within the laboratory across relevant (food) items, while using fewer samples to assess the performance of the method. The factorial approach offers assessment of the precision of quantitative methods. It allows computation of reliable and representative single-laboratory method validation parameters such as in-house reproducibility standard deviation, LOD₅₀ or RLOD values because it provides information on the variability of these values under different measurement conditions. The factorial approach requires fewer test results in order to obtain similar or higher levels of reliability compared to the conventional approach.

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Microbiology of the food chain — Method validation —

Part 4:

Protocol for method validation in a single laboratory

1 Scope

This document specifies the general principles and the technical protocols for single-laboratory validation of methods for microbiology in the food chain. The protocols in this document only validate the method for the laboratory conducting the study.

This document is applicable to single-laboratory validation of:

- methods used in the analysis (detection or quantification) of microorganisms in:
 - products intended for human consumption;
 - products intended for animal feeding;
 - environmental samples in the area of food and feed production, handling;
 - samples from the primary production stage;
- methods for the confirmation or typing of microorganisms. This validation will replace only the confirmation or typing procedure of a specified method (see [Annex G](#));
- A₂ — identification methods of microorganisms (see Annex I). A₂

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

Single-laboratory validation is required if an interlaboratory validation in accordance with ISO 16140-2 is not appropriate. Possible applications are:

- validation of an in-house method;
- method evaluation study in the validation process of a reference method in accordance with ISO 17468;
- extension of the scope of an ISO 16140-2 validated method, e.g. category extension or test portion size;
- modifications of existing methods.

Single-laboratory validation is the second step in the standardization of a reference method (see ISO 17468). It is only applicable to methods that are fully specified with regard to all relevant parameters (including tolerances on temperatures and specifications on culture media) and that have already been optimized.

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 6887 (all parts), *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 16140-1:2016, *Microbiology of the food chain — Method validation — Part 1: Vocabulary*

ISO 16140-2:2016, *Microbiology of the food chain — Method validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method*

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 terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1

block

group of *settings* (3.12) that are conducted in parallel or in a short time interval, and that are used for the same samples

EXAMPLE Block = settings conducted in parallel =

technician “a” + culture medium “b” + temperature “a” + incubation condition “a”

and

technician “b” + culture medium “a” + temperature “b” + incubation condition “b”.

Note 1 to entry: This definition is based on how ISO 3534-3:2013, 3.1.25, defines “block”. In ISO 3534-3:2013, 3.1.25, the definition is more general as it is defining a block as a set of experimental units that are homogenous in some sense. The statistical meaning is the same.

3.2

factor

qualitative or quantitative parameter within the method that can be varied at two or more levels within the limits of the specified method

EXAMPLE Technician.

Note 1 to entry: In this document, only those factors that are in line with the protocol of the method are considered.

3.3

factor level

value of the *factors* (3.2) within the experimental design

EXAMPLE Technician “a”, technician “b”, etc.

Note 1 to entry: In this document, each factor is varied at two factor levels: “a” and “b”.

Note 2 to entry: This definition is based on how ISO 3534-3:2013, 3.1.12, defines “factor level”. In ISO 3534-3:2013, 3.1.12, the definition is more general, but the statistical meaning is the same.

3.4

in-house repeatability

measurement precision under a set of in-house repeatability conditions in a specific laboratory

Note 1 to entry: In-house repeatability conditions include the same measurement procedure, same technicians, same measuring system, same operating conditions, same location and replicate measurements on the same or similar objects over a short period of time in a particular laboratory.

3.5**in-house reproducibility**

measurement precision under a set of in-house reproducibility conditions in a specific laboratory

Note 1 to entry: In-house reproducibility conditions include different technicians, different operating conditions and replicate measurements on the same or similar objects over a longer period of time in a particular laboratory.

3.6**level of detection****LOD_x**

<qualitative methods> measured analyte concentration, obtained by a given measurement procedure, for which the *probability of detection* (3.9) is *x*

EXAMPLE LOD₅₀ is the level of detection for which 50 % of tests give a positive result.

Note 1 to entry: The term “level of detection” is used for qualitative methods in microbiology based on replicate analyses with three different contamination levels of the target analyte in a tested matrix. The replicates are analysed, and the number of positive results is recorded (e.g. 20 %, 70 % and 100 %) respectively at each contamination level. These data are then used to determine the number of cells that would give 50 % positive using a generalized linear model (see ISO 16140-2). This differs from the procedure used for chemical and physical methods for which a “limit of detection” is defined as the lowest quantity of an analyte that can be distinguished from the absence of that analyte with a stated confidence level.

[SOURCE: ISO 16140-1:2016, 2.35, modified — Note 1 to entry has been slightly modified.]

3.7**limit of quantification****LOQ**

limit of determination

<quantitative methods> lowest analyte concentration that can be quantified with an acceptable level of precision and trueness under the conditions of the test

[SOURCE: ISO 16140-1:2016, 2.36]

3.8**orthogonal design**

factorial design, in which for every pair of *factors* (3.2), each combination of *factor levels* (3.3) occurs the same number of times across the possible factor levels

Note 1 to entry: This definition is based on how ISO 3534-3:2013, 3.1.31, defines “orthogonal array”, but for “orthogonal design”, a more general and more theoretical definition is used.

3.9**probability of detection****POD**

proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration

Note 1 to entry: For qualitative methods, POD represents the probability of detection.

[SOURCE: ISO 16140-1:2016, 2.53, modified — Note 1 to entry has been added.]

3.10**relative level of detection****RLOD**

level of detection (3.6) at $P = 0,50$ (LOD₅₀) of the alternative (proprietary) method divided by the level of detection at $P = 0,50$ (LOD₅₀) of the reference method

Note 1 to entry: For purposes of alternative-method acceptance, the derived RLOD is checked with the acceptability limit for conformity.

[SOURCE: ISO 16140-1:2016, 2.61]

3.11 single-laboratory method validation in-house method validation

establishment of the performance characteristics of a method for the one particular laboratory in which the validation is conducted

3.12 setting combination of *factor levels* (3.3)

EXAMPLE Technician “a” + culture medium “b” + temperature “a” + etc.

Note 1 to entry: These conditions can be described by the combination of levels of factors varied within the study.

A₁ 3.13
larger test portion size
measured (volume or mass) representative sample taken from the laboratory sample or test sample for use in the preparation of the initial suspension that is larger than the test portion that has been described in the original method and/or validation document **A₁**

4 General principles of the single-laboratory detection or quantification method validation

4.1 General

A single-laboratory detection or quantification method validation study is the first step in the framework of general method validation and is needed to assess the performance of the method across (food) categories, (food) types and (food) items. The second step in general method validation is an interlaboratory study to assess the performance of the method across laboratories.

A single-laboratory method validation study is used to demonstrate the performance of the method in the laboratory that conducted the study. The results are only valid for that particular laboratory.

A₁ The protocol used to validate a larger test portion size for qualitative methods in a single laboratory shall be as given in Annex H. **A₁**

NOTE [Annex G](#) gives the general principles for single-laboratory validation of alternative methods for microbiological confirmation and typing procedures. **A₂** Annex I gives the general principles for single-laboratory validation of identification methods of microorganisms. **A₂**

This document describes two approaches for single-laboratory method validation:

- a factorial approach, with:
 - performance characteristics derived from ISO 16140-2;
 - an orthogonal, factorial study design (see ISO 3534-3);
 - more routine settings covered and fewer tests required than the conventional approach;
- a conventional approach, with:
 - performance characteristics derived from ISO 16140-2;
 - a stepwise procedure;
 - a study design derived from ISO 16140-2.

Validation protocols are dependent on whether the method is qualitative or quantitative, and on whether a factorial or a conventional approach is chosen. The factorial single-laboratory validation approach can only be used for a fully developed and optimized method. A conventional approach investigates the method for one specific setting (that is, one set of specific conditions under which the method is performed). The main differences in approach for the single-laboratory validation covered in this document are the number of various (food) items and the number of tests required to show that the method performs adequately. Validation of methods with, and without, a reference method is possible with the described protocols.

The scope of the validation protocol shall be determined at the start of the process, e.g. validation of in-house methods, the second step in the validation process in accordance with ISO 17468, extension of the scope of an ISO 16140-2 validated method, modification of existing methods.

For methods that include a PCR-based detection step, an assessment of the performance characteristics for the PCR-based detection step is described in ISO 22118. To ensure the reliable detection of the target organism in the samples tested, the relevant performance parameters of the PCR step should first be assessed (based on ISO 22118), before validation of the complete analytical procedure (e.g. following this document).

The selection of (food) categories and (food) types used in the validation study shall be conducted in accordance with ISO 16140-2:2016, 5.1.3.1. It is recommended that each (food) category relevant to the test method is also considered in the single-laboratory method validation study. Guidance on the selection of (food) categories and (food) types is given in ISO 16140-2:2016, Annex A.

The scope of the validation study, results (tables and calculations) of the different parts and the interpretation of the results, including discrepant results, shall be included in a validation study report.

4.2 Principles of the factorial approach

In a factorial approach, a systematic variation of factors is used to investigate the method performance under a defined range of conditions that are typically encountered in the routine application of the method. Typical factors are the technician or the sample storage, which can vary even within the same laboratory using a given procedure. By investigating the method in a variety of conditions concurrently, the factorial approach allows generalization of the validation to conditions commonly encountered in the laboratory and is not just limited to a single condition.

It is necessary to select four major factors that are expected to reflect the typical variation of conditions encountered in the routine application of the method. A risk analysis of the analytical workflow is recommended for the selection of the factors. Examples of factors are given in [Annex A](#).

The systematic variation of factors ensures that their combined impact on general performance parameters, such as precision and sensitivity, can be derived. This is in contrast to a factorial robustness study, in which the central aim is the detection of specific significant method parameters, so that the performance of the method can be optimized. Compatibility between different factor levels and the impact on precision of non-significant effects are not taken into account in such a study.

Compared to the conventional approach as described in ISO 16140-2, the factorial approach requires a smaller number of (food) items and a smaller number of tests, while allowing for a reliable determination of validation parameters.

4.3 Principles of the conventional approach

The conventional approach principally follows ISO 16140-2. It is conducted in several steps and does not vary factors (see [Table 2](#)). The conventional approach requires more (food) items and test portions to be tested than the factorial approach.

Table 2 — Number of tests required for a method validation per (food) category by the factorial and conventional approach

	Factorial approach	Conventional approach																														
Qualitative method against a reference method	<table><tr><td></td><td>A</td><td>R</td></tr><tr><td>Factorial study (sensitivity + RLOD)</td><td>78</td><td>78</td></tr><tr><td>Inclusivity/exclusivity study^a</td><td>80</td><td>0</td></tr><tr><td>Total number of tests</td><td colspan="2">236</td></tr></table> (see 5.1.1)		A	R	Factorial study (sensitivity + RLOD)	78	78	Inclusivity/exclusivity study ^a	80	0	Total number of tests	236		<table><tr><td></td><td>A</td><td>R</td></tr><tr><td>Sensitivity study</td><td>60</td><td>60</td></tr><tr><td>RLOD study</td><td>30</td><td>30</td></tr><tr><td>Inclusivity/exclusivity study^a</td><td>80</td><td>0</td></tr><tr><td>Total number of tests</td><td colspan="2">260</td></tr></table> (see 6.1.1)		A	R	Sensitivity study	60	60	RLOD study	30	30	Inclusivity/exclusivity study ^a	80	0	Total number of tests	260				
	A	R																														
Factorial study (sensitivity + RLOD)	78	78																														
Inclusivity/exclusivity study ^a	80	0																														
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	A	R																														
Sensitivity study	60	60																														
RLOD study	30	30																														
Inclusivity/exclusivity study ^a	80	0																														
Total number of tests	260																															
Qualitative method without a reference method	<table><tr><td>Factorial study (sensitivity + LOD₅₀)</td><td>256</td></tr><tr><td>Inclusivity/exclusivity study^a</td><td>80</td></tr><tr><td>Total number of tests</td><td>336</td></tr></table> (see 5.1.2)	Factorial study (sensitivity + LOD ₅₀)	256	Inclusivity/exclusivity study ^a	80	Total number of tests	336	<table><tr><td>Specificity</td><td>20</td></tr><tr><td>LOD₅₀ study (LOD₅₀ + sensitivity)</td><td>360</td></tr><tr><td>Inclusivity/exclusivity study^a</td><td>80</td></tr><tr><td>Total number of tests</td><td>460</td></tr></table> (see 6.1.2)	Specificity	20	LOD ₅₀ study (LOD ₅₀ + sensitivity)	360	Inclusivity/exclusivity study ^a	80	Total number of tests	460																
Factorial study (sensitivity + LOD ₅₀)	256																															
Inclusivity/exclusivity study ^a	80																															
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LOD ₅₀ study (LOD ₅₀ + sensitivity)	360																															
Inclusivity/exclusivity study ^a	80																															
Total number of tests	460																															
Quantitative method against a reference method	<table><tr><td></td><td>A</td><td>R</td></tr><tr><td>Factorial study (relative trueness + accuracy profile + in-house precision)</td><td>48</td><td>48</td></tr><tr><td>Inclusivity/exclusivity study^a</td><td>80</td><td>0</td></tr><tr><td>Total number of tests</td><td colspan="2">176</td></tr></table> (see 5.2.1)		A	R	Factorial study (relative trueness + accuracy profile + in-house precision)	48	48	Inclusivity/exclusivity study ^a	80	0	Total number of tests	176		<table><tr><td></td><td>A</td><td>R</td></tr><tr><td>Relative trueness study</td><td>15</td><td>15</td></tr><tr><td>Accuracy profile study</td><td>30</td><td>30</td></tr><tr><td>In-house precision study</td><td>40</td><td>5</td></tr><tr><td>Inclusivity/exclusivity study^a</td><td>80</td><td>0</td></tr><tr><td>Total number of tests</td><td colspan="2">215</td></tr></table> (without LOQ study) (see 6.2.1)		A	R	Relative trueness study	15	15	Accuracy profile study	30	30	In-house precision study	40	5	Inclusivity/exclusivity study ^a	80	0	Total number of tests	215	
	A	R																														
Factorial study (relative trueness + accuracy profile + in-house precision)	48	48																														
Inclusivity/exclusivity study ^a	80	0																														
Total number of tests	176																															
	A	R																														
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Quantitative method without a reference method	<table><tr><td>Factorial study (relative trueness + accuracy profile + in-house precision)</td><td>48</td></tr><tr><td>Inclusivity/exclusivity study^a</td><td>80</td></tr><tr><td>Total number of tests</td><td>128</td></tr></table> (see 5.2.2)	Factorial study (relative trueness + accuracy profile + in-house precision)	48	Inclusivity/exclusivity study ^a	80	Total number of tests	128	<table><tr><td>Relative trueness study</td><td>15</td></tr><tr><td>Accuracy profile study</td><td>30</td></tr><tr><td>In-house precision study</td><td>40</td></tr><tr><td>Inclusivity/exclusivity study^a</td><td>80</td></tr><tr><td>Total number of tests</td><td>165</td></tr></table> (without LOQ study) (see 6.2.2)	Relative trueness study	15	Accuracy profile study	30	In-house precision study	40	Inclusivity/exclusivity study ^a	80	Total number of tests	165														
Factorial study (relative trueness + accuracy profile + in-house precision)	48																															
Inclusivity/exclusivity study ^a	80																															
Total number of tests	128																															
Relative trueness study	15																															
Accuracy profile study	30																															
In-house precision study	40																															
Inclusivity/exclusivity study ^a	80																															
Total number of tests	165																															
Key A: number of tests of the alternative method R: number of tests of the reference method ^a Inclusivity/exclusivity study requires 80 culture strains (130 for <i>Salmonella</i>) and is carried out only once for all approaches irrespective of the number of (food) categories.																																

5 Technical protocol for validation — Factorial approach

5.1 Qualitative methods

5.1.1 Single-laboratory method validation study against a reference method

5.1.1.1 General considerations

The factorial single-laboratory validation can only be used for a fully developed and optimized method. The validation study consists of two parts:

- a factorial, orthogonal comparison study (sensitivity and RLOD);
- an inclusivity/exclusivity study of the alternative method.

See [Annex D](#) for an elaborated example.

5.1.1.2 Factorial, orthogonal method comparison study

5.1.1.2.1 Selection of samples

The method comparison study compares the results obtained by the reference method and that of the alternative method. The study is conducted using naturally and/or artificially contaminated samples: usually, only artificially contaminated samples are used.

The requirements are as follows.

- Twelve different (food) items shall be selected for each (food) category: three (food) types per (food) category shall be selected and four (food) items shall be selected for each (food) type. (Food) items should be representative for the respective (food) type.
- The selection of (food) items shall take into account: background microbiota and food-processing factors, such as heat, pH, freezing, smoking, drying (low a_w); matrix conditions, such as pH value, a_w value, aerobic/anaerobic; special sample preparation requirements, such as high fat content or presence of inhibitors, in accordance with the ISO 6887 series.
- Each (food) item shall be contaminated at a minimum of two levels, consisting of at least the following.
 - A low (fractional) level L_1 : the low level should have fractional recovery by the reference method (fractional recovery at the low level should be between 25 % and 75 % of the number of test portions tested). Ideally, the low level should be close to the theoretical detection level of 0,7 cfu/test portion (e.g. 0,5 cfu/test portion to 0,9 cfu/test portion).
 - A high level L_2 : at the high level (e.g. 5 cfu/test portion to 10 cfu/test portion), 100 % positive results are expected.
- The four (food) items from each (food) type are allocated at random to four different blocks. Each block shall contain three (food) items, each at two contamination levels, from three (food) types.
- Use a different strain per block and/or the same strain subjected to different stress factors [e.g. temperature abuse, acid treatment or chlorination, depending on their relevance for the (food) type]. Where it is not possible to use different strains for each block, the laboratory needs to provide an explanation.
- Points to be considered when selecting strains are provided in ISO 16140-2:2016, Annex E.
- Six (food) items out of the twelve different (food) items shall be tested at zero level L_0 (blank, i.e. no target organism in the test portions).