INTERNATIONAL STANDARD

ISO 16140-3

First edition
2021-01

Microbiology of the food chain — Method validation —

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

Microbiologie de la chaîne alimentaire — Validation des méthodes —

Partie 3: Protocole pour la vérification dans un seul laboratoire de méthodes de référence et de méthodes alternatives validées

Reference number
ISO 16140-3:2021(E)

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Foreword

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

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

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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 six 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.*

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 (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 this document (i.e. 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 17468:2016, 3.5, 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 this document) 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 (qualitative and quantitative) and ISO 16140-5 (quantitative 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.

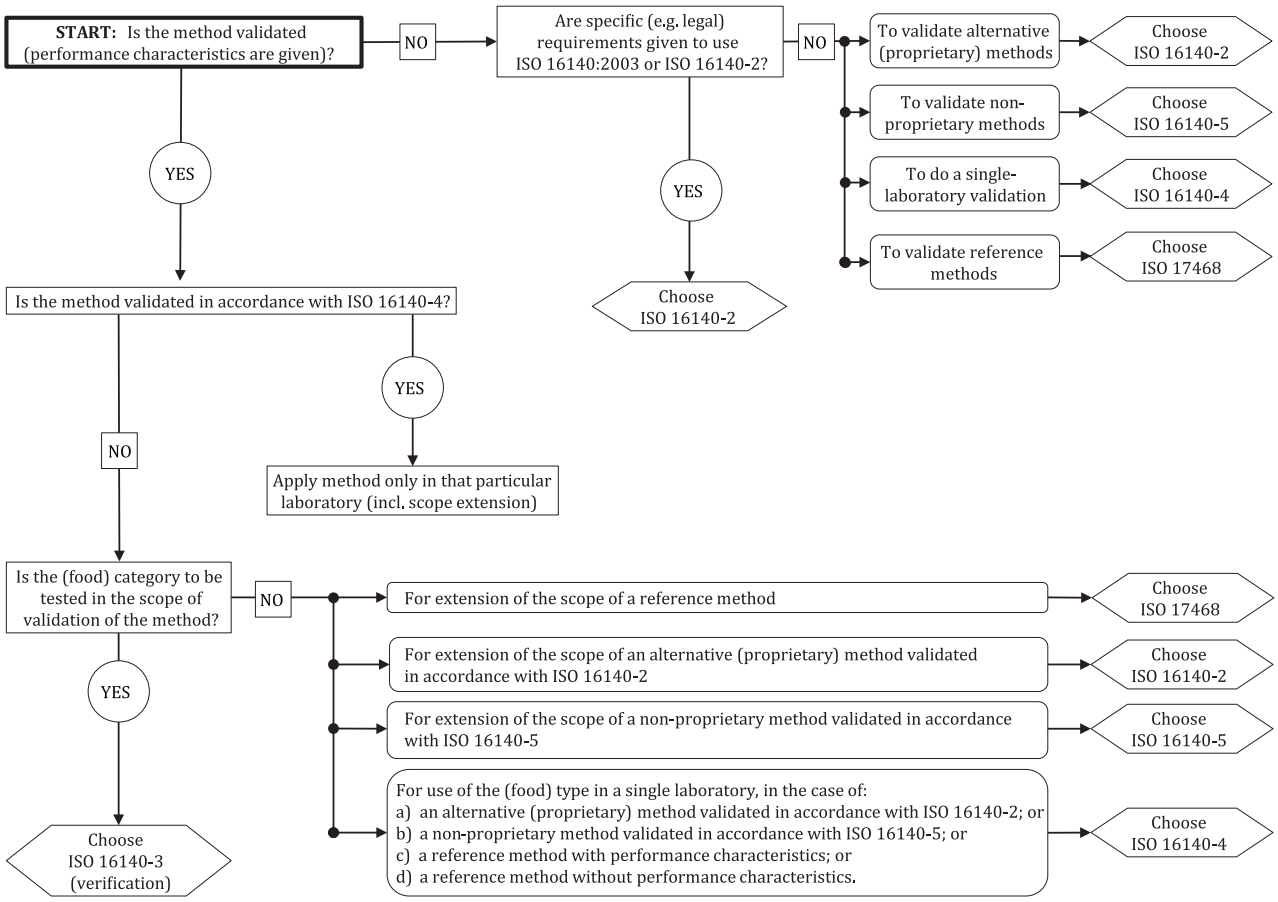

Figure 1 — Flow chart for application of the ISO 16140 series

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.

NOTE 2 The general principle for method verification is that the method to be verified (either alternative or reference) has been validated. However, some reference methods (including ISO or CEN standards) are not yet (fully) validated. For verification of these methods, the protocols are described in Annex F.

ISO 16140-6 is somewhat different from the other parts in the ISO 16140 series in that it relates to a very specific situation where only the confirmation procedure of a method is 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 defines 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).

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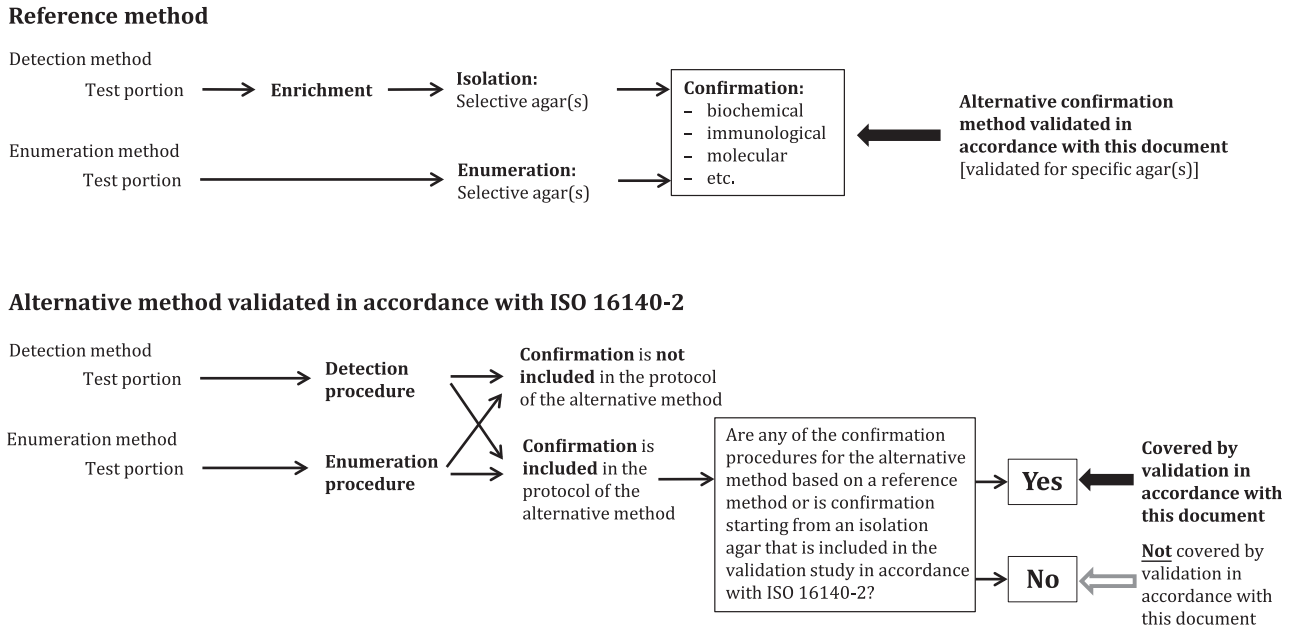

Figure 2 — Use of validated alternative confirmation methods (see ISO 16140-6)

EXAMPLE 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 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.

0.2 Verification versus validation

ISO 16140-1:2016 defines the terms for validation and verification, as follows:

— **validation:** establishment of the performance characteristics of a method and provision of objective evidence that the performance requirements for a specified intended use are fulfilled;

— **verification:** demonstration that a validated method performs, in the user's hands, according to the method's specifications determined in the validation study and is fit for its intended purpose.

NOTE 1 The user's hand means the user laboratory.

Method verification applies to methods that are:

— reference methods, including ISO or CEN standards, that are validated using at least an interlaboratory study;

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NOTE 2 However, some reference methods (including ISO or CEN standards) are not yet (fully) validated. For verification of these methods, the protocols are described in Annex F.

— alternative methods, proprietary or otherwise, when the validation included an interlaboratory study. The method has been validated in accordance with

  — ISO 16140-2 for alternative (proprietary) methods,

  — ISO 16140-5 for non-proprietary methods, or

  — ISO 16140-6 for alternative (proprietary) confirmation and typing methods.

In a validation study, it is not possible to test all existing foods; the diversity and number of samples used in any validation study is limited. In most cases, the validation is based on five different food categories (categories as defined in ISO 16140-1:2016, 2.11, and specified in ISO 16140-2:2016, Annex A). Sometimes the validation is supplemented with additional (other) categories such as pet food and animal feed, environmental samples (food or feed production), and/or primary production samples.

When a minimum of five different food categories are validated, the method is regarded as being validated for a "broad range of foods". And even though only five food categories are tested during the validation study, the method is expected to work for any type of food samples within the 15 food categories in ISO 16140-2: 2016, Annex A. In other words, the "scope" of validation of the method is a broad range of foods, corresponding to the 15 food categories included in ISO 16140-2:2016, Annex A. The scope of validation is important for selecting categories, types and items for the verification.

Two kinds of verification are distinguished:

— The first one is named **implementation verification**. Its purpose is to demonstrate that the user laboratory is able to perform the method correctly. The user laboratory tests a (food) item that was used in the validation study (for qualitative methods) and any (food) item within the scope of validation (for quantitative methods) and then compares the result obtained from the verification to the result obtained from the validation.

— The second one is named **(food) item verification**. Its purpose is to demonstrate that the user laboratory is capable of testing the (food) items it claims in the scope of laboratory application. The user laboratory tests (food) items included in the scope of validation that are commonly examined by the user. As not all (food) items can be included in the verification, the user laboratory is asked to test challenging (food) items.

The scope specifies the (group of) products – categories or types or items – for which the method can be applied. Different scopes are distinguished:

— **scope of the method:** (group of) products – categories or types or items – for which the method is claimed to be applicable.

— **scope of validation:** (group of) products – categories or types or items – for which the applicability of the method is claimed to be validated.

NOTE The claim for the scope of validation is in most cases wider than the products that are included in the validation study itself. For example, in the case of alternative (proprietary) methods validated in accordance with ISO 16140-2:2016: if at least five (≥ 5) food categories – by using a minimum of three different food types per category – were tested in the validation study, then the scope of validation is a "broad range of foods" (so all 15 food categories are claimed in the scope of validation). When less than five (< 5) food categories were tested, the scope of validation is limited to only those food categories included in the validation.

— **scope of laboratory application**: (group of) products – categories or types or items – for which the method is claimed to be used by the laboratory and are within the scope of validation.

The overlap between the different scopes (including an example) is illustrated in Figure 3.

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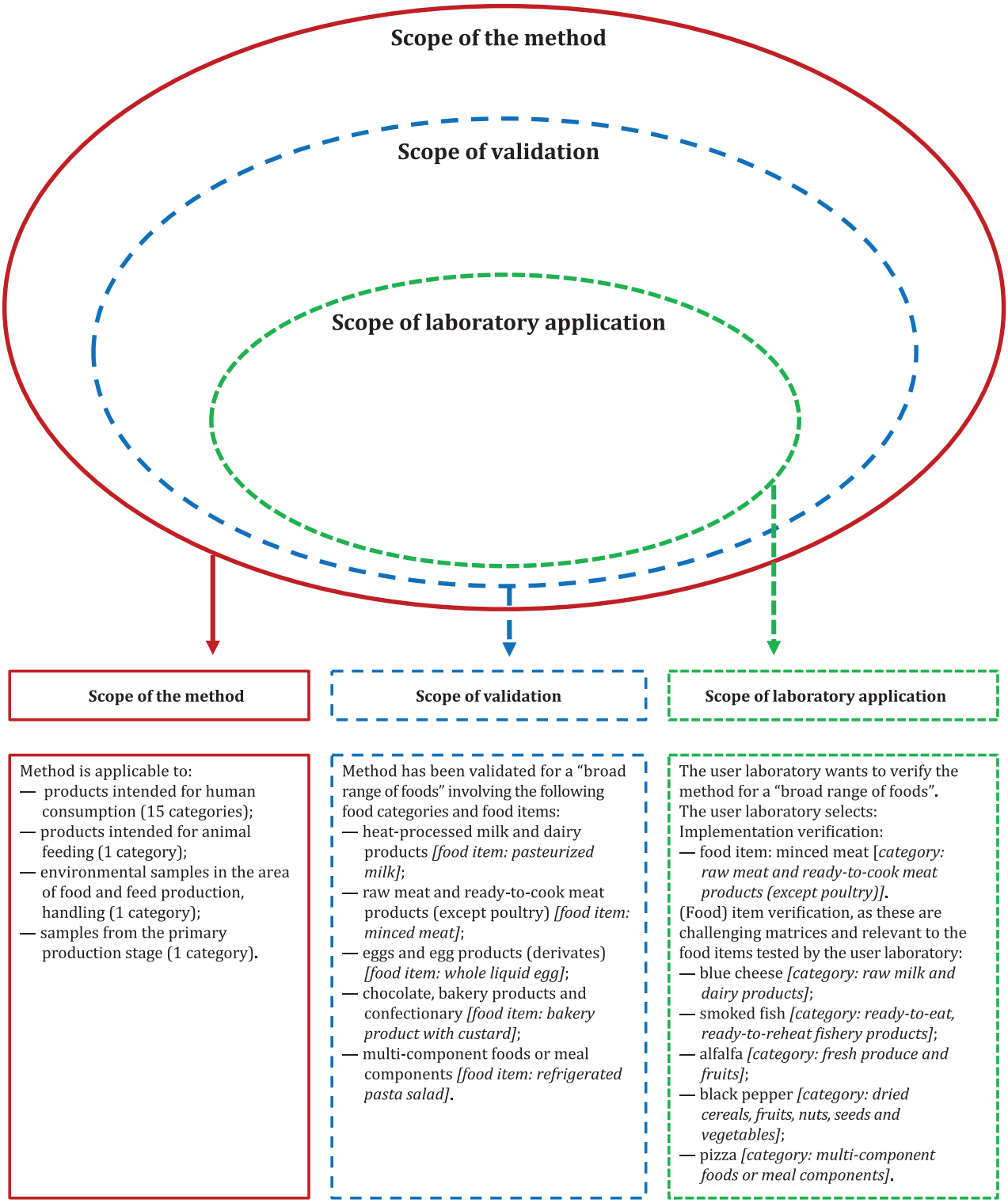

Figure 3 — Overlap between the different scopes (including an example)

At the time of publication of this document (i.e. ISO 16140-3:2021), some reference methods are not yet (fully) validated and would therefore fall outside the scope of this document. It is recognized that standardization organizations (including ISO and CEN committees) will need time to validate their reference methods. Therefore, these non-validated reference methods (including ISO or CEN standards) are verified in a user laboratory according to a specific protocol (see Annex F). This is seen as a temporary situation until these methods are validated by the ISO and/or CEN committees. For further information, see Reference [13].

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In this document:

— “shall” indicates a requirement;

— “should” indicates a recommendation;

— “may” indicates a permission;

— “can” indicates a possibility or a capability.

Information marked “NOTE” is for guidance in understanding or clarifying the associated sentence.

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Microbiology of the food chain — Method validation —

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

1 Scope

This document specifies the protocol for the verification of reference methods and validated alternative methods for implementation in the user laboratory.

This document is applicable to the verification of methods used for the analysis (detection and/or quantification), confirmation and typing 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.

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.

The technical protocols for the verification of validated qualitative methods and validated quantitative methods are described in Clauses 5 and 6. The technical protocol for the verification of validated alternative confirmation and typing methods is described in Clause 7. The protocols for the verification of non-validated reference methods are described in Annex F.

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*

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/

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3.1
alternative confirmation or typing method
confirmation or typing method submitted for validation
method of analysis that confirms or types the same analyte as is confirmed or typed using the corresponding reference method

Note 1 to entry: 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.

[SOURCE: ISO 16140-6:2019, 3.2, modified — Note 2 to entry has been deleted.]

3.2
bias
measurement bias
estimate of a systematic measurement error, or the systematic difference between the quantitative assigned value and the average of measurement replicate results

[SOURCE: ISO 16140-1:2016, 2.9]

3.3
(food) category
group of *(food) types* (3.18) of the same origin

EXAMPLE Food category: heat-processed milk and dairy products. Food type: pasteurized dairy products. Food item: crème brûlée.

Note 1 to entry: The (food) categories are listed in Annex A.

[SOURCE: ISO 16140-1:2016, 2.11, modified — In the term, "(food)" has been added before "category". In the definition, "(food)" has replaced "sample". The example has been modified to align with the terms used in Annex A. Note 1 to entry has been added.]

3.4
estimated bias
eBias
determination of the *bias* (3.2) based on the experimental design described in this document (i.e. ISO 16140-3)

Note 1 to entry: An accurate determination of the bias is not possible as the number of samples tested is small. Therefore, the term "estimated bias" ("eBias") is used in this document.

3.5
estimated LOD_{50}
$eLOD_{50}$
determination of the LOD_{50} (level of detection at 50 % probability of detection) based on the experimental design described in this document

Note 1 to entry: An accurate determination of the LOD_{50} is not possible as the number of samples tested is small in comparison to the number of samples required in ISO 16140-2:2016. Therefore, the term "estimated LOD_{50}" ("$eLOD_{50}$") is used in this document.

Note 2 to entry: LOD_{50} is defined in ISO 16140-1:2016, 2.35.

3.6
exclusivity study
study involving pure *non-target strains* (3.11), which can be potentially cross-reactive, but are not expected to be detected or enumerated by the alternative method

[SOURCE: ISO 16140-1:2016, 2.22]

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3.7
inclusivity study
study involving pure *target strains* (3.15) to be detected or enumerated by the alternative method

[SOURCE: ISO 16140-1:2016, 2.31]

3.8
(food) item
single specified food, feed, environmental or primary production *matrix* (3.10)

EXAMPLE Food category: heat-processed milk and dairy products. Food type: pasteurized dairy products. Food item: crème brûlée.

[SOURCE: ISO 16140-1:2016, 2.34, modified — In the term, “(food)” has been added before “item”. The example has been modified to align with the terms used in Annex A.]

3.9
laboratory sample
sample prepared for sending to the laboratory and intended for inspection or testing

[SOURCE: ISO 6887-1:2017, 3.1]

3.10
matrix
all the components of the sample

[SOURCE: ISO 16140-1:2016, 2.38, modified — In the term, "(product)" has been deleted.]

3.11
non-target strain
strain, defined according to the scope of the reference method that would not reasonably be expected to be confirmed, detected or enumerated by the alternative method

[SOURCE: ISO 16140-1:2016, 2.44, modified — In the definition, “confirmed” has been added to “detected or enumerated”.]

3.12
reference material
material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process

Note 1 to entry: Properties can be quantitative or qualitative, e.g. identity of substances or species.

Note 2 to entry: Uses may include the calibration of a measurement system, assessment of a measurement procedure, assigning values to other materials, and quality control.

[SOURCE: ISO Guide 30:2015, 2.1.1, modified — The original Notes 1 and 4 to entry have been omitted and the notes have been renumbered.]

3.13
scope of laboratory application
categories, matrices, analytes and concentrations for an analytical method that a *user laboratory* (3.19) claims to be capable of satisfactorily testing in its laboratory

Note 1 to entry: A method may have been validated to a broader range (scope) of analytes, matrices and concentrations than the scope that will be claimed by a user laboratory. The scope of laboratory application is ≤ the *scope of validation* (3.14).

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3.14
scope of validation
categories, matrices, analytes and concentrations for which a validated method of analysis can be used satisfactorily

[SOURCE: ISO 16140-1:2016, 2.70, modified — "categories" has been added and "matrices" has been moved before "analytes".]

3.15
target strain
strain, defined according to the scope of the reference method, that is expected to be confirmed, detected or enumerated by the alternative method

[SOURCE: ISO 16140-1:2016, 2.74, modified — In the definition, "confirmed" has been added to "detected or enumerated".]

3.16
test portion
measured (volume or mass) representative sample taken from the *laboratory sample* (3.9) for use in the preparation of the initial suspension

Note 1 to entry: Sometimes preparation of a *test sample* (3.17) from the laboratory sample is required before the test portion is taken, but this is infrequently used in microbiological examinations.

[SOURCE: ISO 6887-1:2017, 3.5, modified — In the Note 1 to entry, "a test sample from" has been added before "the laboratory sample".]

3.17
test sample
sample prepared from the *laboratory sample* (3.9) according to the procedure specified in the test method and from which *test portions* (3.16) are taken

Note 1 to entry: Preparation of the laboratory sample before the test portion is taken is infrequently used in microbiological examinations.

Note 2 to entry: For confirmation and typing methods, the sample is an isolated colony on defined selective or non-selective agar plates.

[SOURCE: ISO 6887-1:2017, 3.4, modified — In the definition, "test method" has replaced "method of test" and Note 2 to entry has been added.]

3.18
(food) type
for a given *(food) category* (3.3), a group of *(food) items* (3.8) processed in a similar way, with similar intrinsic characteristics and a similar microbial ecology

EXAMPLE Food category: heat-processed milk and dairy products. Food type: pasteurized dairy product.

[SOURCE: ISO 16140-1:2016, 2.78, modified — In the term and the definition, "(food)" has been added before "type", "category" and "items".]

3.19
user laboratory
laboratory that implements a validated alternative method and/or a validated reference method

Note 1 to entry: Some reference methods (including ISO or CEN standards) are not yet (fully) validated. For verification of these methods, the protocols are described in Annex F.

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3.20
validation
establishment of the performance characteristics of a method and provision of objective evidence that the performance requirements for a specified intended use are fulfilled

[SOURCE: ISO 16140-1:2016, 2.81]

3.21
verification
demonstration that a validated method performs, in the user's hands, according to the method's specifications determined in the *validation* (3.20) study and is fit for its intended purpose

Note 1 to entry: Some reference methods (including ISO or CEN standards) are not yet (fully) validated. For verification of these methods, the protocols are described in Annex F.

[SOURCE: ISO 16140-1: 2016, 2.83, modified — In the definition, "performs" has replaced "functions" and "intended" has been added before "purpose". Note 1 to entry has been replaced.]

4 General principles of verification of qualitative (detection) methods and quantification methods

4.1 General

The verification of qualitative (detection) methods and quantitative methods is undertaken in two parts:

— implementation verification;

— (food) item verification.

The verification focuses on (food) items that are within the scope of validation and within the scope of laboratory application.

Before performing method verification, the user laboratory shall refer to the validation report(s) published by recognized standards bodies and/or method certification bodies as the source(s) for the scope of validation and to select appropriate (food) items for verification.

Implementation verification occurs before (food) item verification. The technical rules for performing implementation verification and (food) item verification are given in Clause 5 for qualitative methods and Clause 6 for quantitative methods.

For the verification of non-validated reference methods, the user laboratory shall use the technical protocols as described in Annex F.

4.2 Implementation verification

Implementation verification aims to demonstrate the competence of the user laboratory to perform the validated method. This is achieved by its ability to obtain the expected results on a (food) item.

The user laboratory shall:

— review the validation data for the method;

— for qualitative methods:

  — select one (food) item tested during the validation study that belongs within the scope of laboratory application of the user laboratory;

  — when the (food) items included in the validation study do not belong within the scope of laboratory application of the user laboratory, the user laboratory shall obtain one of the (food) items; this is necessary because the limit of detection of the method is affected by the (food) item;

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— use this selected (food) item and the sample size that was used in the validation study to perform the implementation verification;

— for quantitative methods: select any (food) item that belongs within the scope of validation of the method (but not necessarily tested during the validation).

4.3 (Food) item verification

The (food) item verification aims to demonstrate the competence of the user laboratory to perform the validated method with (food) items that are tested in the user laboratory.

The user laboratory shall:

— select one challenging (food) item from each (food) category listed within the scope of validation (see 4.4 for details) that is also a (food) category tested within the scope of laboratory application of the user laboratory;

— use this (food) item and the sample size (or a smaller sample size if routinely used in the user laboratory) used in the validation study to perform the (food) item verification.

4.4 Requirements for implementation verification and (food) item verification

Figures 4, 5 and 6 show the number of (food) items required for implementation verification and (food) item verification under different circumstances. Figures 4 and 5 only refer to food categories. Figure 6 includes other categories.

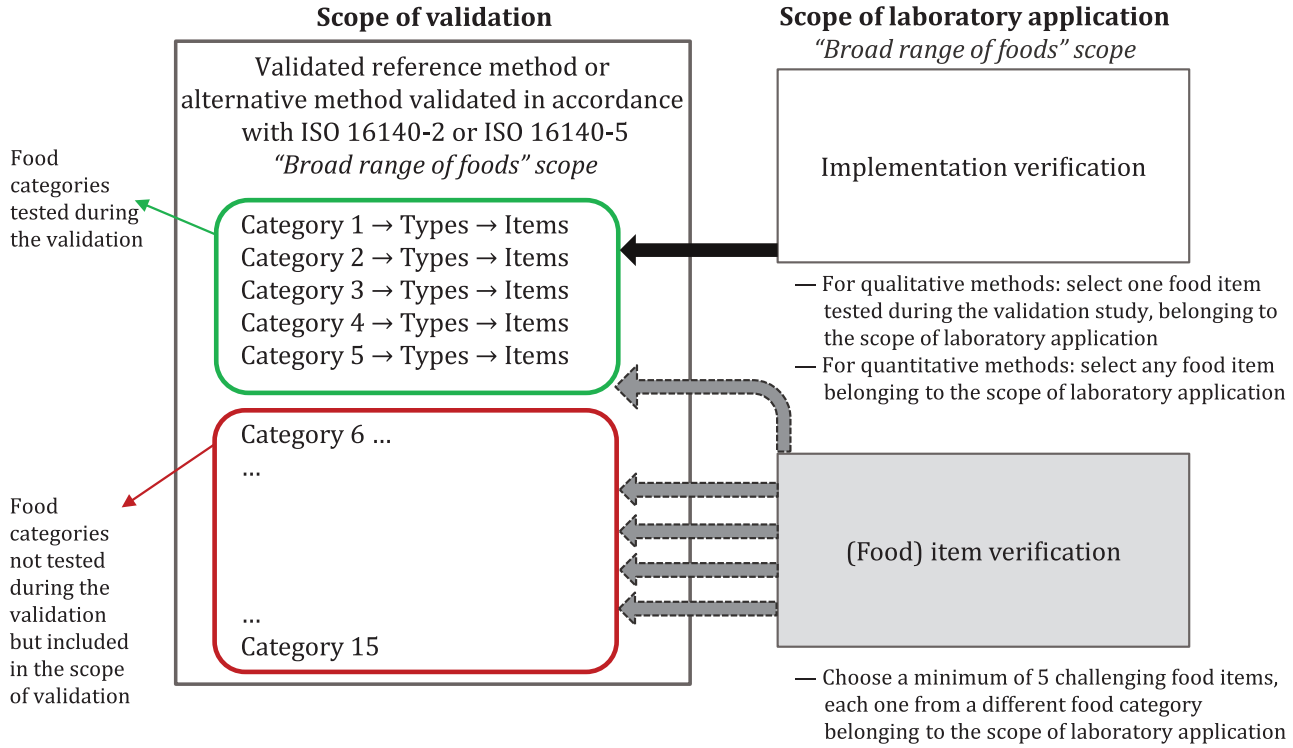

Figure 4 — Food items required when verifying a method for a "broad range of foods" scope

In Figure 4, the selection of the categories for (food) item verification is given only as an example (arrows with dotted outlines). In contrast to implementation verification, there is no obligation to select one food item from a category tested during the validation (in the case of qualitative methods) and four food items from four food categories not tested during the validation. The user laboratory can make its own selection from the 15 food categories.

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The scope of laboratory application shown in Figure 4 is for a "broad range of foods", meaning that the user laboratory has included five or more food categories in its verification study and can therefore claim application for a "broad range of foods". If the scope of laboratory application is smaller than the scope of validation, the user laboratory shall only test food items from its restricted food categories. For example, if the scope of laboratory application is limited to three food categories, then the user laboratory shall verify a minimum of one food item from each of the three food categories.

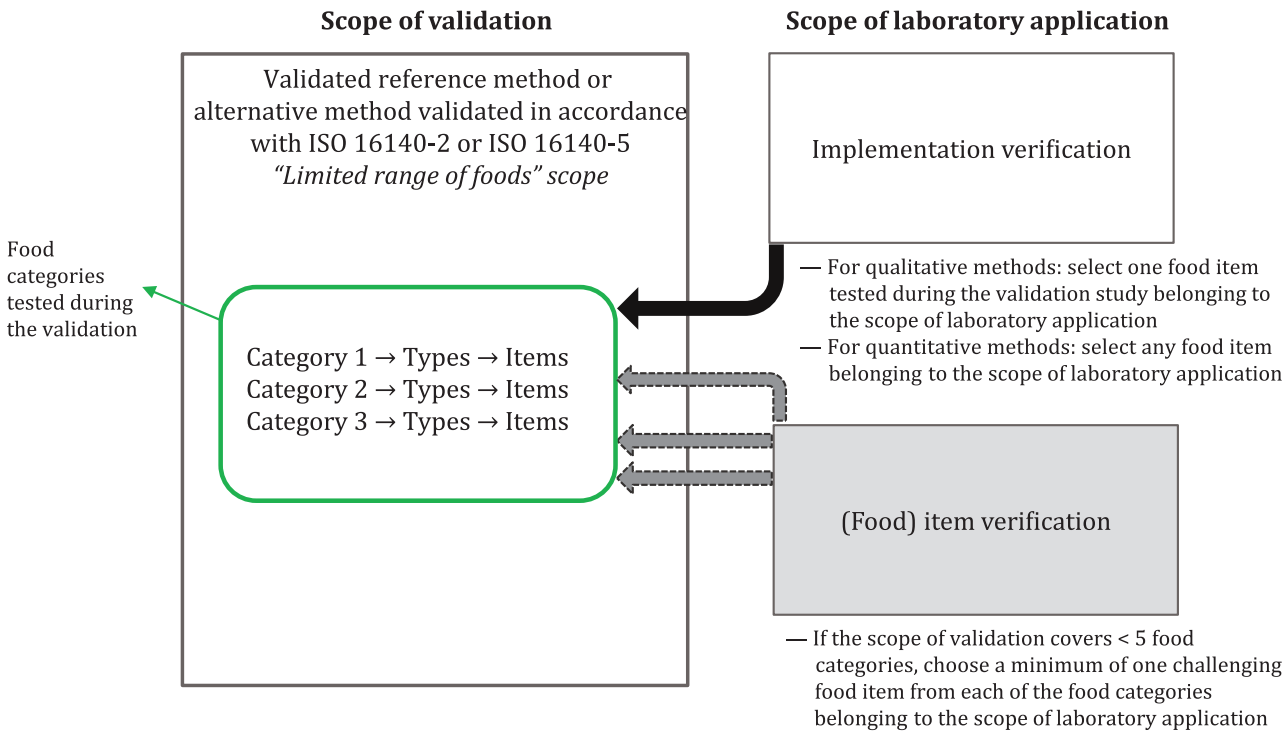

Figure 5 — Food items required when verifying a method for a "limited range of foods" scope

In Figure 5, the selection of the categories for (food) item verification is given only as an example. For the "limited range of foods" scope, a limited number of food categories is tested during the validation. It means the scope of validation is restricted to the tested categories. Consequently, the user laboratory shall not verify the method with categories outside of the limited scope. If the scope of laboratory application is smaller than the scope of validation, the user laboratory shall only test food items from its restricted food categories (arrows with dotted outlines). When the scope of the validation is limited to one category, both implementation verification and (food) item verification shall still be performed, using a minimum of two items from the category: one item for implementation verification and another food item for the (food) item verification.

Figure 6 shows the number of items required when food and other categories are validated and included in the scope of laboratory application. These categories include pet food and animal feed, environmental samples (food or feed production) and primary production samples (PPS). If any of these other categories was included in the validation study and if it is claimed to be within the scope of laboratory application of the user laboratory, then one item from each claimed category shall also be included in the (food) item verification.

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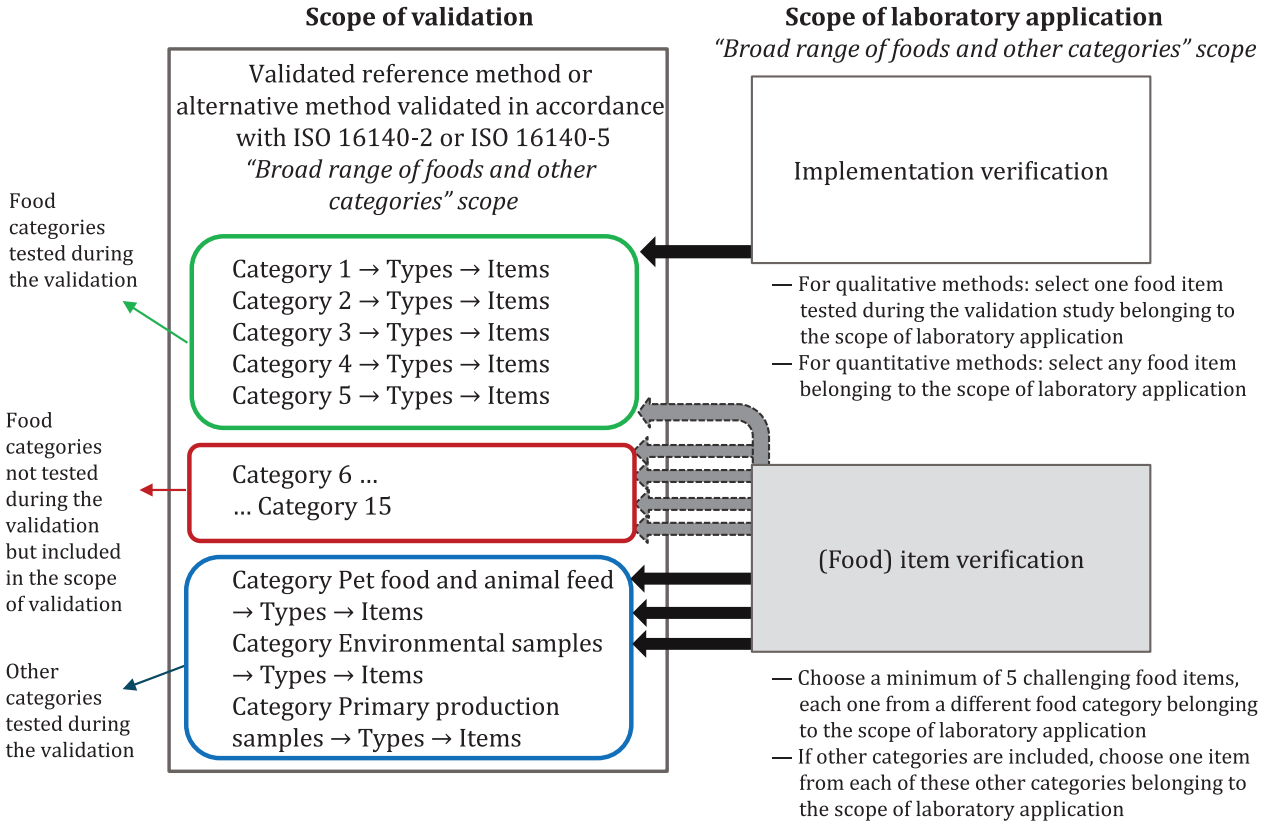

Figure 6 — Items required when verifying a method for a "broad range of foods and other categories" scope

Table 1 summarizes the minimum number of (food) items required for the different scenarios.

Table 1 — Summary of the minimum number of (food) items required for verification

Scope of validation	Number of samples		
	Implementation verification	(Food) item verification	Total
"Broad range of foods" scope ≥ 5 food categories	1	≥ 5	≥ 6
"Limited range of foods" scope N_{food} categories	1	$N_{food} \leq 4$	$(N_{food} + 1) \leq 5$
"Broad range of foods" + other categories (N_{other}) scope	1	≥ 5 food items + 1 item from each of the N_{other} other categories	$\geq 6 + N_{other}$
"Limited range of foods" N_{food} categories + other categories (N_{other}) scope	1	$N_{food} \leq 4$ + 1 item from each of the N_{other} other categories	$(N_{food} + N_{other} + 1) \leq 8$
Other categories (N_{other}) scope only	1	$N_{other} \leq 3$	$(N_{other} + 1) \leq 4$

Table A.1 provides the list of (food) categories and corresponding (food) items. Annex B provides further guidance on the selection of a challenging (food) item from each (food) category for (food) item verification. The (food) items chosen from each (food) category shall be items that reflect the range

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of the laboratory samples received by the user laboratory, and should, as much as possible, be items with components such as natural antimicrobial properties, vitamins, flavours and probiotics that may interfere with the detection of the target microorganism.

4.5 Performance characteristics

Table 2 lists the required performance characteristics for method verification.

Table 2 — Required performance characteristics to be determined for verification

Method	Performance characteristic	Implementation verification	(Food) item verification
Qualitative	Estimated LOD_{50} ($eLOD_{50}$)	✓	✓
Quantitative	Intralaboratory reproducibility standard deviation (S_{IR})	✓	Not applicable
	Estimated bias (eBias)	Not applicable	✓

NOTE 1 The relationship between intralaboratory reproducibility standard deviation (S_{IR}) and ISO 19036 is explained in 6.1.

NOTE 2 For the verification of qualitative method, three protocols are proposed to the user laboratory. The protocol 3 does not require a determination of an $eLOD_{50}$ but to target a concentration of 3 cfu to 5 cfu/test portion.

5 Qualitative methods — Technical protocol for verification

5.1 Estimated LOD_{50} ($eLOD_{50}$) determination

The $eLOD_{50}$ determination is required for both the implementation verification and the (food) item verification.

— The user laboratory first follows one of the selected technical protocols outlined below in its entirety to complete the implementation verification, demonstrating its ability to perform the validated method correctly.

— The user laboratory then applies this same technical protocol for (food) item verification.

During the method verification, run the full procedure of the method as described, including the confirmation procedure (if there is one). A minimum of one individual test portion at each inoculation level needs to be confirmed, and the number of colonies for confirmation may be reduced to one.

5.2 Experimental design

The user laboratory shall select one of the three protocols described in Table 3.

Table 3 — Protocols to determine $eLOD_{50}$ and number of replicates needed per inoculation level

Protocol	Inoculation level of the test portion					
	High level $9 \times LOD_{50}$ / test portion	Intermediate level $3 \times LOD_{50}$ / test portion	Low level $1 \times LOD_{50}$ / test portion	3 cfu to 5 cfu /test portion	Blank	Total number of replicates
1	1	4	4	–	1	10
2	–	3	5	–	1	9
3	–	–	–	7	1	8

NOTE The abbreviation of colony forming units is cfu.

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The choice of protocol depends on the ability of the laboratory to achieve the desired level of contamination of the test portion. Laboratory grown cultures or reference materials can be used for inoculation (see 5.4.1).

— Protocol 1 can be used when there is uncertainty of achieving the desired level of contamination of the test portions. This is relevant when a culture is used, without prior knowledge of the actual level of the inoculum, to inoculate the test portions.

— Protocol 3 can be used when the level of contamination of the inoculum is known, e.g. when using a reference material with known concentration.

— Protocol 2 can be used if the first chosen protocol did not work as anticipated, and the experiment needs to be repeated.

Additional dilutions to that prescribed for any of the protocols can be used to minimize the need to repeat the experiment when inoculation levels do not comply with the requirements or the verification test results cannot be interpreted (see Tables 6 and 8). This is, however, not mandatory, but the decision of the laboratory conducting the experiment.

The protocols shall be performed as follows.

— Prepare cultures of the target microorganisms for inoculating the (food) items.

— At a minimum, prepare the number of test portions of the same (food) item that are required for the selected protocol (see Table 3). Choose a (food) item that should not be naturally contaminated by the target microorganism.

— Inoculate the initial suspensions of the test portions according to the selected protocol in Table 3.

— Determine the level of the target microorganism in the inoculum, at the same time as the test portions are inoculated, by plating on a non-selective medium (e.g. plate count agar) or by performing an MPN (e.g. 3 dilutions × 3 tubes). Enumerate in accordance with ISO 7218.

NOTE If the level of the culture used for inoculation is not known, additional test portions can be inoculated with extra dilutions to ensure that the target levels are included in the verification.

— Analyse the inoculated test portions using the full procedure of the method being verified.

— For protocol 1 and protocol 2: determine the $eLOD_{50}$ using the positive and negative results obtained (see 5.5 for details). For protocol 3, no $eLOD_{50}$ is determined. Instead, the results are evaluated based on the number of positives found out of the seven replicates tested.

See also Annex C for additional guidance and examples.

5.3 Selection of (food) items

One (food) item is required for the implementation verification. Any (food) item that is included in the validation and within the scope of laboratory application can be selected.

For the (food) item verification, the user laboratory shall test a minimum of one (food) item, preferably a challenging one, from each of the required (food) categories. Details on the required number of (food) categories to test according to the scope of validation or to the scope of laboratory application are described in 4.4. Annex B provides guidance on how to choose challenging (food) items.

5.4 Artificial contamination

5.4.1 Selection of strains

Strains can be from:

— culture collections;

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— user laboratory collections;

— reference materials (including commercial reference materials, e.g. a freeze-dried strain with known concentration).

When choosing the test strains, the majority should originate from the (food) categories selected for the verification study and cover the recognized range of the target analyte with respect to the diversity in identification characteristics (e.g. biochemical, serotype, phage type), geographical distribution and incidence (see ISO 16140-2:2016, Annex E).

NOTE Preferably, the strains used in the verification are from sources relevant to the (food) item being verified and a different strain is used for each of the (food) items to be tested.

5.4.2 Inoculation of the test portions

Use the LOD_{50} data of the corresponding (food) categories from the validation study of the method to determine the level of contamination (this should be between one to nine times the LOD_{50}, see Table 3) that will be used to inoculate the test portion. For protocol 3, use 3 cfu to 5 cfu/test portion.

If no corresponding (food) categories are available in the validation study [e.g. for a challenging (food) item tested in (food) item verification], the LOD_{50} value is assumed to be equal to or lower than 1 cfu/test portion.

The following guidance is given as an example of procedures suitable for producing inocula.

— The selected strain is grown in a culture medium under conditions that enable the optimal growth of the strain (e.g. overnight culture). Follow the procedures specified in ISO 11133:2014, 5.4.

  NOTE In this document, overnight culture is specified as 16 h to 24 h of incubation.

— Enumerate the culture on a non-selective medium to determine the concentration of the strain in cfu/ml. It is assumed that this level will be consistently achieved when the same culture conditions are used.

— Repeat the culture under the same conditions and take into account the previously determined concentration to prepare dilutions to cover the range for inoculation. This step is not required if the stability of the strain is known by the user laboratory (e.g. viability after storage at 4 °C overnight).

If the user laboratory works with ready-to-use target strains with known levels (e.g. reference material), the steps described above are not required.

The prepared inoculum is introduced directly into the initial suspension of the individual test portions. After inoculation, the suspension is mixed thoroughly. The use of stressed cultures is recommended but is not required (see ISO 16140-2:2016, Annex C).

Table 4 provides a guide on how to achieve the inoculation levels for each protocol.

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Table 4 — Inoculation levels for each protocol

Protocol	High level 9 × LOD_{50}/test portion	Intermediate level 3 × LOD_{50}/test portion	Low level 1 × LOD_{50}/test portion	3 cfu to 5 cfu/test portion
1	This should be at a maximum of nine times the expected LOD_{50}.	From the high inoculation level, perform a 1:3 dilution to achieve the intermediate level.	From the intermediate inoculation level, perform 1:3 dilution to achieve the low level.	–
2	–	This should be at a maximum of three times the expected LOD_{50}.	From the intermediate inoculation level, perform 1:3 dilution to achieve the low level.	–
3	–	–	–	The level of contamination of the inoculum is known, (e.g. reference material with known concentration).

More dilutions can be tested to ensure that the target levels are included. Use as many dilutions as needed but always take into account a 1:3 dilution factor between the levels.

To determine the inoculum level, enumerate, at the time the test portions are inoculated, the high-level inoculum when using protocol 1, the intermediate-level inoculum when using protocol 2 or the 3 cfu to 5 cfu/test portion inoculum when using protocol 3, in accordance with ISO 7218 (using a non-selective medium, e.g. plate count agar). Take into account the fact that the level of contamination of the inoculum is very low and thus more replicates and/or a larger volume of the inoculum shall be analysed to obtain a valid result in accordance with ISO 7218. The concentration of the low and intermediate levels using protocol 1 or 2 will be calculated using the counts obtained and the dilution factors used.

Alternatively, an MPN determination of the inocula can be performed using a 3 dilutions × 3 tubes MPN approach; the use of a non-selective medium for enrichment (e.g. Brain Heart Infusion broth or Tryptone Soy Broth) is suitable (see also Annex C for more information). In this case, the results are determined according to Table C.1.

EXAMPLE A user laboratory wants to verify the *Salmonella* method (see ISO 6579-1) using protocol 1.

— Based on the LOD_{50} (2,5 cfu/test portion) determined in the validation study, the range of contamination for (food) item A will, theoretically, be 22,5 cfu/test portion, 7,5 cfu/test portion and 2,5 cfu/test portion.

— An overnight culture is prepared. Based on the preliminary enumeration, this is assumed to contain 6×10^8 cfu/ml.

— As the actual count of the new overnight culture is unknown, the user laboratory can use several dilutions to cover the three target levels, using each dilution and test portions required in Table 5. In this case:

  — Dilution A (60 cfu/ml): use 1 ml of 10^{-7} dilution of the overnight culture;

  — Dilution B (20 cfu/ml): use 1 ml of 1:3 dilution of A;

  — Dilution C (6,7 cfu/ml): use 1 ml of 1:3 dilution of B;

  — Dilution D (2,2 cfu/ml): use 1 ml of 1:3 dilution of C;

  — Dilution E (0,7 cfu/ml): use 1 ml of 1:3 dilution of D;

  — Dilution F (0,2 cfu/ml): use 1 ml of 1:3 dilution of E.

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In this particular example, six dilutions are used to make sure the right dilutions are included. In total, 21 inoculated test portions will be examined together with one blank test portion. Only the three relevant levels and the blank level (not inoculated) will be retained for the $eLOD_{50}$ determination.

Table 5 — Example of dilutions and corresponding number of replicates for protocol 1, 2, and 3 using more than the minimum number of required dilutions

Protocol	Dilution A (10^{-7})	Dilution B (1:3 of A)	Dilution C (1:3 of B)	Dilution D (1:3 of C)	Dilution E (1:3 of D)	Dilution F (1:3 of E)
1	1	4	4	4	4	4
2	–	3	5	5	5	5
3	–	–	7	7	7	7

— In this example, the count making the final average contamination level for this dilution is 54 cfu/ml.

— Using this selected dilution (A) for the preparation of the dilutions according to Table 5 will result in dilution B containing 18 cfu/ml; dilution C (1:3 dilution of dilution B) containing 6 cfu/ml and dilution D (1:3 dilution C) containing 2 cfu/ml. Dilutions B, C and D are considered to be the three relevant levels as a 1 ml inoculum of dilution D is closest to the LOD_{50} (2,5 cfu/test portion) of the method. See also Annex C for additional guidance and examples.

5.5 Evaluation of results

5.5.1 Determination of $eLOD_{50}$ using protocol 1

Record the number of positive results obtained at each inoculum level and use Table 6 to determine the $eLOD_{50}$. The blank level shall not produce a positive result. If a positive result is obtained for the blank level, the experiment shall be repeated for all levels.

For the evaluation of the results using protocol 1, the high-level inoculum ($9 \times LOD_{50}$) shall produce only positive results. If negative results are obtained, the experiment shall be repeated for all levels. Some of the MPN combinations indicated as “unreliable MPN result” (see Tables 6 and 7) are very unlikely to occur and the experiment shall therefore be repeated.

When more dilutions are used, the three dilutions, with the low inoculation level closest to the LOD_{50} level, shall be used to evaluate the data according to Table 6.

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Table 6 — Determination of $eLOD_{50}$ based on the number of positive results per level of contamination using protocol 1

High inoculation level targeted 9 × LOD_{50}/ test portion	Intermediate inoculation level targeted 3 × LOD_{50}/ test portion	Low inoculation level targeted 1 × LOD_{50}/ test portion	Blank level	$eLOD_{50}$ cfu/test portion
1/1	4/4	4/4	0/1	< 1,0 × LIL[a]
1/1	4/4	3/4	0/1	= 0,5 × LIL
1/1	4/4	2/4	0/1	= 0,7 × LIL
1/1	4/4	1/4	0/1	= 1,0 × LIL
1/1	4/4	0/4	0/1	= 1,5 × LIL
1/1	3/4	4/4	0/1	= 0,7 × LIL
1/1	3/4	3/4	0/1	= 1,0 × LIL
1/1	3/4	2/4	0/1	= 1,3 × LIL
1/1	3/4	1/4	0/1	= 1,7 × LIL
1/1	3/4	0/4	0/1	= 2,3 × LIL
1/1	2/4	4/4	0/1	= 1,1 × LIL
1/1	2/4	3/4	0/1	= 1,5 × LIL
1/1	2/4	2/4	0/1	= 1,9 × LIL
1/1	2/4	1/4	0/1	= 2,6 × LIL
1/1	2/4	0/4	0/1	= 3,7 × LIL
1/1	1/4	4/4	0/1	Unreliable MPN result[b]
1/1	1/4	3/4	0/1	= 2,1 × LIL
1/1	1/4	2/4	0/1	= 2,8 × LIL
1/1	1/4	1/4	0/1	= 4,0 × LIL
1/1	1/4	0/4	0/1	= 6,3 × LIL
1/1	0/4	4/4	0/1	Unreliable MPN result[b]
1/1	0/4	3/4	0/1	= 3,0 × LIL
1/1	0/4	2/4	0/1	= 4,3 × LIL
1/1	0/4	1/4	0/1	= 6,7 × LIL
1/1	0/4	0/4	0/1	= 14,0 × LIL

[a] LIL = low inoculation level.

[b] Unreliable MPN result: MPN combination is very unlikely to occur. The experiment shall be repeated.

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