
**Microbiology of the food chain —
Method validation —**

Part 3:

**Protocol for the verification of
reference methods and validated
alternative methods in a single
laboratory**

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Microbiologie de la chaîne alimentaire — Validation des méthodes —

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

[ISO 16140-3:2021](#)

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Contents

Page

Foreword	v
Introduction	vi
1 Scope	1
2 Normative references	1
3 Terms and definitions	1
4 General principles of verification of qualitative (detection) methods and quantification methods	5
4.1 General	5
4.2 Implementation verification	5
4.3 (Food) item verification	6
4.4 Requirements for implementation verification and (food) item verification	6
4.5 Performance characteristics	9
5 Qualitative methods — Technical protocol for verification	9
5.1 Estimated LOD ₅₀ (eLOD ₅₀) determination	9
5.2 Experimental design	9
5.3 Selection of (food) items	10
5.4 Artificial contamination	10
5.4.1 Selection of strains	10
5.4.2 Inoculation of the test portions	11
5.5 Evaluation of results	13
5.5.1 Determination of eLOD ₅₀ using protocol 1	13
5.5.2 Determination of eLOD ₅₀ using protocol 2	16
5.5.3 Use of protocol 3	17
5.6 Acceptability limits	18
5.7 Root cause analysis	18
6 Quantitative methods — Technical protocol for verification	19
6.1 Intralaboratory reproducibility standard deviation determination	19
6.1.1 General	19
6.1.2 Experimental design	19
6.1.3 Selection of the (food) item	21
6.1.4 Natural contamination	21
6.1.5 Artificial contamination	21
6.1.6 Evaluation of results	22
6.1.7 Acceptability limit	23
6.1.8 Root cause analysis	24
6.2 Estimated bias (eBias) determination	25
6.2.1 General	25
6.2.2 Experimental design	25
6.2.3 Selection of (food) items	25
6.2.4 Artificial contamination	25
6.2.5 Evaluation of results	27
6.2.6 Acceptability limit	27
6.2.7 Root cause analysis	28
7 Validated alternative confirmation and typing methods — Technical protocol for verification	28
7.1 General	28
7.2 Implementation verification	28
7.3 Experimental design	28
7.3.1 General	28
7.3.2 Strain selection	29
7.4 Evaluation of results	29
7.5 Acceptability limit	30

7.6 Root cause analysis	30
8 Summary of acceptability limits for the verification of validated methods	30
Annex A (informative) Classification of (food) categories and suggested target combinations for verification studies	31
Annex B (informative) Guidance on how to choose challenging (food) item(s) for (food) item verification	45
Annex C (informative) Qualitative method verification — Example	47
Annex D (informative) Quantitative method verification — Example	55
Annex E (informative) Validated alternative confirmation or typing method verification — Examples	60
Annex F (normative) Protocol for the verification of non-validated reference methods in a single laboratory	63
Bibliography	70

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO 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).

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

A list of all parts in the ISO 16140 series can be found on the ISO website.
<a href="https://standards.iteh.ai/catalog/standards/iso.html?part=1&part=2&part=3&part=4&part=5&part=6&part=7&part=8&part=9&part=10&part=11&part=12&part=13&part=14&part=15&part=16&part=17&part=18&part=19&part=20&part=21&part=22&part=23&part=24&part=25&part=26&part=27&part=28&part=29&part=30&part=31&part=32&part=33&part=34&part=35&part=36&part=37&part=38&part=39&part=40&part=41&part=42&part=43&part=44&part=45&part=46&part=47&part=48&part=49&part=50&part=51&part=52&part=53&part=54&part=55&part=56&part=57&part=58&part=59&part=60&part=61&part=62&part=63&part=64&part=65&part=66&part=67&part=68&part=69&part=70&part=71&part=72&part=73&part=74&part=75&part=76&part=77&part=78&part=79&part=80&part=81&part=82&part=83&part=84&part=85&part=86&part=87&part=88&part=89&part=90&part=91&part=92&part=93&part=94&part=95&part=96&part=97&part=98&part=99&part=100&part=101&part=102&part=103&part=104&part=105&part=106&part=107&part=108&part=109&part=110&part=111&part=112&part=113&part=114&part=115&part=116&part=117&part=118&part=119&part=120&part=121&part=122&part=123&part=124&part=125&part=126&part=127&part=128&part=129&part=130&part=131&part=132&part=133&part=134&part=135&part=136&part=137&part=138&part=139&part=140&part=141&part=142&part=143&part=144&part=145&part=146&part=147&part=148&part=149&part=150&part=151&part=152&part=153&part=154&part=155&part=156&part=157&part=158&part=159&part=160&part=161&part=162&part=163&part=164&part=165&part=166&part=167&part=168&part=169&part=170&part=171&part=172&part=173&part=174&part=175&part=176&part=177&part=178&part=179&part=180&part=181&part=182&part=183&part=184&part=185&part=186&part=187&part=188&part=189&part=190&part=191&part=192&part=193&part=194&part=195&part=196&part=197&part=198&part=199&part=200&part=201&part=202&part=203&part=204&part=205&part=206&part=207&part=208&part=209&part=210&part=211&part=212&part=213&part=214&part=215&part=216&part=217&part=218&part=219&part=220&part=221&part=222&part=223&part=224&part=225&part=226&part=227&part=228&part=229&part=230&part=231&part=232&part=233&part=234&part=235&part=236&part=237&part=238&part=239&part=240&part=241&part=242&part=243&part=244&part=245&part=246&part=247&part=248&part=249&part=250&part=251&part=252&part=253&part=254&part=255&part=256&part=257&part=258&part=259&part=260&part=261&part=262&part=263&part=264&part=265&part=266&part=267&part=268&part=269&part=270&part=271&part=272&part=273&part=274&part=275&part=276&part=277&part=278&part=279&part=280&part=281&part=282&part=283&part=284&part=285&part=286&part=287&part=288&part=289&part=290&part=291&part=292&part=293&part=294&part=295&part=296&part=297&part=298&part=299&part=300&part=301&part=302&part=303&part=304&part=305&part=306&part=307&part=308&part=309&part=310&part=311&part=312&part=313&part=314&part=315&part=316&part=317&part=318&part=319&part=320&part=321&part=322&part=323&part=324&part=325&part=326&part=327&part=328&part=329&part=330&part=331&part=332&part=333&part=334&part=335&part=336&part=337&part=338&part=339&part=340&part=341&part=342&part=343&part=344&part=345&part=346&part=347&part=348&part=349&part=350&part=351&part=352&part=353&part=354&part=355&part=356&part=357&part=358&part=359&part=360&part=361&part=362&part=363&part=364&part=365&part=366&part=367&part=368&part=369&part=370&part=371&part=372&part=373&part=374&part=375&part=376&part=377&part=378&part=379&part=380&part=381&part=382&part=383&part=384&part=385&part=386&part=387&part=388&part=389&part=390&part=391&part=392&part=393&part=394&part=395&part=396&part=397&part=398&part=399&part=400&part=401&part=402&part=403&part=404&part=405&part=406&part=407&part=408&part=409&part=410&part=411&part=412&part=413&part=414&part=415&part=416&part=417&part=418&part=419&part=420&part=421&part=422&part=423&part=424&part=425&part=426&part=427&part=428&part=429&part=430&part=431&part=432&part=433&part=434&part=435&part=436&part=437&part=438&part=439&part=440&part=441&part=442&part=443&part=444&part=445&part=446&part=447&part=448&part=449&part=450&part=451&part=452&part=453&part=454&part=455&part=456&part=457&part=458&part=459&part=460&part=461&part=462&part=463&part=464&part=465&part=466&part=467&part=468&part=469&part=470&part=471&part=472&part=473&part=474&part=475&part=476&part=477&part=478&part=479&part=480&part=481&part=482&part=483&part=484&part=485&part=486&part=487&part=488&part=489&part=490&part=491&part=492&part=493&part=494&part=495&part=496&part=497&part=498&part=499&part=500&part=501&part=502&part=503&part=504&part=505&part=506&part=507&part=508&part=509&part=510&part=511&part=512&part=513&part=514&part=515&part=516&part=517&part=518&part=519&part=520&part=521&part=522&part=523&part=524&part=525&part=526&part=527&part=528&part=529&part=530&part=531&part=532&part=533&part=534&part=535&part=536&part=537&part=538&part=539&part=540&part=541&part=542&part=543&part=544&part=545&part=546&part=547&part=548&part=549&part=550&part=551&part=552&part=553&part=554&part=555&part=556&part=557&part=558&part=559&part=5510&part=551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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.

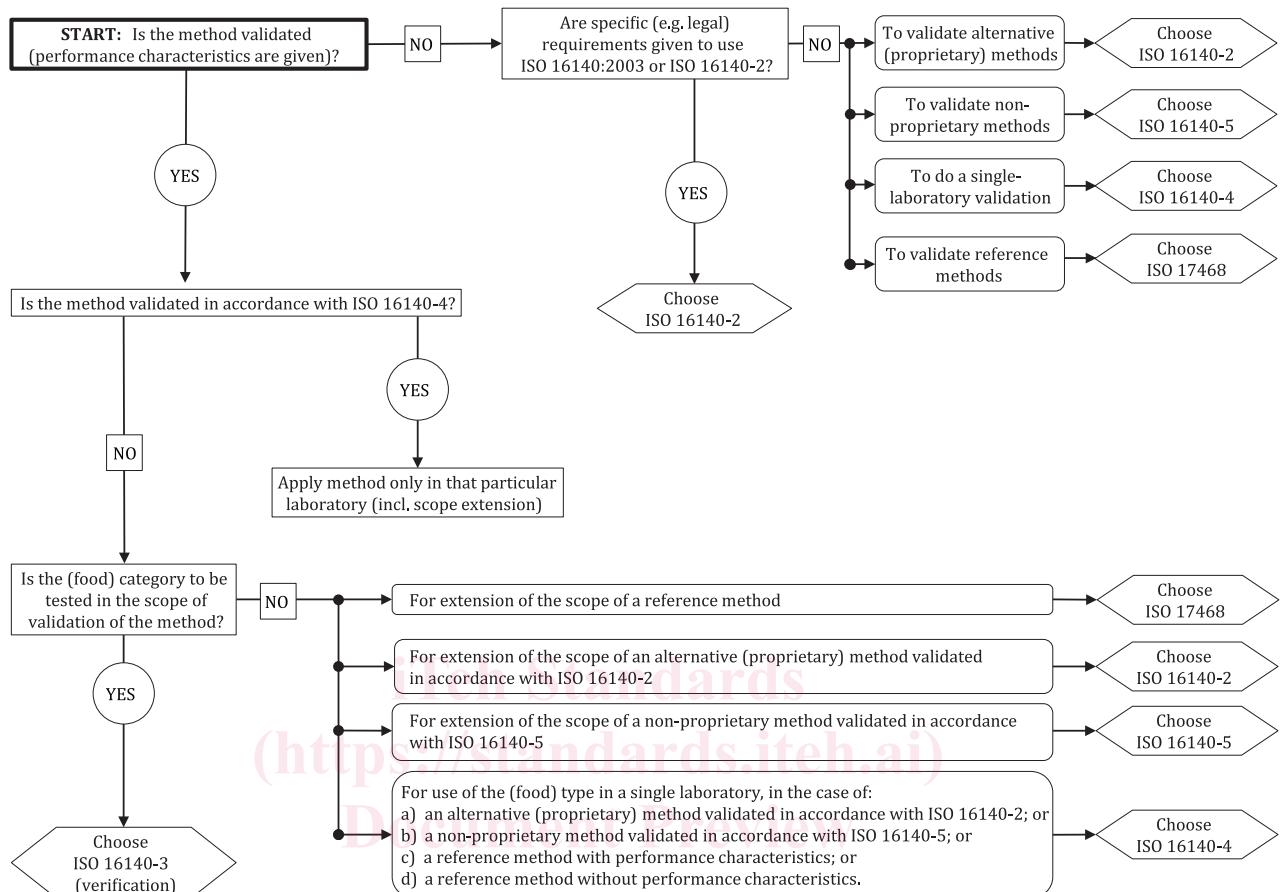
A reference method is defined in ISO 16140-1:2016, 2.59, as an “internationally recognized and widely accepted method”. The note to entry clarifies that “these are ISO standards and standards jointly published by ISO and CEN or other regional/national standards of equivalent standing”.

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.

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.

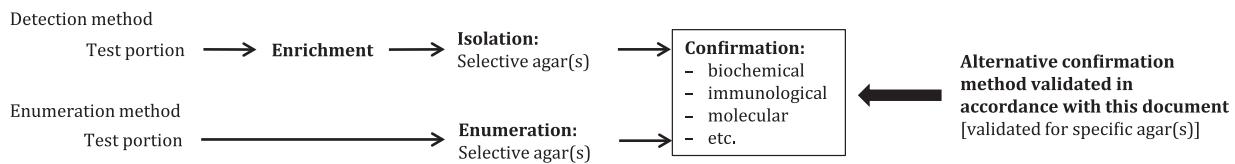
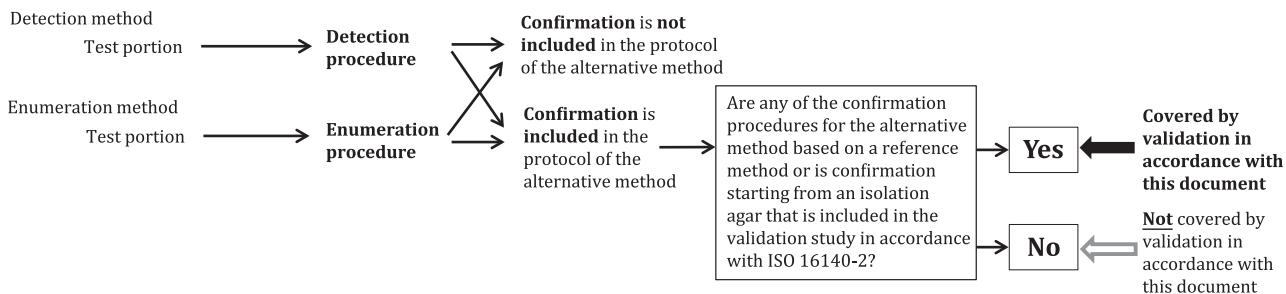


<https://standards.iteh.ai> **Figure 1 — Flow chart for application of the ISO 16140 series** ISO 16140-3:2021

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).

Reference method**Alternative method validated in accordance with ISO 16140-2****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 <https://standards.iteh.ai/catalog/standards/iso/48baa79a-a57f-4d5a-b73f-fda5fd4254e2/iso-16140-3-2021>
- 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;

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](#).

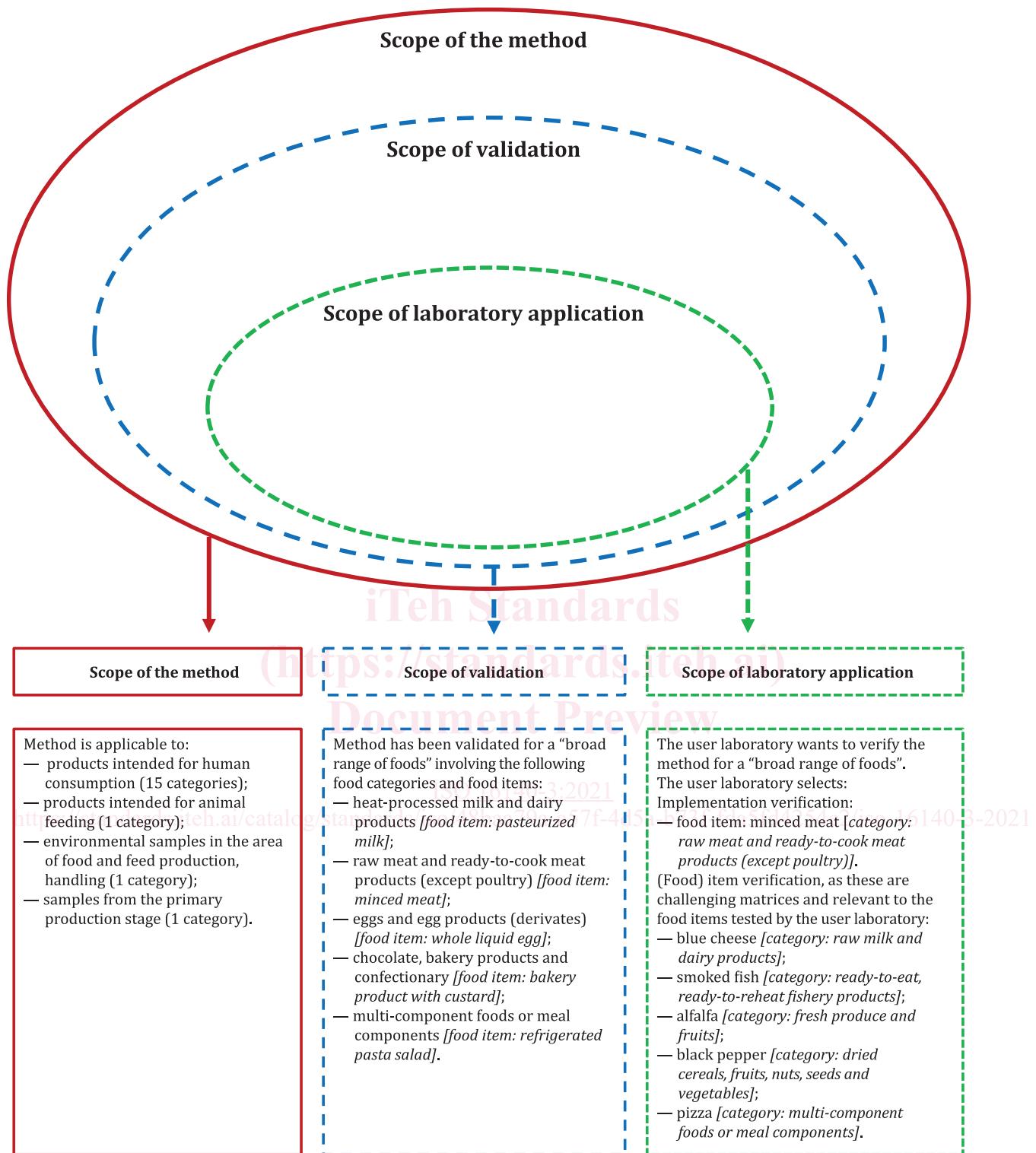


Figure 3 — Overlap between the different scopes (including an example)

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].

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.

iTeh Standards (<https://standards.iteh.ai>) Document Preview

[ISO 16140-3:2021](#)

<https://standards.iteh.ai/catalog/standards/iso/48baa79a-a57f-4d5a-b73f-fda5fd4254e2/iso-16140-3-2021>

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](#).

<https://standards.iec.ch/catalog/standard/iso/16140-3-2021/16140-3-2021-1-b73f-fda5fd4254e2/iso-16140-3-2021>

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/>

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

[ISO 16140-3:2021](#)

eBias <https://standards.iteh.ai/catalog/standards/iso/48baa79a-a57f-4d5a-b73f-fda5fd4254e2/iso-16140-3-2021>
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 LOD₅₀

eLOD₅₀

determination of the LOD₅₀ (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 LOD₅₀ 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 LOD₅₀” (“eLOD₅₀”) is used in this document.

Note 2 to entry: LOD₅₀ 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]