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Microbiology of the food chain — Method validation —

Part 5:
Protocol for factorial interlaboratory validation of non-proprietary methods

Microbiologie de la chaîne alimentaire — Validation des méthodes —

Partie 5: Protocole pour la validation interlaboratoires de méthodes non propriétaires par plan factoriel

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Contents

Foreword **5**
Introduction **6**
1 Scope **9**
2 Normative references **9**
3 Terms and definitions **9**
4 General principles for the factorial interlaboratory validation of non-proprietary methods **10**
4.1 General 10
4.2 Validation against a reference method 11
4.3 Validation without reference method 11
5 Qualitative methods - Technical protocol for factorial interlaboratory validation **11**
5.1 In-house validation study 11
5.2 Interlaboratory study 12
5.2.1 General considerations 12
5.2.2 Measurement protocol 12
5.2.3 Experimental design 14
5.3 Calculations and summary of data 14
5.4 Interpretation of data 16
5.4.1 Paired study 16
5.4.2 Unpaired study 16
5.4.3 Analysis of factorial effects 17
6 Quantitative methods - Technical protocol for factorial interlaboratory validation **17**
6.1 In-house validation study 17
6.2 Interlaboratory study 18
6.2.1 General considerations 18
6.2.3 Measurement protocol 18
6.2.3 Experimental design 19
6.3 Calculations and summary of data 19
6.4 Precision study without a reference value 22
Annex A (normative) — List of factors for factorial study design **23**
Annex B (informative) — Example of a factorial interlaboratory study for a quantitative method **25**
B.1 Study design 25
B.2 Calculations and summary of data 27
B.2.1 Summary of the results of laboratories 1 to 5 of the interlaboratory study 27
B.2.2 Precision data 28
B.2.3 Accuracy profile 30
Annex C (informative) — Example of a factorial interlaboratory study for a qualitative method **34**
Bibliography **42**

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).

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For an explanation on 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 the following URL: www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products,* Subcommittee SC 9, *Microbiology* (Working Group WG 3, *Method validation).*

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

Introduction

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

— *Part 1: Vocabulary*

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

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

— *Part 4: Protocol for single-laboratory (in-house) method validation*

— *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, *Microbiology of the food chain — Technical requirements and guidance on establishment or revision of a standardized reference method*[2], is a closely linked International Standard. This International Standard, which establishes technical rules for the development and validation of standardized methods, is intended for the development of standardized methods by ISO/TC 34, *Food products,* Subcommittee SC 9, *Microbiology* and CEN/TC 275/WG 6, *Microbiology of the food chain.*

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

— The first stage is the validation of the method. This is either conducted in several laboratories (parts 2 and 5 of ISO 16140) or in one laboratory (part 4 of ISO 16140).

— The second stage is method verification, where a laboratory demonstrates that it can satisfactorily perform a validated method. This is described in part 3 of ISO 16140 (method verification). In part 3, a separation is made between verification of (food) items that are included in the validation study and (food) items that are not tested in the validation study but belong within the scope of validation.

NOTE 1 Standardized reference methods (with and without published validation data) only require verification before implementation in the laboratory.

NOTE 2 In this part of ISO 16140, the words 'category', 'type' and 'item' are sometimes combined with 'food' to improve the readability of this document. However, the word 'food' is interchangeable with 'feed' and the other areas of the food chain as mentioned in the Scope of ISO 16140-5.

Part 4 of ISO 16140 addresses validation within a single laboratory. The results are therefore only valid in the laboratory which conducted the study. In this case, verification (part 3 of ISO 16140) is not required.

Part 5 of ISO 16140 describes protocols for situations where a more rapid validation is required or when the method to be validated is highly specialised, and, the number of participating laboratories required by ISO 16140-2 cannot be reached.

The flow chart in Figure 1 gives an overview of the links between the different parts mentioned above. It also guides the users in selecting the right part of the ISO 16140 series, taking into account the purpose of the study and the remarks given above. For this, it is important to distinguish between 'reference method' and 'standardized reference method'. A reference method is an internationally recognized and widely accepted method (term 2.59 of ISO 16140-1:2016) and a standardized reference method is a reference method described in a standard (term 3.5 of ISO 17468:2016). In the ISO 16140 series, reference method includes standardized reference method. The flow diagram acknowledges that published validation data may not be available for some standardized reference methods.

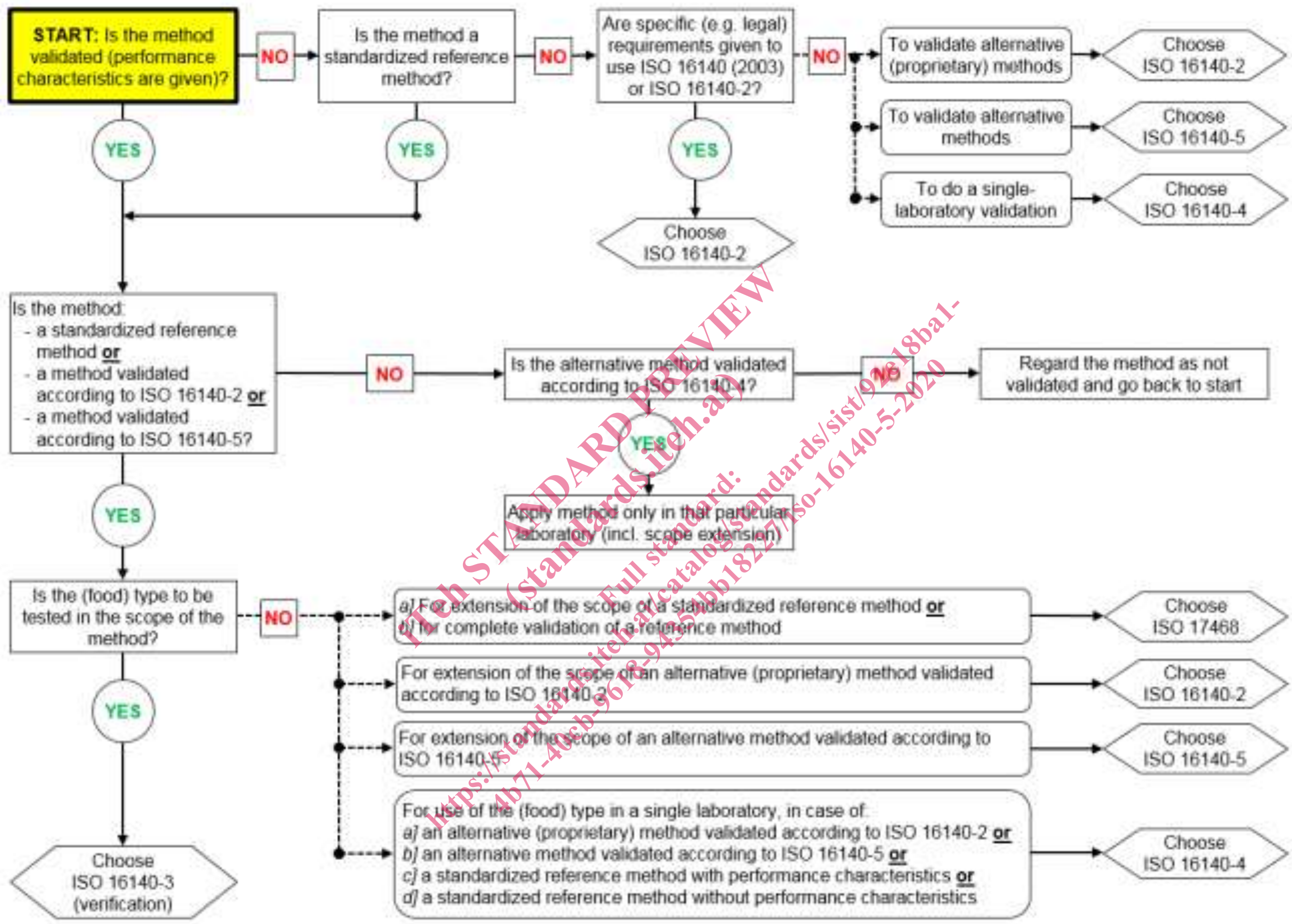
START: Is the method validated (performance characteristics are given)?
NO
Is the method a standardized reference method?
NO
Are specific (e.g. legal) requirements given to use ISO 16140 (2003) or ISO 16140-2?
NO
To validate alternative (proprietary) methods
Choose ISO 16140-2
To validate alternative methods
Choose ISO 16140-5
To do a single-laboratory validation
Choose ISO 16140-4
YES
YES
YES
Choose ISO 16140-2
Is the method:
- a standardized reference method or
- a method validated according to ISO 16140-2 or
- a method validated according to ISO 16140-5?
NO
Is the alternative method validated according to ISO 16140-4?
NO
Regard the method as not validated and go back to start
YES
Apply method only in that particular laboratory (incl. scope extension)
YES
Is the (food) type to be tested in the scope of the method?
NO
a) For extension of the scope of a standardized reference method or
b) for complete validation of a reference method
Choose ISO 17468
For extension of the scope of an alternative (proprietary) method validated according to ISO 16140-2
Choose ISO 16140-2
For extension of the scope of an alternative method validated according to ISO 16140-5
Choose ISO 16140-5
For use of the (food) type in a single laboratory, in case of:
a) an alternative (proprietary) method validated according to ISO 16140-2 or
b) an alternative method validated according to ISO 16140-5 or
c) a standardized reference method with performance characteristics or
d) a standardized reference method without performance characteristics
Choose ISO 16140-4
YES
Choose ISO 16140-3 (verification)

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

Part 6 of ISO 16140, 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 validated. The confirmation procedure advances a suspected (presumptive) result to a confirmed positive result. The typing of pure strains (e.g. serotyping of *Salmonella*) is included in part 6 of ISO 16140.

An interlaboratory study, according to ISO 16140-2 (proprietary methods), requires at least 8 laboratories for quantitative methods and 10 laboratories for qualitative methods. ISO 16140-5 is intended to be used for interlaboratory studies comprising 4-7 laboratories for quantitative methods and 4-9 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 ISO 16140

Number of laboratories	With reference method	Without reference method
1	Part 4 of ISO 16140: factorial or conventional	Part 4 of ISO 16140: factorial or conventional
4 to 7 (quantitative method)/ 4 to 9 (qualitative method)	Part 5 of ISO 16140: for non-proprietary methods only	Part 5 of ISO 16140: for non-proprietary methods only
≥ 8 (quantitative method)/ ≥ 10 (qualitative method)	Part 2 of ISO 16140 (for the interlaboratory study part)	Not available

This part of ISO 16140 provides a protocol that addresses the special case where the number of laboratories (8 for quantitative methods and 10 for qualitative methods) required in an interlaboratory validation of a method by ISO 16140-2 cannot be achieved. The protocol allows a method validation based on only 4 laboratories. It applies, for example, in situations where there is an urgent need for a validated method but the protocols of ISO 16140-2 take too long to complete. This part of ISO 16140 also addresses the problem of method validation of highly specialised methods, for which only a few laboratories might be available for a validation study.

In order to reduce the number of required laboratories to 4 while maintaining the generalizability of the validation to all laboratories, the protocol is based on a factorial experimental design. In the factorial design, several factors such as the technician or culture medium are altered simultaneously and the method is used in a range of different factor settings. This approach allows a more detailed analysis of the precision parameters of the method while, at the same time, requiring a smaller number of laboratories and tests.

Microbiology of the food chain — Method validation — Part 5: Protocol for factorial interlaboratory validation of non-proprietary methods

1 Scope

This part of ISO 16140 describes the general principle and the technical protocol (based on orthogonal, factorial studies) for the validation of non-proprietary methods for microbiology of the food chain.

This part of ISO 16140 is applicable to the validation of non-proprietary 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, and

— samples from the primary production stage.

This part of ISO 16140 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.

This part of ISO 16140 only applies to the validation of methods that are fully specified with regard to all relevant parameters (including tolerances on temperatures and specifications on culture media) and which have already been optimised.

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 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:

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

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

3.1
block
group of settings which have to be conducted in parallel or in a short time interval, and which are used for the same samples

EXAMPLE Block = settings conducted in parallel =
Technician 'a' + culture medium 'b' + temperature 'a' + incubator 'a'
AND
Technician 'b' + culture medium 'a' + temperature 'b' + incubator 'b'

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 part of ISO 16140, only those factors that are in line with the prescription of the method are considered.

3.3
factor level
value of the factors within the experimental design

EXAMPLE Technician 'a', technician 'b', etc.

Note 1 to entry: In this part of ISO 16140, each factor is varied at two factor levels 'a' and 'b'.

3.4
setting
combination of factor levels

EXAMPLE Technician 'a' + culture media 'b' + temperature 'a' + etc.

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

4 General principles for the factorial interlaboratory validation of non-proprietary methods

4.1 General

This part of ISO 16140 uses a protocol based on orthogonal, factorial studies. A high certainty of the determined method validation parameters is obtained, by focussing on suitable factors (e.g. technician, culture media, sample preparation, temperature, duration) that can influence the test result. This allows the number of required laboratories to be reduced to a minimum of 4. General concepts and considerations given in ISO 16140-2 shall be used, unless explicitly excluded. The validation can be conducted against a reference method or by the use of appropriate reference materials.

This validation protocol can be used in different ways:

— **For all laboratories**: if 4 laboratories can be considered a 'random sample' of independent laboratories from different organizations, the test method can be considered to be validated for all laboratories in that accurate and precise measurements are to be expected from any laboratory.

— **For laboratories within an organization**: if 4 laboratories can be considered a 'random sample' of independent laboratories from one organization, the test method can be considered to be validated

for this organization in that accurate and precise measurements are to be expected from any laboratory in the organization.

4.2 Validation against a reference method

The validation protocol comprises two phases:

— an in-house validation study of the non-proprietary method against the reference method carried out in the organizing laboratory;

— an interlaboratory study of the non-proprietary method against the reference method carried out in different laboratories.

The technical protocol for performing the in-house validation study and the interlaboratory study are given in Clause 5 and 6, depending upon whether the test method is qualitative or quantitative in nature.

The selected factors for the factorial interlaboratory study should be relevant to both the reference and the alternative method.

4.3 Validation without reference method

The validation protocol comprises two phases:

— an in-house validation study of the non-proprietary method against the reference value carried out in the organizing laboratory;

— an interlaboratory study of the non-proprietary method against the reference value carried out in different laboratories.

The technical protocol for performing the in-house validation study and the interlaboratory study for quantitative test methods are given in Clause 6.

Qualitative test methods cannot be validated without a reference method according to this part of ISO 16140.

5 Qualitative methods - Technical protocol for factorial interlaboratory validation

5.1 In-house validation study

The in-house validation study is the part of the validation process that is performed in the organizing laboratory. It can be conducted according to the conventional approach or the factorial approach, as described in part 4 of ISO 16140.

An in-house validation study can be used in order to demonstrate the performance of the method for the laboratory that conducted the study. In the framework of general method validation, it is the first step and is needed for assessing the performance of the method across a large number of (food) types and samples, whereas in the interlaboratory study, the performance of the method is assessed across a larger number of laboratories.

5.2 Interlaboratory study

5.2.1 General considerations

The aim of the interlaboratory study is to compare the performance of the reference method to the alternative method in terms of the RLOD obtained by different laboratories, using the same set of samples examined under reproducibility conditions and to compare these results with pre-set criteria for the acceptable difference between the reference method and the alternative method. The interlaboratory study is planned by the organizing laboratory.

The organizing laboratory prepares the samples and a data sheet for the recording of all experimental data and critical experimental conditions used by each laboratory. It is necessary for each laboratory to demonstrate its competence in the use of the alternative and the reference method according to ISO 7218 prior to participating in the study.

Technicians, involved in the preparation of the samples used in the interlaboratory study, shall not take part in the testing of the samples of the interlaboratory study.

5.2.2 Measurement protocol

Four or more independent laboratories are required to conduct the measurement series. These laboratories shall be representative of the population of laboratories and shall belong to a minimum of 3, but preferably 4 different organizations, excluding the organizing laboratory.

NOTE 1 Laboratories in different locations, but belonging to one company or institute, are accepted as different organizations.

NOTE 2 If the 4 laboratories are independent and representative of the population of laboratories within one organization, the outcome of the validation study applies only to laboratories within that organization.

If possible, more than 4 laboratories should participate, so that results from at least 8 technicians remain even if, for some reason, certain data cannot be used.

The protocol is as follows:

— In cases where different enrichment protocols for the alternative method exist, the most challenging enrichment protocol shall be selected, e.g. the protocol having the shortest incubation time or the most selective enrichment conditions. The selected (food) item shall be relevant for the chosen enrichment protocol. This relevant (food) type, which is used to prepare the test samples, should contain a natural background microflora.

— The selected (food) item shall be inoculated with the target organism. The protocol for inoculation of the samples shall be appropriate for the selected food. Samples shall be prepared by the organizing laboratory to ensure homogeneity between and within samples using preparation protocols contained in Annex B and Annex C of ISO 16140-2:2016.

— At least three different levels of contamination shall be used: a negative control (L_0) and two levels (L_1 and L_2). Level L_1 should be between the LOD_{50} of the reference method ($LOD_{50,ref}$) and the LOD_{50} of the alternative method ($LOD_{50,alt} = RLOD \cdot LOD_{50,ref}$). Level L_2 should be 1 $\log_{10}$ level above L_1.

— For the selected (food) item, for each setting and for each laboratory, 4 replicates shall be conducted at level L_1. Only 1 test is conducted at the two other levels L_0 and L_2.

— According to the factorial, orthogonal design, 8 settings have to be conducted by each laboratory for both test methods and for all levels of contamination (L_0, L_1 and L_2). In total 96 results (1 + 4 + 1 tests × 8 settings × 2 methods) are conducted in each laboratory.

— All samples should be blind-coded to ensure that the analysts are not aware of their level of contamination.

— For tests which give paired results (paired result occurs when the primary enrichment is the same for the alternative and reference method), one test portion is required to obtain a result for the alternative and the reference method. For tests which give unpaired results (unpaired result occurs when the alternative and reference methods start from different primary enrichments), two sets of test portions are required. One test portion is analysed by the alternative method and another test portion by the reference method.

— The data are reported in two tables, giving the results from the reference method and from the alternative method before and after confirmation of the results. If the results for alternative and reference methods have been obtained from the same initial enrichment broth (paired data), there is no need to confirm the results of the alternative method if the results agree with that obtained with the reference method. In cases when the reference method gives a negative result and the alternative method gives a positive result, then confirmation of the positive result is required. If the results for alternative and reference methods have been obtained from different enrichments (unpaired data), then all enrichments obtained with the alternative method shall be taken forward for confirmation. Confirmation pathways are described in 5.1.3.3 of ISO 16140-2:2016.

— The organizing laboratory can indicate that broths, plates, and/or isolates shall be retained for a certain period of time to be able to confirm results obtained by a laboratory, if needed.

— The analysis of samples shall be performed by each laboratory on the stipulated date.

To predict accuracy and precision in the single laboratory under routine conditions, relevant method factors that are difficult to control shall be selected and varied systematically, for example personnel, media and incubation conditions. The choice of these factors and factor levels is crucial to the reliability of the test result. Four relevant method factors shall be varied simultaneously, each on 2 levels.

For methods for culturable microorganisms, the factors 'technicians' and 'culture media' have the greatest significance and shall be included in all studies.

— Technicians: tests shall be conducted in the single laboratory by 2 technicians independently.

— Culture media: use media from 2 different manufacturers if available, or pre-prepared versus prepared from dehydrated media, otherwise use 2 different batches [lots]; if available, each laboratory should use manufacturers/batches that are different from the ones used in the other laboratories.

Three further factors have to be selected for use in the study. Decision on the most suitable additional factors for the particular study should be based on expert knowledge. The following list gives examples for the selection of further factors and factor levels:

— sample preparation if appropriate (e.g. thawing of frozen samples at room temperature = 'a' or at 4 °C = 'b');

— different incubators (use two different incubators; if only one incubator is available, vary incubation conditions at two levels, e.g. 'a' = shortest time and lowest temperature permitted by the method tolerance, and 'b' = longest time and highest temperature).

