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Microbiology of the food chain — Method validation —

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

Microbiologie de la chaîne alimentaire — Validation des méthodes —

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

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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).

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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 16140-6, and as described in this document). In the case when a method is validated within one laboratory (see ISO 16140-4), no interlaboratory study is conducted.

— The second stage is method verification, where a laboratory demonstrates that it can satisfactorily perform a validated method. This is described in ISO 16140-3. Verification is only applicable to methods that have been validated using an interlaboratory study.

In general, two types of methods are distinguished: reference methods and alternative methods.

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

In the ISO 16140 series, reference methods include standardized reference (ISO and CEN) methods as defined in ISO 17468:2016, 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 ISO 16140-3) is not applicable. This document, 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 this document can be used for validation against a reference method. ISO 16140-4 (regarding qualitative and quantitative methods) and this document (regarding quantitative methods 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.

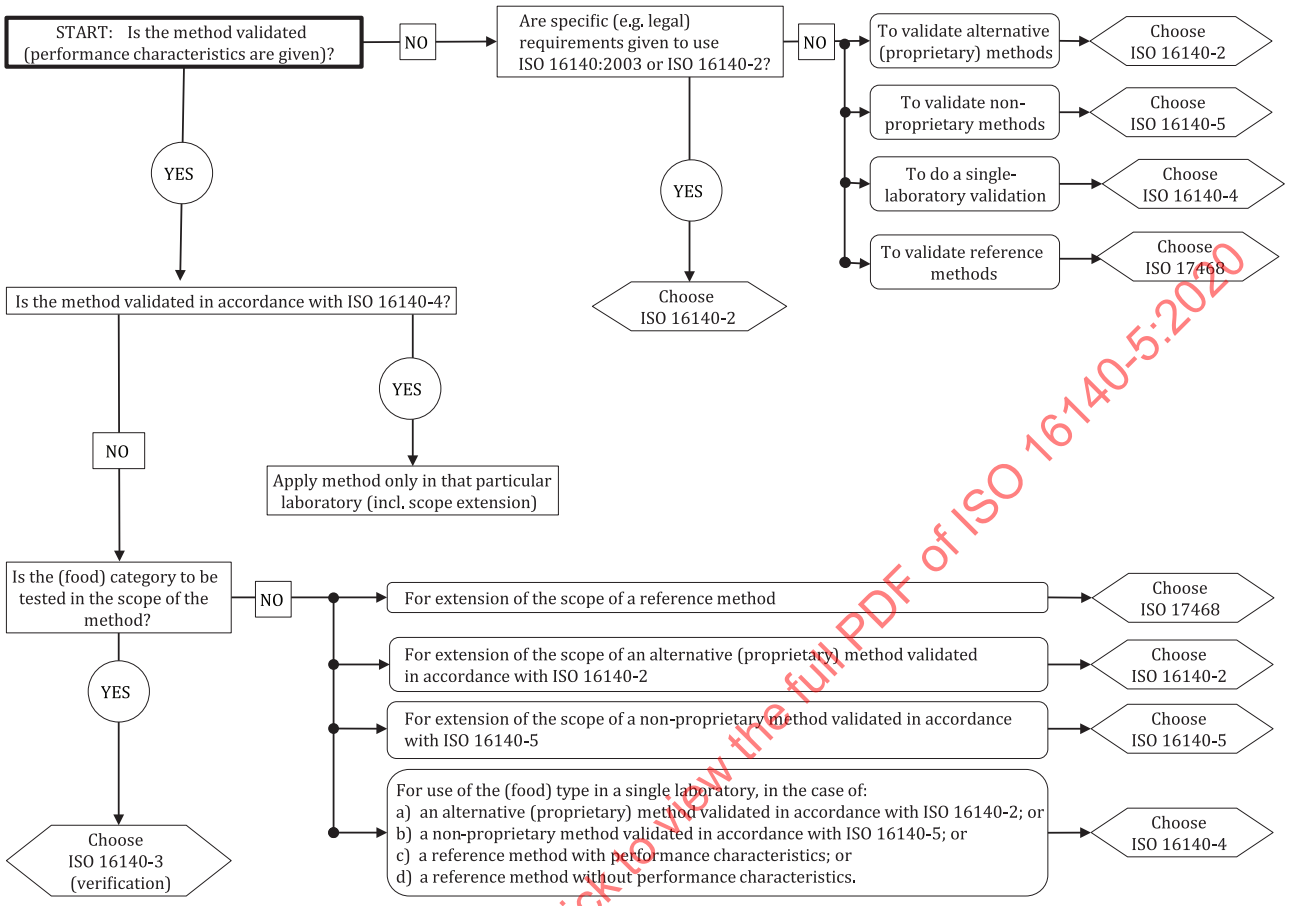

Figure 1 — Flow chart for application of the ISO 16140 series

NOTE In this document, the words "category", "type" and/or "item" are sometimes combined with "(food)" to improve readability. However, the word "(food)" is interchangeable with "(feed)" and other areas of the food chain as mentioned in Clause 1.

ISO 16140-6 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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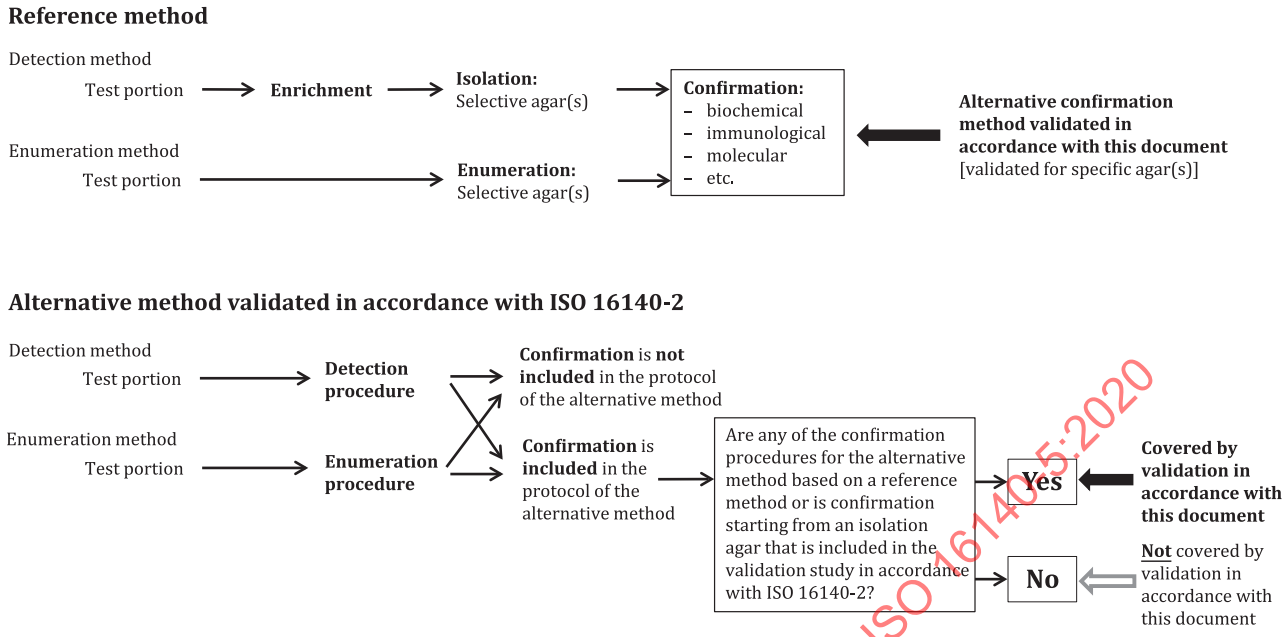

Figure 2 — Use of validated alternative confirmation methods (see ISO 16140-6)

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

An alternative confirmation method based on ELISA has been validated (in accordance with ISO 16140-6) to replace the biochemical confirmation for *Salmonella* as described in ISO 6579-1. In the validation study, XLD (mandatory agar in accordance with ISO 6579-1) plus BGA and a specified chromogenic agar (two optional agars for second plating in accordance with ISO 6579-1) were used as the agars to start the confirmation. The validated confirmation method can be used to replace the biochemical confirmation under the following conditions:

— by laboratories using the ISO 6579-1; or

— by laboratories using an ISO 16140-2 validated alternative method that refers to ISO 6579-1 for confirmation; or

— by laboratories using an ISO 16140-2 validated alternative method that starts the confirmation from XLD and/or BGA agar and/or the specified chromogenic agar.

The validated confirmation method cannot be used under the following conditions:

— by laboratories using an ISO 16140-2 validated alternative method that refers only to agars other than those included in the validation to start the confirmation (e.g. Hektoen agar and SS agar only); or

— by laboratories using an ISO 16140-2 validated alternative method that refers only to a confirmation procedure that does not require isolation on agar.

0.2 Validation protocols in the ISO 16140 series

An interlaboratory validation study, in accordance with ISO 16140-2, requires at least eight laboratories for quantitative methods and at least ten laboratories for qualitative methods.

This document provides a protocol that addresses the special case where the number of laboratories required in an interlaboratory validation of a method by ISO 16140-2 cannot be achieved. The protocol allows a method validation based on a minimum of four laboratories. It applies, for example, in situations where there is an urgent need for a validated method but the in-house and interlaboratory studies in accordance with ISO 16140-2 take too long to complete. This document also addresses the problem of method validation of highly specialized methods, for which only a few laboratories might be available for a validation study. This document can only be used for non-proprietary methods. Table 1 provides an overview of the different protocols.

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

Number of participating laboratories	With reference method	Without reference method
1	ISO 16140-4	ISO 16140-4
4 to 7 (quantitative method)/ 4 to 9 (qualitative method)	This document: for non-proprietary methods only	This document: for non-proprietary quantitative methods only
≥ 8 (quantitative method)/ ≥ 10 (qualitative method)	ISO 16140-2: for the interlaboratory study part	Not applicable

In order to reduce the number of required laboratories to a minimum of four, while maintaining the applicability of the validation to all laboratories, the protocol is based on a factorial experimental design. In the factorial design, 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.

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Microbiology of the food chain — Method validation —

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

1 Scope

This document specifies the general principles and the technical protocols (based on orthogonal, factorial studies) for the validation of non-proprietary methods for microbiology of the food chain.

This document is applicable to the validation of methods used for the analysis (detection or quantification) of microorganisms in:

— products intended for human consumption;

— products intended for animal feeding;

— environmental samples in the area of food and feed production, handling;

— samples from the primary production stage.

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.

This document specifies protocols for the validation against a reference method for both quantitative and qualitative methods. This document also provides a protocol for the validation of quantitative methods without a reference method. Qualitative methods cannot be validated without a reference method in accordance with this document.

NOTE ISO 16140-2 specifies the general principle and the technical protocol for the validation of alternative, mostly proprietary, methods against a reference method.

This document is only applicable 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 that have already been optimized.

Methods that have been validated in accordance with this document can be used by the laboratories of the specified population of laboratories.

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*

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

EXAMPLE Technician.

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

3.2
factor level
value of the *factors* (3.1) within the experimental design

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

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

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

3.3
orthogonal design
factorial design, in which for every pair of *factors* (3.1), each combination of *factor levels* (3.2) occurs the same number of times across the possible factor levels

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

3.4
reference value
<reference method is available> estimated concentration level obtained with the reference method

3.5
reference value
<no reference method is available, for artificially contaminated samples only> estimated concentration level obtained from the concentration level of the inoculum

3.6
setting
combination of *factor levels* (3.2)

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.

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

4.1 General

This document uses a protocol based on orthogonal, factorial studies. A high certainty of the determined method validation parameters is achieved by focusing on suitable factors (e.g. technician, culture media, sample preparation, temperature, test duration) that can influence the test result. This allows the number of required laboratories to be reduced to a minimum of four. General concepts and considerations given in ISO 16140-2 shall apply, unless explicitly excluded. The validation can be conducted against a reference method or, in the case of a quantitative method, without a reference method.

The outcome of the validation study applies to:

— any laboratory: if the four laboratories can be considered a "random sample" of independent laboratories from different organizations;

— all laboratories within an organization: if the four laboratories can be considered a "random sample" of laboratories at different sites from one 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 (see ISO 16140-1:2016, 2.45);

— 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 Clauses 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 and applied to both the reference and the alternative method.

4.3 Validation without a reference method

The validation protocol applies to the validation of quantitative methods only. It 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 methods are given in Clause 6.

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 in accordance with the conventional approach or the factorial approach, as described in ISO 16140-4.

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An in-house validation study can be used to demonstrate the performance of the method for the laboratory that conducted the study. It is the first step in the framework of general method validation. The in-house validation study assesses the performance of the method across (food) categories, (food) types and (food) items, whereas the interlaboratory study assesses the performance of the method across laboratories.

5.2 Interlaboratory validation study against a reference method

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. The results of the same set of samples, examined under reproducibility conditions, are compared with pre-set criteria for the acceptable difference between the reference method and the alternative method. Wherever possible, the study conditions should reflect the normal variation between laboratories.

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. Each laboratory shall demonstrate its competence in the use of the alternative and the reference method in accordance with 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 in the interlaboratory study.

5.2.2 Measurement protocol

A minimum of four independent laboratories are required to conduct the test series.

The laboratories shall belong to different organizations or shall be located at different sites.

If all laboratories belong to one organization or network, results of the validation study can only be used by laboratories belonging to this organization or network.

EXAMPLE 1 Laboratories belonging to one public or private organization.

EXAMPLE 2 Network of federal, state and/or provincial laboratories.

EXAMPLE 3 Network of national reference laboratories coordinated by a European Union reference laboratory.

If possible, more than four laboratories should participate, so that results from at least eight technicians are available for analysis 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) type shall be relevant for the chosen enrichment protocol. The (food) item, which is used to prepare the test samples, should contain natural background microbiota.

— The selected (food) item shall be artificially contaminated with the target organism. The protocol for inoculation of the samples shall be appropriate for the selected (food) item. Samples shall be prepared by the organizing laboratory to ensure homogeneity between and within samples using preparation protocols contained in ISO 16140-2:2016, Annex C. In general, liquid samples (compared to solid samples) give greater assurance of homogeneity. The samples should be shown to be homogeneous by the organizing laboratory. Homogeneity tests and criteria for acceptance are described in ISO 22117.

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— At least three different levels of contamination shall be used: a blank (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 · $LOD_{50,ref}$). Level L_2 should be 1 $\log_{10}$ above level L_1. Level L_1 shall produce fractional positive results.

— Inocula should be enumerated using a non-selective medium. Enumeration shall be performed as described in ISO 7218.

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

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

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

— Settings shall be common to both test methods (paired and unpaired). Paired results are obtained when the primary enrichment is the same for the alternative and reference method. That is, one test portion is used to conduct tests according to the alternative and the reference method. Unpaired results are obtained when the alternative and reference methods use different primary enrichments. That is, 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 presumptive results of the alternative method if the results agree with that obtained with the reference method. However, confirmation of the positive result is required when the reference method gives a negative result and the alternative method gives a positive result.

  — 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 ISO 16140-2:2016, 5.1.3.3.

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

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

5.2.3 Selection of the factors to be studied

Decisions on the most suitable factors for the particular study should be based on expert knowledge. For example, optimal conditions are specified in each method, e.g. incubation temperature and duration at 37° C and 24 h, and these will give the best results. However, ranges around these, due to inaccuracies of the instrument, but which provide still acceptable conditions (e.g. 37 °C ± 1 °C, 24 h ± 1 h), are permitted and the study design should test the extremes of these. Acceptable operating conditions for equipment are described in ISO 7218. Other influences such as stress conditions can also be taken into account.

To estimate the accuracy under routine conditions, relevant method factors that are difficult to control shall be selected and varied systematically, e.g. technicians, culture media and incubation conditions. The choice of these factors and factor levels is crucial to the reliability of the test result. For unpaired study designs, chosen settings biased against the reference method cannot be used.

Five relevant method factors shall be varied simultaneously, each on two levels.

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

— Technicians: Tests shall be independently conducted in each of the laboratories by two technicians.

— Culture media: Use culture media from two different manufacturers, if available, or two different batches of culture media (lots), or pre-prepared versus prepared from dehydrated media. If possible, each laboratory should use manufacturers/batches that are different from the ones used in the other laboratories.

Three other factors shall be included in the study. A non-comprehensive list of grouped potential factors is provided in Annex A. Depending on the method, one factor from each of the most relevant groups shall be selected.

Factors and factor levels shall reflect the normal variation within routine testing laboratories. Factors are studied simultaneously using the study design described in 5.2.4.

5.2.4 Experimental design

Each setting is a combination of levels of five method factors. At each setting, each of the laboratories conducts (1 + 4 + 1) tests at levels L_0, L_1 and L_2 for both test methods. Eight different settings (1 to 8) shall be considered. The study design is shown in Table 2, where “a” and “b” represent the levels of the respective factors.

NOTE This study design (5 factors) is different from the design used in ISO 16140-4 for single-laboratory validation studies (4 factors).

Table 2 — Study design for qualitative methods to be conducted by each of at least four laboratories

Setting	Factor 1: technician	Factor 2: culture medium	Factor 3: e.g. thawing process	Factor 4: e.g. incubation condition	Factor 5: e.g. background microbiota
1	a	a	a	a	a
2		b	b	b	b
3		a	a	b	b
4		b	b	a	a
5	b	a	b	a	b
6		b	a	b	a
7		a	b	b	a
8		b	a	a	b

With four laboratories, eight settings, three levels of contamination and two test methods (reference and alternative) the minimum number of individual tests is 4 × 8 × (1 + 4 + 1) × 2 = 384.

The organizing laboratory shall examine the raw data and other information requested in the data sheet to ascertain that all laboratories have performed the analyses in accordance with both the alternative and reference methods as written. When there is evidence that results might be obtained under inappropriate conditions and/or that the methods have not been strictly followed, these or all results from the laboratory are excluded for further analysis.

Annex C shows an example of a factorial interlaboratory study for a qualitative method.

5.3 Calculations and summary of data

The results obtained by the individual laboratories in the interlaboratory study are summarized in Tables 3 and 4.

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Table 3 — Fraction of positive results by the reference method

Laboratory	Contamination level		
	L_0	L_1	L_2
Laboratory 1	/8	/32	/8
Laboratory 2	/8	/32	/8
Laboratory 3	/8	/32	/8
Etc....	/8	/32	/8
Total			

Table 4 — Fraction of positive results (before and after confirmation) by the alternative method

Laboratory	Contamination level					
	L_0		L_1		L_2	
	Presumptive	**Confirmed**	**Presumptive**	**Confirmed**	**Presumptive**	**Confirmed**
Laboratory 1	/8	/8	/32	/32	/8	/8
Laboratory 2	/8	/8	/32	/32	/8	/8
Laboratory 3	/8	/8	/32	/32	/8	/8
Etc....	/8	/8	/32	/32	/8	/8
Total						

Subsequent calculations, using the data from the fractional recovery level L_1 only, are carried out in accordance with ISO 16140-2:2016, 5.2.3. These calculations shall be conducted across all factor settings, 1 to 8, and also separately for each subgroup of factor settings that belongs to one of the five factors and one of the two factor levels, as shown in Table 5.

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Table 5 — Summary of results

Factor levels	Settings	PA	NA	ND	PD	FP	N[a]	$SE_{(alt)}$ %	$SE_{(ref)}$ %	RT %	$SP_{(alt)}$ %	$SP_{(ref)}$ %	FPR[b] %
All settings	1 to 8												
Factor 1 (technician) "a" – see NOTE 2	1,2,3,4												
Factor 1 (technician) "b" – see NOTE 2	5,6,7,8												
Factor 2 (culture medium) "a"	1,3,5,7												
Factor 2 (culture medium) "b"	2,4,6,8												
Factor 3 (e.g. thawing process) "a"	1,3,6,8												
Factor 3 (e.g. thawing process) "b"	2,4,5,7												
Factor 4 (e.g. incubation condition) "a"	1,4,5,8												
Factor 4 (e.g. incubation condition) "b"	2,3,6,7												
Factor 5 (e.g. background microbiota) "a"	1,4,6,7												
Factor 5 (e.g. background microbiota) "b"	2,3,5,8												

Key

PA: positive agreement, NA: negative agreement, ND: negative deviation, PD: positive deviation, FP: false positive, N: sum, $SE_{(alt)}$: sensitivity alternative method, $SE_{(ref)}$: sensitivity reference method, RT: relative trueness, $SP_{(alt)}$: relative specificity alternative method, $SP_{(ref)}$: relative specificity reference method, FPR: false positive ratio

[a] N = NA + PA + PD + ND.

[b] FPR = FP/NA × 100 %.

NOTE 1 Definitions and the derivation of the parameters can be found in ISO 16140-2:2016, 5.2.3.

NOTE 2 In most cases, factor levels for the factor "technician" are different from one laboratory to the next (except cases where technician "a" and "b" represent specific training levels). Then the sensitivity analysis cannot be meaningfully interpreted for this factor, and the corresponding rows should be left empty. This holds also for any other factor when sensitivity analysis for its factor levels cannot be meaningfully interpreted.

5.4 Interpretation of data

5.4.1 Paired study

For a paired study, calculate the difference, (ND – PD), and the sum, (ND + PD), for the fractional recovery level L_1. The values found for (ND – PD) and (ND + PD) shall not be higher than the acceptability limits (ALs) given in Table 6 with respect to the number of participating laboratories (N_{lab}).

Table 6 — Acceptability limits (ALs) for a paired study design in relation to the number of participating laboratories

N_{lab}	Acceptability limits (ALs)	
	(ND – PD)	ND + PD
4	3	4
5	4	5
6	4	6
7	5	7
8	5	8
9	6	9

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The AL is not met when the observed value is higher than the AL. When the AL is not met, investigations should be conducted (e.g. root cause analysis) in order to provide an explanation for the observed results. The decision on whether the alternative method is regarded as fit for purpose is based on the AL and the findings of the investigations. The reasons for acceptance of the alternative method when the AL is not met shall be stated in the validation study report.

5.4.2 Unpaired study

For an unpaired study, calculate the difference, (ND – PD), for the fractional recovery level L_1. The observed value shall not be higher than the AL. Calculate AL as shown by Formula (1):

$$AL = 4\sqrt{6N_{lab}\left((p_+)_{ref} + (p_+)_{alt} - 2\left((p_+)_{ref} \times (p_+)_{alt}\right)\right)} \quad (1)$$

where

N_{lab} is the number of laboratories;

$(p_+)_{ref}$ is the number of samples with a positive result obtained with the reference method at level L_1 for all laboratories divided by the number of samples tested at level L_1;

$(p_+)_{alt}$ is the number of samples with a confirmed positive result obtained with the alternative method at level L_1 for all laboratories divided by the number of samples tested at level L_1.

The AL is not met when the observed value is higher than the AL. When the AL is not met, investigations should be conducted (e.g. root cause analysis) in order to provide an explanation for the observed results. The decision on whether the alternative method is regarded as fit for purpose is based on the AL and the findings of the investigations. The reasons for acceptance of the alternative method when the AL is not met shall be stated in the validation study report.

5.4.3 Analysis of factorial effects with respect to RLOD

In addition, effects of the individual factors (e.g. technician) and of the interactions between factors (e.g. culture medium and incubation conditions) can be calculated from the factorial design based on the RLOD values calculated for each laboratory.

NOTE An Excel®-based program[1)] is available for performing the RLOD calculations in an interlaboratory study from https://standards.iso.org/iso/16140/-5/ed-1/en.

The following notation is used: k refers to the laboratory and p is the number of laboratories ($1 \le k \le p$). For a specific factor of interest, calculate the RLOD using all test results of the four settings of the laboratory k in which the factor is set to level "a", and denote the $\log_{10}$ value $Y_k^{(a)} = \log_{10}(\text{RLOD(level a)})$. Calculate the RLOD using all test results of the four settings of the laboratory k in which the factor is set to level "b" and denote the $\log_{10}$ value $Y_k^{(a)} = \log_{10}(\text{RLOD(level b)})$.

Calculate the average of the difference of these $\log_{10}$ RLOD values across all laboratories, as shown by Formula (2):

$$d = \frac{1}{p}\sum_{k=1}^{p}\left(Y_k^{(b)} - Y_k^{(a)}\right) \quad (2)$$

If this difference (d) is smaller than −0,3 or larger than +0,3, the factor is considered to have a substantial influence on the RLOD and shall be reported in the validation study report. Further analysis techniques can be found in Reference [9].

1) Excel® is the trade name of a product supplied by Microsoft and is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

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6 Quantitative methods — Technical protocol for factorial interlaboratory validation

6.1 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 described in ISO 16140-4.

An in-house validation study can be used to demonstrate the performance of the method for the laboratory that conducted the study. It is the first step in the framework of general method validation. The in-house validation study assesses the performance of the method across (food) categories, (food) types and (food) items, whereas the interlaboratory study assesses the performance of the method across laboratories.

6.2 Interlaboratory validation study against a reference method

6.2.1 General considerations

The aim of the interlaboratory study is to compare the performance (accuracy and precision) of the reference method to the alternative method across different laboratories. The results of the same set of samples, examined under reproducibility conditions are compared with pre-set criteria for the acceptable difference between the reference method and the alternative method. Wherever possible, the study conditions should reflect the normal variation between laboratories.

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. Each laboratory shall demonstrate its competence in the use of the alternative and the reference method in accordance with 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 in the interlaboratory study.

6.2.2 Measurement protocol

A minimum of four independent laboratories are required to conduct the test series.

The laboratories shall belong to different organizations or shall be located at different sites.

If all laboratories belong to one organization or network, results of the validation study can only be used by laboratories belonging to this organization or network.

EXAMPLE 1 Laboratories belonging to one public or private organization.

EXAMPLE 2 Network of federal, state and/or provincial laboratories.

EXAMPLE 3 Network of national reference laboratories coordinated by a European Union reference laboratory.

If possible, more than four laboratories should participate, so that results from at least eight technicians are available for analysis even if, for some reason, certain data cannot be used.

The protocol is as follows.

— A relevant (food) item is used to prepare the test samples. The (food) item should contain natural background microbiota.

— The selected (food) item may be artificially contaminated with the target organism. The protocol for inoculation of the samples shall be appropriate for the selected (food) item. Samples shall be prepared by the organizing laboratory to ensure homogeneity between and within samples using

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preparation protocols contained in ISO 16140-2:2016, Annexes B and C. In general, liquid samples (compared to solid samples) give greater assurance of homogeneity. The samples should be shown to be homogeneous by the organizing laboratory. Homogeneity tests and criteria for acceptance are described in ISO 22117.

— At least three different levels of contamination shall be used. The analyte concentrations should be chosen to cover at least the lower, middle and upper levels of the entire range of the alternative method. A negative control level should also be included, especially in cases where artificially contaminated samples are used.

— Analyses are carried out by each laboratory for each enumeration method at the three levels of contamination, in accordance with the factorial, orthogonal design described in 6.2.3.

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

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

The method factors shall be selected as described in 5.2.3. Five factors are studied simultaneously using the study design described in 6.2.3.

6.2.3 Experimental design

The study design for quantitative methods requires participation by at least four laboratories. Eight different settings (1 to 8) shall be considered. Each setting is a combination of levels of five method factors, where "a" and "b" represent the levels of respective factors. At each setting, each laboratory conducts one single measurement at each contamination level L_1, L_2 and L_3 for both test methods. The study design is shown in Table 7 and requires 4 laboratories × 3 contamination levels × 8 settings × 2 methods = 192 individual tests. If no reference method is available, the minimum number of individual tests is 4 × 3 × 8 = 96 if available, or in cases of artificial contamination, a negative control at each laboratory should be included. The results of the negative control can provide information on the background microbiota and may be used to explain unexpected test results.

Table 7 — Study design for quantitative methods to be conducted by each of at least four laboratories

Setting	Factor 1: technician	Factor 2: culture medium	Factor 3: e.g. thawing process	Factor 4: e.g. incubation condition	Factor 5: e.g. background microbiota
1	a	a	a	a	a
2		b	b	b	b
3		a	a	b	b
4		b	b	a	a
5	b	a	b	a	b
6		b	a	b	a
7		a	b	b	a
8		b	a	a	b

The organizing laboratory shall examine the raw data and other information requested in the data sheet to ascertain that all laboratories have performed the analyses in accordance with both the alternative and reference methods as written. When there is evidence that results might be obtained under inappropriate conditions and/or that the methods have not been followed strictly, these or all results from the laboratory shall be excluded for further analysis. No outlier tests are performed on the selected data.

Annex B shows an example of a factorial interlaboratory study for a quantitative method.

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6.3 Calculations, summary, and interpretation of data

6.3.1 Summary of test results

The log-transformed test results from the different laboratories for both the reference and alternative method are entered in Table 8. Calculations are performed as a sequence of operations starting with the log-transformation of all test results. The following notation is used: i refers to the level and q is the number of levels ($1 \leq i \leq q$); j refers to the setting ($1 \leq j \leq 8$); k refers to the laboratory and p is the number of laboratories ($1 \leq k \leq p$). Note the data as follows:

— x_{ijk}, is the $\log_{10}$ transformed test result on level i for setting j of laboratory k using the reference method (if available);

— y_{ijk}, is the $\log_{10}$ transformed test result on level i for setting j of laboratory k using the alternative method.

In Table 8, each square represents a single test result. Results of additional laboratories ($p > 4$) can be added.

Table 8 — Summary of the results of the interlaboratory study for a quantitative method

		Setting/method															
		1	1	2	2	3	3	4	4	5	5	6	6	7	7	8	8
Laboratory	**Contamination level**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**
1	Negative control																
2																	
3																	
4																	
⋮																	
1	Low																
2																	
3																	
4																	
⋮																	
1	Medium																
2																	
3																	
4																	
⋮																	
1	High																
2																	
3																	
4																	
⋮																	

Key

R: reference method

A: alternative method

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6.3.2 Precision data

Precision data (reproducibility standard deviations) can be derived by means of restricted maximum likelihood (REML) using a random effect model with random factors: “laboratory”, “laboratory × technician”, “laboratory × culture medium”, “laboratory × thawing process”, “laboratory × incubation condition”, “laboratory × background microbiota”. The calculation can be conducted with software packages such as “R”. Details on the statistical method can be found in Reference [10].

NOTE “R” is a programming language and free software environment for statistical computing and graphics that is supported by the R Foundation for Statistical Computing. The R language is widely used among statisticians. It can be downloaded from https://cran.r-project.org.

An alternative relatively simple calculation method for the precision data is described in the following steps. This method is statistically less efficient and should be used only if the REML method is not available.

Step 1: If a reference method is available, calculate for each level i of contamination, the median of the log-transformed counts X_i obtained with the reference method, x_{ijk}. These medians are called “reference values” of the samples used in the validation study: $X_i = \text{median}(x_{ijk})$.

If no reference method is available, use log-transformed counts obtained with the method to be validated, $Y_i = \text{median}(y_{ijk})$.

Step 2: Calculate, for each level i (using y_{ijk}), the repeatability (r) standard deviation, s_r, as shown by Formula (3):

$$s_{ri}^2 = \frac{1}{8p}\sum_{k=1}^{p}\left(y_{i1k}+y_{i2k}-y_{i3k}-y_{i4k}\right)^2+\frac{1}{8p}\sum_{k=1}^{p}\left(y_{i5k}+y_{i6k}-y_{i7k}-y_{i8k}\right)^2 \quad (3)$$

$$s_{ri} = \sqrt{s_{ri}^2}$$

Step 3: For each level i (using y_{ijk}), the intermediate standard deviation, s_A, can be obtained by using the calculation shown in Formula (4):

$$s_{Ai}^2 = s_{ri}^2 + s_{1i}^2 + s_{2i}^2 + s_{3i}^2 + s_{4i}^2 + s_{5i}^2 \quad (4)$$

$$s_{Ai} = \sqrt{s_{Ai}^2}$$

with

$$s_{1i}^2 = \frac{1}{32p}\sum_{k=1}^{p}\left(y_{i1k}+y_{i2k}+y_{i3k}+y_{i4k}-y_{i5k}-y_{i6k}-y_{i7k}-y_{i8k}\right)^2-\frac{1}{4p}s_{ri}^2$$

$$s_{2i}^2 = \frac{1}{32p}\sum_{k=1}^{p}\left(y_{i1k}-y_{i2k}+y_{i3k}-y_{i4k}+y_{i5k}-y_{i6k}+y_{i7k}-y_{i8k}\right)^2-\frac{1}{4p}s_{ri}^2$$

$$s_{3i}^2 = \frac{1}{32p}\sum_{k=1}^{p}\left(y_{i1k}-y_{i2k}+y_{i3k}-y_{i4k}-y_{i5k}+y_{i6k}-y_{i7k}+y_{i8k}\right)^2-\frac{1}{4p}s_{ri}^2$$

$$s_{4i}^2 = \frac{1}{32p}\sum_{k=1}^{p}\left(y_{i1k}-y_{i2k}-y_{i3k}+y_{i4k}+y_{i5k}-y_{i6k}-y_{i7k}+y_{i8k}\right)^2-\frac{1}{4p}s_{ri}^2$$

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$$s_{5i}^2 = \frac{1}{32p}\sum_{k=1}^{p}\left(y_{i1k} - y_{i2k} - y_{i3k} + y_{i4k} - y_{i5k} + y_{i6k} + y_{i7k} - y_{i8k}\right)^2 - \frac{1}{4p}s_{ri}^2$$

If one of these terms $s_{1i}^2, s_{2i}^2, s_{3i}^2, s_{4i}^2, s_{5i}^2$ is a negative number, replace the negative value with 0.

Step 4: For each level i, the residual laboratory standard deviation, s_B, can be obtained using the calculation shown in Formula (5):

$$s_{Bi}^2 = \frac{1}{p-1}\sum_{k=1}^{p}\left(\bar{y}_{ik} - \bar{y}_i\right)^2 - \frac{1}{8}s_{ri}^2 - \frac{1}{2}\left(s_{1i}^2 + s_{2i}^2 + s_{3i}^2 + s_{4i}^2 + s_{5i}^2\right) \quad (5)$$

$$s_{Bi} = \sqrt{s_{Bi}^2}$$

with the laboratory mean values:

$$\bar{y}_{ik} = \frac{1}{8}\sum_{j=1}^{8} y_{ijk}$$

and the overall mean:

$$\bar{y}_i = \frac{1}{p}\sum_{k=1}^{p}\bar{y}_{ik}$$

Step 5: Calculate, for each level i, the reproducibility (R) standard deviation, s_R, as shown in Formula (6):

$$s_{Ri} = \sqrt{s_{Ai}^2 + s_{Bi}^2} \quad (6)$$

6.3.3 Accuracy profile

The accuracy profile for comparing the alternative method with the reference method shall be calculated in accordance with ISO 16140-2:2016, 6.2.3: the β-ETI (range of values within which a stated proportion of the population is expected to lie (see ISO 16140-1:2016, 2.8) is calculated from the median X_i of the reference values and the reproducibility standard deviation above. The necessary intermediate parameters G and H are based in the repeatability standard deviation of Step 1 and the residual laboratory standard deviation of Step 4. The β-ETI then defines the upper and lower limit for the ALs.

The interpretation of results shall also be carried out in accordance with ISO 16140-2:2016, 6.2.3: the alternative method is regarded as equivalent to the reference method when the values for the β-ETI fall within the ALs for all levels of contamination. The AL is set at ±0,5 $\log_{10}$. However, if any of the values falls outside these limits, an adjustment to the AL, based on the reproducibility standard deviation of the reference method as calculated in the above Step 5 for the reference test results, is performed in accordance with ISO 16140-2:2016, 6.2.3, and the results are then re-evaluated.

6.4 Interlaboratory validation study without a reference method

Factorial interlaboratory studies can be conducted without a reference method. The calculation of precision data for the alternative method is not dependent on a reference value and can be conducted in accordance with 6.3.

For the calculation of the accuracy profile, the selected (food) item shall be artificially contaminated so that the reference concentration of the contamination is known. The results of the reference method are replaced by the corresponding reference values in the calculations outlined in 6.3.

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Annex A
(informative)

List of factors and factor levels for factorial validation

The following list can be used to select factors and factor levels for the factorial study designs provided in this document. Factors and factor levels that are expected to have a noticeable effect on the end result should be selected from the following list.

- Culture media and incubation:
  - supplier of non-selective/primary broth;
  - in-house versus ready-made non-selective/primary broth;
  - supplier of selective/secondary broth;
  - in-house versus ready-made selective/secondary broth;
  - supplier of agar plate;
  - in-house versus ready-made agar plate;
  - storage time of broths or plates after incubation before subsequent subculturing or counting (as long as specified within the procedure of the method);
  - age of the medium (within the expiry period of the medium);
  - batches of media (e.g. media for *Enterobacteriaceae* containing bile salts);
  - time of incubation of different steps (e.g. 20 h versus 28 h when 24 h ± 4 h is allowed).
- Background microbiota [at least two levels of background microbiota per (food) item, e.g. fresh and at the expiry of the best-before date]:
  - background microbiota associated with storage (refrigerated/room temperature, stored/not stored);
  - contamination levels (add different levels artificially);
  - interference organisms;
  - batches of (food) items, where background microbiota is known to vary.
- Sample preparation and storage:
  - blender or vortex;
  - resuscitation procedure [e.g. thawing of frozen samples (“a”) at room temperature or (“b”) at 4 °C];
  - immediate use or freezing/thawing of samples;
  - transport conditions: storage or transport of samples.
- Equipment and technicians:
  - technician (use technicians with different level of experience, if possible);
  - different pipetting systems;

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  - different incubation conditions [use two different types of incubators, e.g. different 37 °C air incubators, air incubator versus water bath incubation; if only one type of incubator is available, vary incubation conditions at two levels, e.g. level "a" = shortest time permitted by the method tolerance and lowest position in the incubator (= lowest temperature), and level "b" = longest time permitted by the method tolerance and highest position in the incubator (= highest temperature)];
  - spread plates versus pour plates, if relevant.
- Stress factors (if applicable):
  - low/normal temperature;
  - high/normal temperature;
  - chemical stress (e.g. smoking and curing);
  - pH-stress;
  - high pressure stress;
  - low-dose radiation/no radiation (e-beam);
  - dry/non-dry, e.g. low a_w (≤0,60)/high a_w (≥0,95).

NOTE A setting is a specific combination of levels for all factors. Two examples of a setting for the factors from Table A.1 are:

- setting 1 (a,a,b,b,a): technician "a"; in-house agar plates; refrigerated storage; incubation condition "b"; fresh product;
- setting 2 (b,b,a,a,b): technician "b"; ready-made agar plates; no storage; incubation condition "a"; stored product.

Table A.1 — Examples of factors and their levels

Factor	Level "a"	Level "b"
Technician	Technician "a"	Technician "b"
Preparation of agar plates	Made in-house	Purchased ready-made
Storage of inoculated samples (increases background levels)	None	Hold refrigerated 2 days
Incubation condition	Incubation condition "a"	Incubation condition "b"
Background microbiota	Fresh product	Stored product

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Annex B
(informative)

Example of a factorial interlaboratory study for a quantitative method

B.1 General

This annex provides an example to illustrate the procedure for the computation of validation parameters in accordance with 6.2.

Twenty-four test portions (eight settings at three contamination levels) of naturally contaminated instant non-fat dry milk powder (NFMP) were shipped to five laboratories located in three countries (Canada, France and United States). Two technicians from each laboratory performed the alternative method and the reference method [see Standard Methods for the Examination of Dairy Products (SMEDP)][11] for the aerobic plate count analysis of the NFMP. Incubation times for the alternative method and the reference method were 48 h ± 3 h and 72 h ± 3 h, respectively.

B.2 Study design

Five influencing factors were evaluated in the study:

— technician (technician “a”, technician “b”);

— culture medium (agar medium pre-made, agar medium prepared from dehydrated media);

— pipette (1,0 ml serological, 1 000 µl micropipette);

— incubation condition (incubation condition “a”, incubation condition “b”);

— incubation time (lowest, highest).

See Table B.1 for a detailed summary of the factors to be evaluated.

All 24 test portions (for 8 settings, 3 contamination levels each) were evaluated in a paired study design: the same test portions were used for both the alternative and the reference method. The variations in technicians, culture media, pipettes, incubation conditions and incubation times in accordance with the experimental design in Table 7 and summarized in Table B.1 were applied to both the alternative and the reference method. Table B.2 provides a summary of the study.

Each laboratory analysed all test portions. Test portions were prepared so that the technician did not know the contamination level. They were randomized and blind-coded with a 3-digit reference number, followed by the test setting number. For example, 125-1 or 145-3. Samples were clearly identified with the factor levels that needed to be adhered to for each test portion (i.e. incubation condition, incubation time, etc.). Detailed instructions were provided for the analysis, including a flowchart for each of the two technicians (see Figures B.1 and B.2).

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Table B.1 — Experimental design

Setting	Factor 1: Technician	Factor 2: Agar medium	Factor 3: Incubation time		Factor 4: Incubation condition	Factor 5: Pipette
			Alternative	Reference		
1	"a"	Agar medium (pre-made)	45 h	69 h	Incubation condition "a"	1,0 ml serological pipette
2		Agar medium (prepared from dehydrated media)	51 h	75 h	Incubation condition "b"	1 000 µl micropipette
3		Agar medium (pre-made)	45 h	69 h	Incubation condition "b"	1 000 µl micropipette
4		Agar medium (prepared from dehydrated media)	51 h	75 h	Incubation condition "a"	1,0 ml serological pipette
5	"b"	Agar medium (pre-made)	51 h	75 h	Incubation condition "a"	1 000 µl micropipette
6		Agar medium (prepared from dehydrated media)	45 h	69 h	Incubation condition "b"	1,0 ml serological pipette
7		Agar medium (pre-made)	51 h	75 h	Incubation condition "b"	1,0 ml serological pipette
8		Agar medium (prepared from dehydrated media)	45 h	69 h	Incubation condition "a"	1 000 µl micropipette

Table B.2 — Overview of study design per participating laboratory

Matrix/test portion size	Target contamination level	# Test portions	Alternative method	Reference method
NFMP 11 g	Low	8	Plating of appropriate dilutions per test portion incubated at 30 °C ± 1 °C for 48 h ± 3 h[a]	Plating of appropriate dilutions per test portion incubated at 30 °C ± 1 °C for 72 h ± 3 h[a]
	Medium	8		
	High	8		

[a] With variations through different settings.

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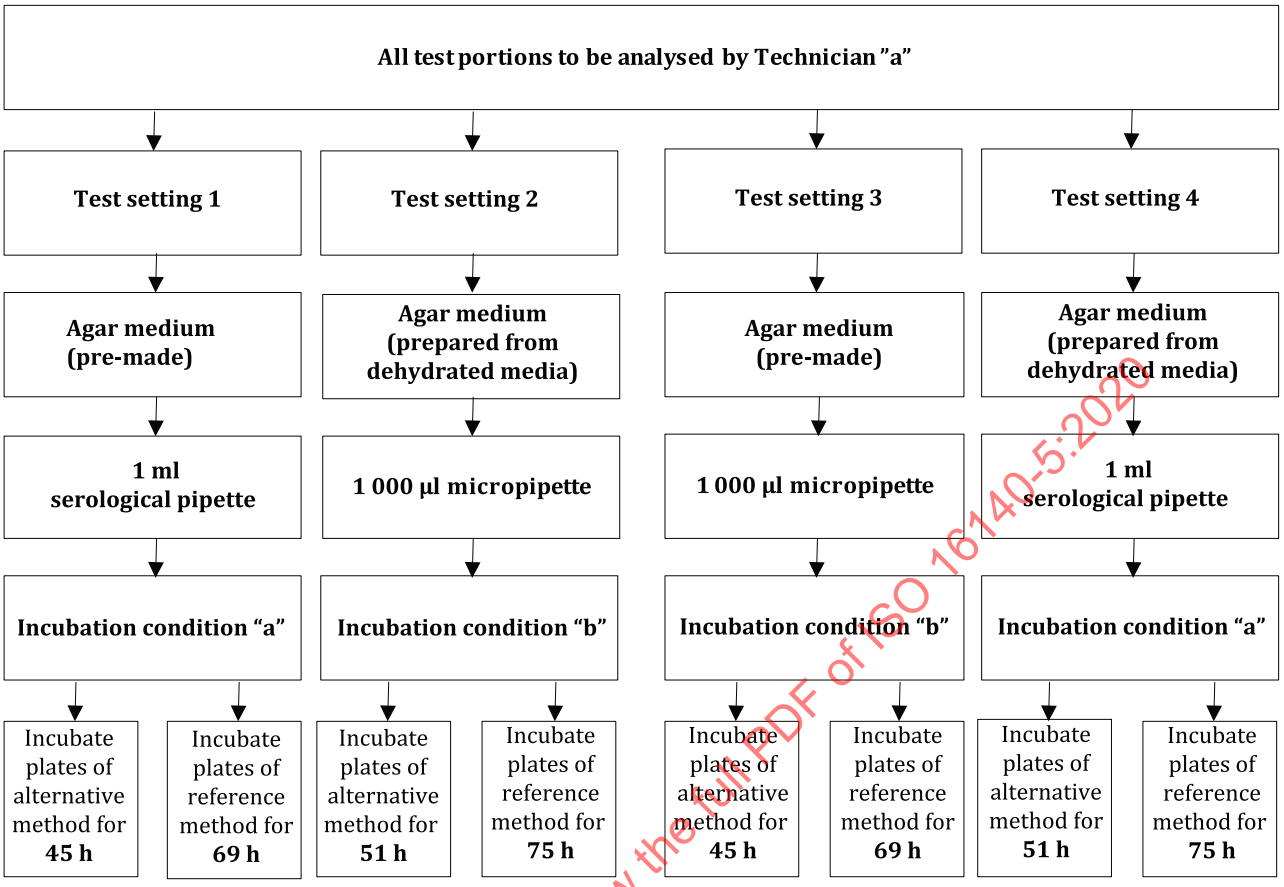

Figure B.1 — Instructions for technician "a"

NOTE The order of factors, as presented in Figure B.1 from top to bottom, is the order of the microbiological work flow and not the order of the factors as numbered in the study (see also Table B.1).

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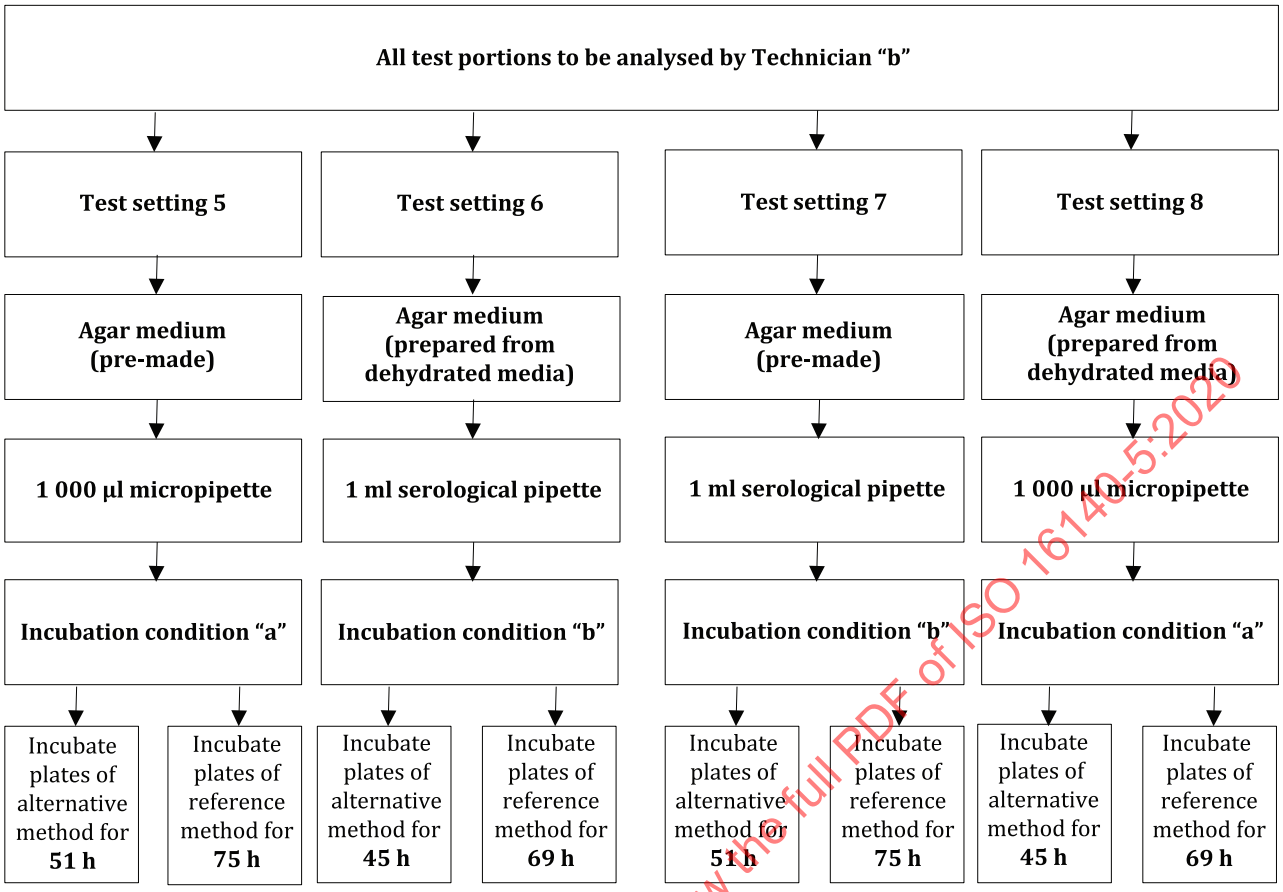

Figure B.2 — Instructions for technician "b"

NOTE The order of factors, as presented in Figure B.2 from top to bottom, is the order of the microbiological work flow and not the order of the factors as numbered in the study (see also Table B.1).

B.3 Calculations and summary of data

B.3.1 Summary of the results of laboratories 1 to 5 of the interlaboratory study

Upon receipt of the data, it was verified that all laboratories performed the analyses in accordance with the methods as indicated in the study design. No outlier tests were performed on the data and no data points were excluded.

All data points (aerobic plate count results per test portion and method in cfu/g) were $\log_{10}$ transformed. The results are shown in Table B.3 (to only two decimal places for better readability).

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Table B.3 — Results of interlaboratory study, $\log_{10}$ transformed data

		Setting/method															
		1	1	2	2	3	3	4	4	5	5	6	6	7	7	8	8
Laboratory	**Level**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**	**R**	**A**
1	Low	2,08	2,11	2,15	2,15	2,04	2,15	2,11	2,20	1,90	2,00	2,04	2,08	2,00	2,00	2,00	2,08
2	Low	2,52	2,41	2,41	2,38	2,46	2,41	2,59	2,51	2,34	2,45	2,32	2,30	2,49	2,53	2,45	2,46
3	Low	2,69	2,36	2,73	2,38	2,66	2,45	2,53	2,53	2,04	2,53	2,62	2,45	2,49	2,45	2,20	2,48
4	Low	2,62	2,40	2,62	2,40	2,58	2,60	2,72	2,45	2,64	2,54	2,78	2,51	2,72	2,41	2,91	2,66
5	Low	2,15	2,45	2,57	2,45	2,41	2,60	2,84	2,85	2,26	2,45	2,64	2,45	2,34	2,43	2,75	1,90
1	Medium	3,28	3,26	3,20	3,20	3,36	3,18	3,08	3,15	3,26	3,08	3,08	3,15	3,11	3,08	3,38	3,38
2	Medium	2,97	2,91	2,94	2,91	2,91	2,93	2,99	2,90	3,08	2,94	2,99	2,97	2,98	2,94	2,99	2,99
3	Medium	2,83	2,93	2,91	3,11	2,89	2,88	2,81	2,66	2,38	2,96	2,82	2,95	2,78	2,90	2,85	3,04
4	Medium	2,93	2,98	2,96	2,97	2,94	2,86	3,04	3,08	2,98	3,04	2,97	2,99	3,00	3,00	3,08	3,08
5	Medium	2,58	2,91	2,70	2,92	2,65	2,79	2,73	2,91	2,66	2,97	2,59	2,97	2,70	2,96	2,85	3,04
1	High	3,94	3,79	4,08	3,70	4,08	3,79	3,66	3,43	3,95	3,61	3,89	3,58	4,15	3,72	3,98	3,74
2	High	4,34	4,20	4,15	4,08	4,38	4,08	4,08	3,89	4,34	4,08	4,49	4,18	4,34	4,11	4,30	4,08
3	High	4,26	4,23	4,56	4,20	4,56	4,36	4,58	4,40	4,00	3,86	4,08	4,00	4,08	3,80	4,04	4,15
4	High	4,11	3,89	4,52	4,11	4,18	4,04	4,26	4,08	4,49	4,30	4,28	4,08	4,18	4,00	4,26	4,08
5	High	3,73	3,70	3,80	3,89	3,80	3,58	3,81	3,66	3,60	3,68	3,92	3,97	3,69	3,62	3,97	3,79

Key

R: reference method

A: alternative method

B.3.2 Precision data

Calculation of precision data was conducted in accordance with the steps described in 6.3. For the alternative method, results, at each step in the calculation, are provided below. For the reference method, the final results are provided.

Step 1: Median of the reference values X_i and the values of the alternative method Y_i, as shown in Table B.4.

Table B.4 — Median values

Reference method			Alternative method		
Low	**Medium**	**High**	**Low**	**Medium**	**High**
X_1	X_2	X_3	Y_1	Y_2	Y_3
2,490	2,950	4,095	2,450	2,970	3,985

Step 2: Repeatability variance and standard deviation for each level i (using y_{ijk}), as shown in Table B.5.

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Table B.5 — Repeatability standard deviations

Alternative method			
	Low	**Medium**	**High**
s_{ri}^2	0,020 825	0,008 607	0,016 208
s_{ri}	0,144	0,093	0,127

Step 3: Intermediate variance and standard deviation for each level i (using y_{ijk}), as shown in Table B.6, with Table B.7.

Table B.6 — Intermediate standard deviations

Alternative method			
	Low	**Medium**	**High**
s_{Ai}^2	0,037 876	0,015 615	0,040 516
s_{Ai}	0,195	0,125	0,201

Table B.7 — Components of intermediate variances

		Alternative method		
Factor		**Low** $i = 1$	**Medium** $i = 2$	**High** $i = 3$
Technician	s_{1i}^2	0,008 751	0,001 667	0,012 418
Culture medium	s_{2i}^2	0,000 026	0,000 762	0,005 855
Incubation time	s_{3i}^2	0,004 314	0,001 367	0,003 968
Incubation condition	s_{4i}^2	0,000 011	0,001 419	0,000 653
Pipette	s_{5i}^2	0,003 949	0,001 794	0,001 415

Step 4: Mean values, residual variance, residual standard deviation for each level i, as shown in Tables B.8 and B.9.

Table B.8 — Laboratory mean values and overall mean values

		Alternative method		
		Low $i = 1$	**Medium** $i = 2$	**High** $i = 3$
Laboratory mean values	$\bar{y}_{i1}$	2,096	3,185	3,670
	$\bar{y}_{i2}$	2,431	2,936	4,088
	$\bar{y}_{i3}$	2,454	2,929	4,125
	$\bar{y}_{i4}$	2,496	3,000	4,073
	$\bar{y}_{i5}$	2,448	2,934	3,736
Overall mean	$\bar{y}_i$	2,385	2,997	3,938

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Table B.9 — Residual laboratory standard deviation

	Alternative method		
	Low	**Medium**	**High**
s_{Bi}^2	0,015 503	0,007 35	0,032 803
s_{Bi}	0,125	0,086	0,181

Step 5: Reproducibility variance and standard deviation for each level *i*, as shown in Table B.10.

Table B.10 — Reproducibility standard deviation

	Alternative method		
	Low	**Medium**	**High**
s_{Ri}^2	0,053 379	0,022 961	0,073 320
s_{Ri}	0,231	0,152	0,271

Summary: As shown in Table B.11.

Table B.11 — Summary

	Reference method			Alternative method		
	Low	**Medium**	**High**	**Low**	**Medium**	**High**
Median $\log_{10}$	2,490	2,950	4,095	2,450	2,970	3,985
Repeatability standard deviation, s_r	0,114	0,093	0,109	0,144	0,093	0,127
s_1	0,114	0,051	0,144	0,094	0,041	0,111
s_2	0,135	0,049	0,080	0,005	0,028	0,077
s_3	0,023	0,052	0,079	0,066	0,037	0,063
s_4	0,082	0,049	0,062	0,003	0,038	0,026
s_5	0,055	0,054	0,057	0,063	0,042	0,038
Intermediate standard deviation, s_A	0,234	0,147	0,229	0,195	0,125	0,201
Residual laboratory standard deviation, s_B	0,189	0,188	0,179	0,125	0,086	0,181
Reproducibility standard deviation, s_R	0,301	0,239	0,291	0,231	0,152	0,271
Pooled average reproducibility standard deviation	0,278			0,223		
NOTE For reasons of readability, the index *i* is not used in this table.						

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B.3.3 Accuracy profile

The calculation of the accuracy profile was conducted in accordance with the steps described in ISO 16140-2:2016, 6.2.3.

Step 1: Average of the reference values X_i for each level *i*, as shown in Table B.12.

In accordance with 6.3, as average of the reference values, the respective median will be used.

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Table B.12 — Median values of reference method

Reference method		
Low	**Medium**	**High**
X_1	X_2	X_3
2,490	2,950	4,095

Step 2: Reproducibility variance s_{Ri}^2 for each level *i*, as shown in Table B.13.

Taken from Step 5 (see 6.3) calculated above.

Table B.13 — Reproducibility variances for reference method

Alternative method		
Low	**Medium**	**High**
s_{R1}^2	s_{R2}^2	s_{R3}^2
0,053 379	0,022 961	0,073 320

Step 3: Global average $\bar{y}_i$ of measurements made with the alternative method, as shown in Table B.14.

Table B.14 — Mean values of alternative method

Alternative method		
Low	**Medium**	**High**
$\bar{y}_1$	$\bar{y}_2$	$\bar{y}_3$
2,385	2,997	3,938

Step 4: Absolute bias $\bar{y}_i - X_i$, as shown in Table B.15.

Table B.15 — Bias of alternative method

Bias		
Low	**Medium**	**High**
$\bar{y}_1 - X_1$	$\bar{y}_2 - X_2$	$\bar{y}_3 - X_3$
−0,105	0,047	−0,157

Step 5 and Step 6: Limits of the β-ETI, as shown in Table B.16.

Table B.16 — Limits of the β-ETI

	Low $i = 1$	**Medium** $i = 2$	**High** $i = 3$
n	8	8	8
p	5	5	5
s_{ri}^2	0,020 8	0,008 6	0,016 2
$s_{Li}^2 = s_{Ri}^2 - s_{ri}^2$	0,032 5	0,014 3	0,057 1
s_{Ri}^2	0,053 4	0,022 9	0,073 3
H (intermediate parameter)	0,744	0,853	2,024
G (intermediate parameter)	0,501	0,487	0,419
s_{TIi}^2	0,058 7	0,025 4	0,083 7

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Table B.16 *(continued)*

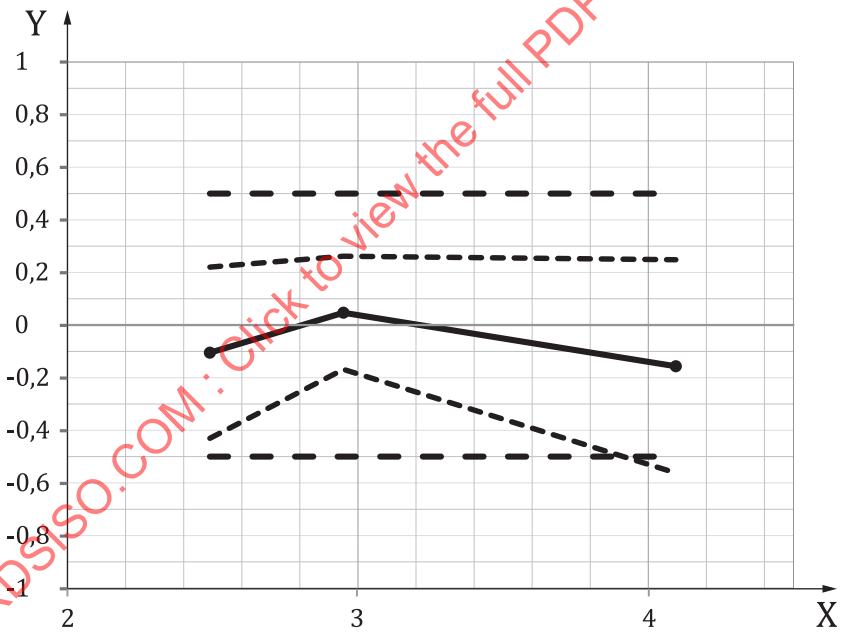
		Low $i = 1$	Medium $i = 2$	High $i = 3$
v		14,432	13,151	7,773
k_{Mi}		1,343	1,349	1,400
Limits of β-ETI	U_i	2,695	3,201	4,317
	L_i	2,075	2,793	3,559

NOTE Table B.16 provides the correct k_{Mi} values. An Excel®-based program[2)] does not allow the calculation of correct quantile values for non-integer degrees of freedom. Only a rough approximation is possible.

Step 7: Graphical representation of computed results (see Figure B.3), as shown in Table B.17.

Table B.17 — Limits of accuracy profile

	Low $i = 1$	Medium $i = 2$	High $i = 3$
$L_i - X_i$	−0,415	−0,157	−0,536
$U_i - X_i$	0,205	0,251	0,222



Key

X reference value ($\log_{10}$)

Y accuracy: difference $\log_{10}$(alt) – $\log_{10}$(ref)

absolute bias

upper and lower β-ETI

line of zero bias

upper and lower acceptability limits

Figure B.3 — Accuracy profile of the alternative method using β = 80 % and λ = 0,5 $\log_{10}$

2) Excel® is the trade name of a product supplied by Microsoft and is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

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Step 8: Additional evaluation procedure.

For the high target contamination level, the lower β-ETI falls outside the ±0,5 $\log_{10}$ AL. Hence, an additional evaluation procedure is followed.

Pooled average reproducibility standard deviation of the reference method, as shown by Formula (B.1):

$$s_{R,\text{ref}} = 0{,}278 \tag{B.1}$$

Step 9: New ALs (see Figure B.4), as shown by Formula (B.2):

$$\text{AL}_\text{s} = 3{,}3 \cdot s_{R,\text{ref}} = 0{,}918 \tag{B.2}$$

Summary: As shown in Table B.18.

Table B.18 — Summary

Step			Low $i = 1$	Medium $i = 2$	High $i = 3$
1	X_i		2,490	2,950	4,095
2	s_{Ri}^2		0,053 4	0,022 9	0,073 3
3	$\overline{y}_i$		2,385	2,997	3,938
4	$\overline{y}_i - X_i$		−0,105	0,047	−0,157
5/6	n		8	8	8
	p		5	5	5
	s_{ri}^2		0,020 8	0,008 6	0,016 2
	$s_{Li}^2 = s_{Ri}^2 - s_{ri}^2$		0,032 5	0,014 3	0,057 1
	H		0,744	0,853	2,024
	G		0,501	0,487	0,419
	s_{Tli}^2		0,058 7	0,025 4	0,083 7
	ν		14,432	13,151	7,773
	k_{Mi}		1,343	1,349	1,400
	Limits of β-ETI	U_i	2,695	3,201	4,317
		L_i	2,075	2,793	3,559
7	$L_i - X_i$		−0,415	−0,157	−0,536
	$U_i - X_i$		0,205	0,251	0,222
8	$s_{R,\text{ref}}$			0,278	
9	AL_s			0,918	

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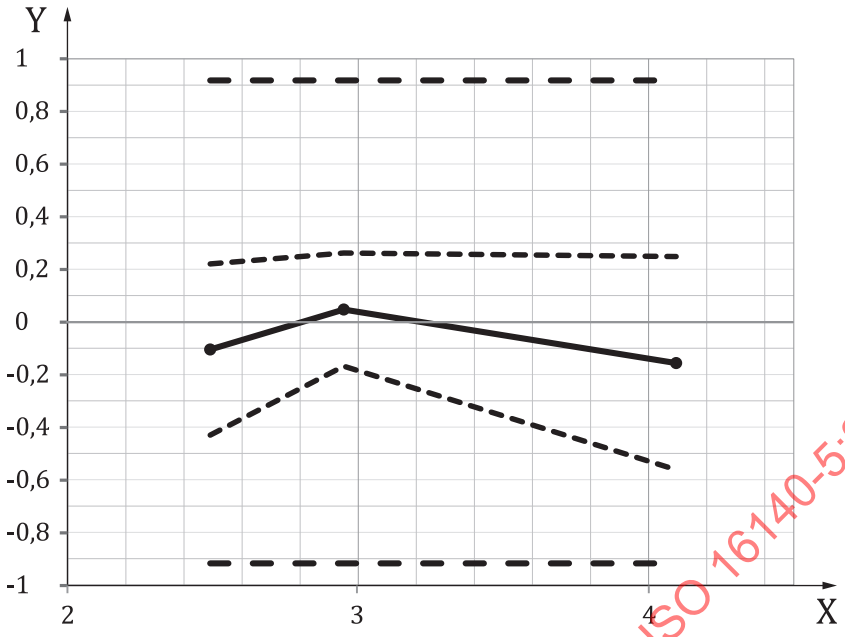

Key

X	reference value ($\log_{10}$)
Y	accuracy: difference $\log_{10}$(alt) – $\log_{10}$(ref)
—●—	absolute bias
- - - - - -	upper and lower β-ETI
———	line of zero bias
- - - -	upper and lower acceptability limits

Figure B.4 — Accuracy profile of the alternative method using β = 80 % and corrected ALs based on the reproducibility standard deviation of the reference method in accordance with steps 8 and 9

$U_i - X_i \leq AL_s$ and $L_i - X_i \geq -AL_s$ for all i in the accuracy profile. Therefore, the alternative method is accepted as being equivalent to the reference method.

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