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AMENDMENT 1
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Microbiology of the food chain — Method validation —

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

AMENDMENT 1: Revision of qualitative method comparison study data evaluation, relative level of detection calculations in the interlaboratory study, calculation and interpretation of the relative trueness study, and inclusion of a commercial sterility testing protocol for specific products

Microbiologie de la chaîne alimentaire — Validation des méthodes —

Partie 2: Protocole pour la validation de méthodes alternatives (commerciales) par rapport à une méthode de référence

AMENDEMENT 1: Révision de l'évaluation des données des études de comparaison de méthodes qualitatives, des calculs du niveau de détection de l'étude interlaboratoires et de l'interprétation de l'étude de justesse relative, et ajout d'un protocole pour la détermination de la stérilité commerciale pour des produits spécifiques

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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 document 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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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.

Microbiology of the food chain — Method validation —

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

AMENDMENT 1: Revision of qualitative method comparison study data evaluation, relative level of detection calculations in the interlaboratory study, calculation and interpretation of the relative trueness study, and inclusion of a commercial sterility testing protocol for specific products

Introduction

Replace the text with the following:

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 this document, ISO 16140-5 and ISO 16140-6). In the case when a method is validated within one laboratory (see ISO 16140-4), no interlaboratory study is conducted.

— The second stage is method verification, where a laboratory demonstrates that it can satisfactorily perform a validated method. This is described in 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 17468:2023, 3.7, 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. 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 this document cannot be reached. ISO 16140-4 and ISO 16140-5 can be used for validation against a reference method. ISO 16140-4 (regarding qualitative and quantitative methods) and ISO 16140-5 (regarding quantitative methods only) can also be used for validation without a reference method.

The flow chart in Figure 0.1 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.

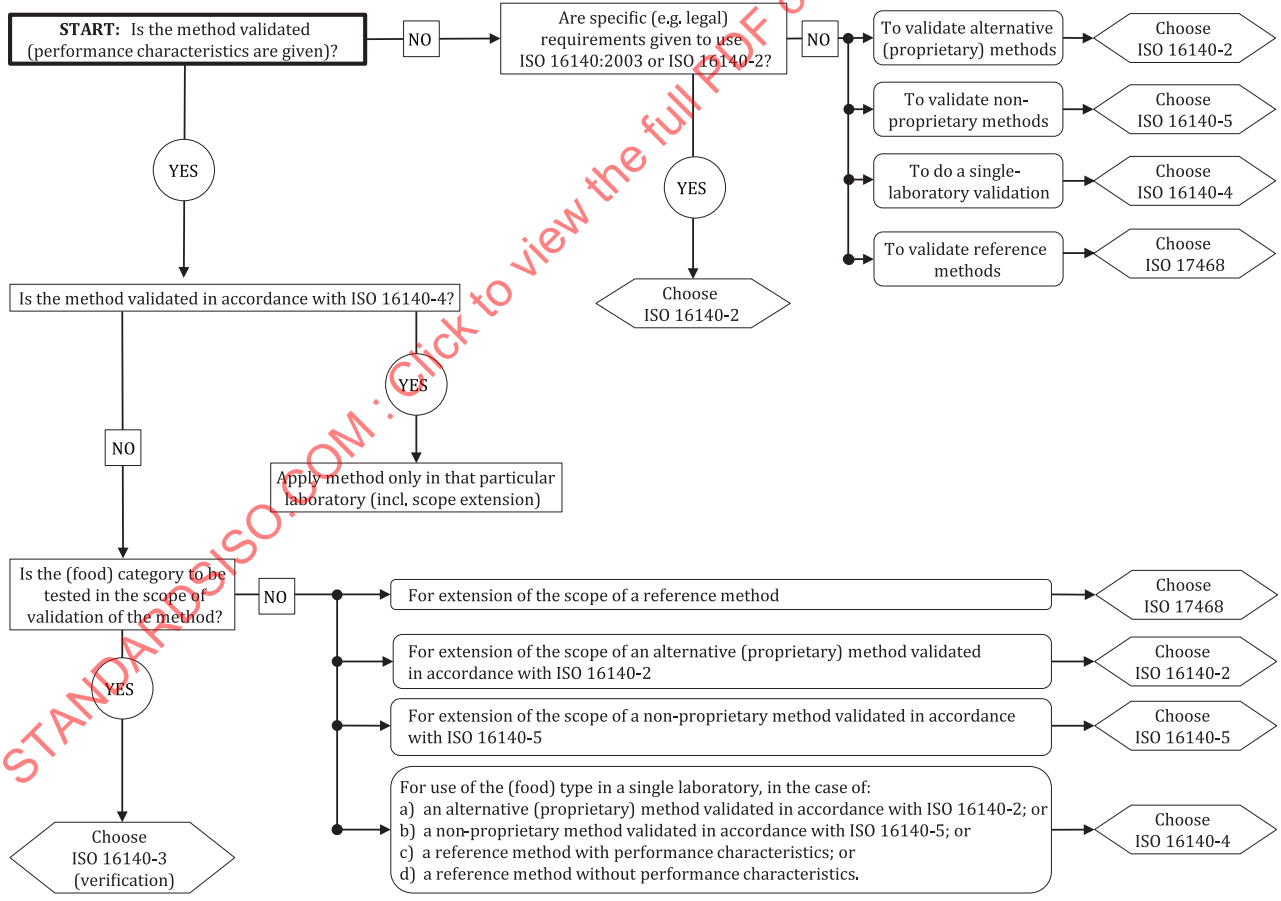


Figure 0.1 — Flow chart for application of the ISO 16140 series

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 0.2 shows the possibilities where an alternative confirmation method validated in accordance with ISO 16140-6 can be applied (see text in the boxes).

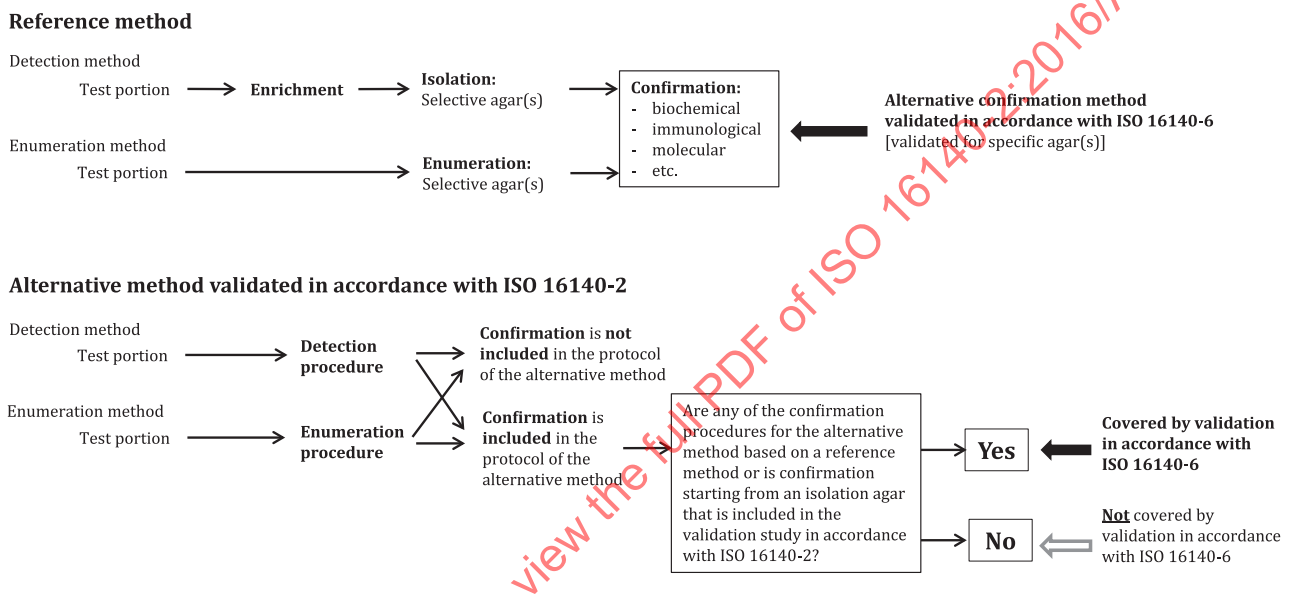


Figure 0.2 — Use of validated alternative confirmation methods (see ISO 16140-6)

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 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

This document describes the general approach to method validation in the field of microbiology of the food chain and serves as a fundamental basis to the other parts of the ISO 16140 series, which cross-reference to it. An understanding of the performance characteristics, the (food) categories, the technical protocol and data analysis as outlined in this document provides support in the application of the ISO 16140 series in general.

Clause 4

Add the following text at the end of the clause:

For the validation of an alternative qualitative method, a corresponding qualitative reference method is selected for carrying out the validation study. This is commonly done using test portions of 10 gram, 25 gram or higher. In some cases, it can be of interest to validate a qualitative alternative method against a quantitative reference method, using smaller test portion sizes.

EXAMPLE 1 *Enterobacteriaceae* criterion for pasteurized milk and other liquid pasteurized products in Regulation (EC) No 2073/2005 is < 10 cfu/ml and refers to the quantitative method ISO 21528-2.

In such situations, it is of interest to validate the performance of qualitative alternative methods against the specified (quantitative) reference method. To that end, the technical protocol for the validation of qualitative methods (see Clause 5) is to be used. For such a validation study, the quantitative results of the reference method have to be converted into qualitative results prior to interpretation according to Clause 5.

EXAMPLE 2 When one or more colonies are observed on a plate using 1 ml of a 10^{-2} dilution, this result corresponds to a positive detection in 0,01 gram.

NOTE Annex J provides the special case of validation of a method for commercial sterility testing for specific products [sterilized or ultra high temperature (UHT) dairy and plant-based liquid products].

If a technical change in a validated alternative (proprietary) method is evaluated as being major, a re-validation of this alternative method in accordance with this document is needed.

When the re-validation of the alternative method is conducted, the impact on the performance characteristics shall be evaluated to determine if the changes are to be regarded as major (performance characteristics have substantially changed) or minor (no or minor impact on performance characteristics observed). In certain cases, a major technical change in the method can be considered to be minor, if the re-validation study shows that it has no significant impact on the performance characteristics or test results. A major (technical) change that, after re-validation, has a major impact on the performance characteristics of the alternative method, requires re-verification of the method by the user laboratory in accordance with ISO 16140-3.

5.1.1

Add the following text at the end of the subclause:

The organizing laboratory shall be competent to perform both the reference method as well as the alternative method.

NOTE Competence can be demonstrated in different ways (e.g. for the reference method, a documented proof of meeting the requirements of ISO/IEC 17025, and for the alternative method, a documented training).

5.1.2

Add the following text at the end of the subclause:

When the reference and alternative methods are based on two different principles and are performed with the same test portion, but do not share a common enrichment procedure, an unpaired data study is performed. For example, when a qualitative alternative method is validated against a quantitative reference method at a limit of 100 cfu/g. In this case, a suspension of the (food) item can be used to inoculate both culture media for the reference method and the alternative method before any enrichment/multiplication of the microorganism.

5.1.3.3

Add the following text at the end of the subclause:

The alternative method shall be evaluated for a defined test portion size (e.g. 25 g, 200 g, 375 g) during the validation study. The method is considered to be validated for any test portion size up to the validated test portion size if the testing protocol (dilution ratio, incubation time and incubation temperature) is the same as that used during the validation study.

EXAMPLE A reference method used in a validation study of this document was validated for a "broad range of foods" using a 25 g test portion and a 1:10 dilution ratio. The alternative method was validated for a "broad range of foods" using a 375 g test portion and a 1:5 dilution ratio at a determined incubation time. In practice, a user laboratory can use the alternative method for all food items (broad range of foods) using a test portion size up to 375 g test portion and a 1:5 dilution ratio at the validated incubation time (unless stated differently by the organization involved in the method validation).

5.1.3.4

Add the following text before the last sentence of the second paragraph:

The interpretation of the results (positive agreement, negative agreement, etc.) is based on a comparison of the reference method result (column 1 in Tables 1 and 2) and the alternative method result, including any confirmations as described in the alternative method protocol (column 2 in Tables 1 and 2). When positive or negative deviations are obtained, a footnote should be included at the end of each table to provide additional explanations for the interpretation of the deviations. The footnotes indicate if the result is due to a false positive or false negative result of the alternative method. The footnote is a comparison of the results of the alternative method (including any confirmations as described in the alternative-method protocol) (column 2 in Tables 1 and 2) and the confirmed alternative method (by any means) (column 3 in Tables 1 and 2).

5.1.3.4, after the second paragraph

Replace the text with the following:

Table 1 — Comparison and interpretation of sample results between the reference and alternative methods for a paired study

Result of the (reference or alternative) method per sample			
Reference method	**Alternative method** (including any confirmations as described in the alternative-method protocol)	**Confirmed alternative method** (by any means)[a]	**Interpretation** (based on the confirmed alternative-method result)
+	+	Not needed[b]	Positive Agreement (PA)
-	-	Not needed[b]	Negative Agreement (NA)
+	-	Not needed[b]	Negative Deviation due to false negative alternative-method result ($ND_{FN(alt)}$)
-	+	+	Positive Deviation (PD)
-	+	-	Positive Deviation due to false positive alternative-method result ($PD_{FP(alt)}$)[c]

[a] Confirmation of the alternative-method result is done according to 5.1.3.3.

[b] No need for additional confirmation test(s). Confirmed alternative-method result is the same as the alternative-method result.

[c] This false positive result (FP) shall also be used to calculate the false positive ratio.

Table 2 — Comparison and interpretation of sample results between the reference and alternative methods for an unpaired study

Result of the (reference or alternative) method per sample			
Reference method	**Alternative method** (including any confirmations as described in the alternative-method protocol)	**Confirmed alternative method** (by any means)[a,b]	**Interpretation** (based on the confirmed alternative-method result)
+	+	+	Positive Agreement (PA)
+	+	-	Positive Agreement due to false positive alternative-method result ($PA_{FP(alt)}$)[c]
-	-	-	Negative Agreement (NA)
-	-	+	Negative Agreement due to false negative alternative-method result ($NA_{FN(alt)}$)
+	-	-	Negative Deviation (ND)
+	-	+	Negative Deviation due to false negative alternative-method result ($ND_{FN(alt)}$)
-	+	+	Positive Deviation (PD)
-	+	-	Positive Deviation due to false positive alternative-method result ($PD_{FP(alt)}$)[c]

[a] Confirmation of the alternative-method result is done according to 5.1.3.3

[b] Confirmation by any means is only required when the result of the alternative method does not produce viable organisms. This is used as the confirmed alternative method result in comparison to the reference method result.

[c] These false positive results (FP) shall also be used to calculate the false positive ratio.

Determine the Total Negative Deviation (TND) and Total Negative Agreement (TNA) for the validation study.

Paired evaluation: Total Negative Deviation: TND = $ND_{FN(alt)}$

Total Negative Agreement: TNA = NA + $PD_{FP(alt)}$

Unpaired evaluation: Total Negative Deviation: TND = ND + $ND_{FN(alt)}$ + $PA_{FP(alt)}$

Total Negative Agreement: TNA = NA + $NA_{FN(alt)}$ + $PD_{FP(alt)}$

Table 3 — Summary of results obtained with the reference and alternative methods (after confirmation) of all samples for each category

	Reference-method positive (R+)	**Reference-method negative** (R-)
Alternative-method positive (A+)	+/+ Positive Agreement (PA)	-/+ Positive Deviation (PD)
Alternative-method negative (A-)	+/– Total Negative Deviation (TND)	–/– Total Negative Agreement (TNA)

Based on data summarized in Table 3 for the combined categories per category and per type, calculate the values for sensitivity of the alternative method (see Formula (1)) and of the reference method (see Formula (2)), as well as the relative trueness (see Formula (3)) and false positive ratio and false negative ratio for the alternative method after the additional confirmation of the results (see Formula (4)).

Sensitivity for the alternative method:

$$SE_{alt} = \frac{(PA + PD)}{(PA + TND + PD)} \times 100\,\% \quad (1)$$

Sensitivity for the reference method:

$$SE_{ref} = \frac{(PA + TND)}{(PA + TND + PD)} \times 100\,\% \quad (2)$$

Relative trueness:

$$RT = \frac{(PA + TNA)}{N} \times 100\,\% \quad (3)$$

False positive ratio (FPR) and false negative ratio (FNR) for the alternative method:

$$\text{Paired evaluation: } FPR = \frac{PD_{FP(alt)}}{TNA} \times 100\,\%$$

$$\text{Unpaired evaluation: } FPR = \frac{PA_{FP(alt)} + PD_{FP(alt)}}{TNA} \times 100\,\% \quad (4)$$

$$\text{False negative ratio: } FNR = \frac{NA_{FN(alt)} + ND_{FN(alt)}}{PA + TND + PD}$$

where

N is the total number of samples (PA + PD + TND + TNA);

FP is the false positive results;

FN is the false negative results.

For explanation of the abbreviated terms used, see Tables 1 to 3.

The confirmed alternative-method results shall be used to determine whether the alternative method produces comparable results to the reference method.

Calculate the difference between (TND – PD) for both paired and unpaired studies and the sum of (TND + PD) for paired studies. Check whether the difference and/or sum of PD and TND conform to the Acceptability Limit (AL) stated in Table 4 with respect to the type of study (paired or unpaired) and the number of categories used in the evaluation.

NOTE 1 Acceptability Limits (AL) are based on data and consensus expert opinion. The AL are not based on statistical analysis of the data.

The interpretation of results shall be done per category and for all categories used in the validation study. An interpretation of results shall also be done per enrichment protocol in case different protocols are used for different types of samples. A sensitivity study can also exist of a partly paired and unpaired study. In that case the results for (TND + PD) shall be evaluated based on the number of positive samples obtained for the categories tested using the paired study design. The results for (TND – PD) shall be evaluated based on the number of positive samples obtained for the full study (so all categories belonging to both the paired and unpaired study design).

The AL is not met when the observed value is higher than the AL. When the AL is not met, if the number of positive samples is higher than expected according to the number of categories (e.g. having 60 or more positive samples for one single category), it is possible to use the second column of Table 4 and switch to higher AL. When the AL is not met, investigations should be made (e.g. root cause analysis) in order to provide an explanation of the observed results. Based on the AL and the additional information, it is decided whether the alternative method is regarded as not fit for purpose for the category or categories involved. The reasons for acceptance of the alternative method in case the AL is not met shall be stated in the study report.

Table 4 — Acceptability limit parameters and values for a paired and unpaired study design in relation to the number of positive samples obtained

Number of categories	Number of positive samples (N+)	Paired study		Unpaired study	Mixed study[c]	
		(TND[a] - PD[b])	(TND + PD)	(TND - PD)	(TND - PD)	(TND + PD)
1	30 to 59	3	6	3	3	6
2	60 to 89	4	8	4	4	8
3	90 to 119	5	10	5	5	10
4	120 to 149	5	12	5	5	12
5	150 to 179	5	14	5	5	14
6	180 to 209	6	16	6	6	16
7	210 to 239	6	18	7	7	18
8	240 to 269	6	20	7	7	20
9	270 to 299	7	22	8	8	22
10	300 to 329	7	24	8	8	24
11	330 to 359	7	26	9	9	26
12	360 to 389	8	28	9	9	28
13	390 to 419	8	30	10	10	30
14	420 to 449	8	32	10	10	32
15	450 to 479	9	34	11	11	34
16	480 to 509	9	36	11	11	36
17	510 to 539	9	38	12	12	38
18	540 to 569	10	40	12	12	40

[a] TND = total number of samples with Negative Deviation results.

[b] PD = number of samples with Positive Deviation results.

[c] Mixed study includes both paired and unpaired study design.

Table 4 *(continued)*

Number of categories	Number of positive samples (N+)	Paired study		Unpaired study	Mixed study[c]	
		(TND[a] - PD[b])	(TND + PD)	(TND - PD)	(TND - PD)	(TND + PD)
19	570 to 599	10	42	13	13	42
20	600 to 629	10	44	13	13	44
21	630 to 659	11	46	14	14	46
22	660 to 689	11	48	14	14	48
23	690 to 719	11	50	15	15	50
24	720 to 749	12	52	15	15	52
25	750 to 779	12	54	16	16	54

[a] TND = total number of samples with Negative Deviation results.

[b] PD = number of samples with Positive Deviation results.

[c] Mixed study includes both paired and unpaired study design.

NOTE 2 A negative value for (TND – PD) is acceptable as this indicates a better performance of the alternative method compared to the reference method.

NOTE 3 Information on differences observed between results of the alternative method before and after confirmation of the results (step 1 and step 2) according to the alternative-method protocol is commonly presented in the validation report as additional information but is not used in the overall assessment of the alternative-method performance.

5.1.4, last sentence

Replace the text with the following:

The level of contamination shall be determined. This allows calculation of the LOD_{50} of the alternative method, which is required in order to verify the performance of the alternative method upon implementation of the validated method in a laboratory in accordance with ISO 16140-3. The level of contamination is determined by performing a most probable number (MPN) analysis on the (stabilized) inoculated samples (preferably) and/or through the enumeration of the inoculum at the time of inoculation, see 5.1.4.3.

5.1.4.1, second paragraph

Replace the text with the following:

A minimum of three levels per type shall be prepared consisting of at least a negative control level, a low level and a higher level. Ideally, the low level shall be the theoretical detection level (i.e. 0,7 cfu per test portion) and the higher level just above the theoretical detection level (e.g. 1 cfu to 1,5 cfu per test portion). For fastidious bacteria, the low level can significantly exceed the theoretical detection level; thus, the low and high level should be adjusted appropriately. A fixed ratio (e.g. 1:2) between the low- and high-level contamination should be used to aid in determining the final contamination levels. At least the low level should provide fractional recovery by either the reference method or the alternative method (fractional recovery at the low level should be between 25 % and 75 % of the number of samples tested). An evaluation shall be performed to ensure the relationship and consistency of the number of positives of the intermediate and high level. In cases where the alternative method produces fractional recovery at the low level and the reference method produces all positive results, the results of the RLOD study are not valid and a root cause analysis shall be performed.

The level of contamination of the sample used (except for the negative control) shall be determined as in 5.1.4. At the negative control level, at least five replicate samples should be tested by both methods. For the second (low) level (theoretical detection level), at least 20, and for the third (higher) level, at least five replicate samples should be tested by both methods. The negative control level shall not produce positive (by isolation of the target organism) results. When positive results are obtained, the experiments have to be repeated for all levels.

5.1.4

Add the following text at the end of the subclause:

5.1.4.3 Calculation of the LOD_{50}

LOD_{50} shall be calculated for each category for the alternative method and optionally for the reference method. The LOD_{50} is used in method verification (see ISO 16140-3).

For each category evaluated, determine the contamination of the low level by performing a 3-level MPN for the particular (food) item tested using the reference method (preferably) at the time of the RLOD experiment and/or by the enumeration of the inoculum at the time of inoculation using a non-selective medium. When enumeration is performed, the inoculum for the low and high contamination levels should be determined.

The MPN and 95 % confidence interval shall be determined for the fractional level only. For the low (or fractional) contamination level, analyse 20 test portions plus 5 test portions at approximately 2 times the test portion size and 5 test portions at approximately half of the test portion size evaluated in the validation study (e.g. if the reference method test portions were 25 g, evaluate 5 test portions at 50 g and 5 test portions at 10 g). To each test portion, add a proportionate amount of enrichment broth as described in the reference method to maintain the enrichment volume to mass ratio (e.g. a reference method with a 1:10 enrichment ratio, add 450 ml to the 50 g test portions and 90 ml to the 10 g test portions). Analyse the test portions following the reference method from enrichment to confirmation. Use the number of positive results per test portion size to calculate the MPN value. An Excel®-based program[1)] for calculating MPN values is freely available for download at https://standards.iso.org/iso/7218 (download the file "MPN calculation Excel program").

Enumerate the inoculum at the time of inoculation by plating onto non-selective agar (see ISO 7218 for guidance on plating). Agar plates should be incubated under conditions to allow for optimal growth of the target microorganism.

Use the number of positive results per test portion size and the MPN value or the results of the enumeration of the inoculum to calculate the LOD_{50} and 95 % confidence interval. An Excel®-based program for calculating LOD_{50} values is freely available for download at https://standards.iso.org/iso/16140/-2/ed-1/en/amd/1/ (download the file "PODLOD_ver12"). The LOD_{50} value is calculated per category tested in the RLOD study and shall be expressed as cfu/test portion.

NOTE The 20 test portions from the low level are the same as the 20 test portions used in the RLOD study. Therefore, only 5 test portions at 2 times and 5 at approximately half of the test portion size are analysed in addition to the RLOD study.

1) Excel® 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.

5.2.1

Add the following text after the second sentence of the first paragraph:

The interlaboratory study shall be conducted with collaborators belonging to more than one country.

The collaborators shall be competent to perform both the reference method as well as the alternative method.

NOTE Competence can be demonstrated in different ways (e.g. for the reference method, a documented proof of meeting the requirements of ISO/IEC 17025, and for the alternative method, a documented training).

5.2.2, third bullet

Replace the text with the following:

— at least three different levels of contamination shall be used: a negative control (L_0) and two levels (L_1 and L_2). At least one of these shall produce fractional positive results. The level of contamination needed to obtain fractional recovery shall be based on the RLOD study data of the reference method in the method comparison study. Theoretically, an average level of contamination of 1 cfu/sample is adequate to obtain fractional recovery. The level of contamination shall be determined. This allows calculation of the LOD_{50} of the alternative method, which is required in order to verify the performance of the alternative method upon implementation of the validated method in a laboratory in accordance with ISO 16140-3. The level of contamination is determined by performing an MPN analysis at the time of the start of the interlaboratory study, see 5.2.4.4.

5.2.3, title

Replace the text with the following:

Summary of data and trueness calculations

5.2.3, after the third paragraph

Replace the text with the following:

Table 9 — Summarized results for all collaborators for a paired study

Result of the (reference or alternative) method per sample			
Reference method	**Alternative method[a]**	**Confirmed alternative method[b]**	**Interpretation** (based on the confirmed alternative-method result)
+	+	Not needed[c]	Positive Agreement (PA)
-	-	Not needed[c]	Negative Agreement (NA)
+	-	Not needed[c]	Negative Deviation due to false negative alternative-method result ($ND_{FN(alt)}$)
-	+	+	Positive Deviation (PD)
-	+	-	Positive Deviation due to false positive alternative-method result ($PD_{FP(alt)}$)[d]

[a] The alternative-method results includes any confirmations as described in the alternative-method protocol.

[b] The confirmed alternative-method result is the result after additional confirmation as described in the protocol for the validation study.

[c] No need for additional confirmation test(s). Confirmed alternative-method result is the same as the alternative-method result.

[d] This false positive result (FP) shall also be used to calculate the false positive ratio.

Table 10 — Summarized results for all collaborators for an unpaired study

Result of the (reference or alternative) method per sample			
Reference method	**Alternative method[a]**	**Confirmed alternative method[b]**	**Interpretation** (based on the confirmed alternative-method result)
+	+	+	Positive Agreement (PA)
+	+	-	Positive Agreement due to false positive alternative-method result ($PA_{FP(alt)}$)[c]
-	-	-	Negative Agreement (NA)
-	-	+	Negative Agreement due to false negative alternative-method result ($NA_{FN(alt)}$)
+	-	-	Negative Deviation (ND)
+	-	+	Negative Deviation due to false negative alternative-method result ($ND_{FN(alt)}$)
-	+	+	Positive Deviation (PD)
-	+	-	Positive Deviation due to false positive alternative-method result ($PD_{FP(alt)}$)

[a] The alternative-method result includes any confirmations as described in the alternative-method protocol.

[b] The confirmed alternative-method result is the result after additional confirmation as described in the protocol for the validation study.

Determine the Total Negative Deviation (TND) and Total Negative Agreement (TNA) for the validation study.

Paired evaluation: Total Negative Deviation: $TND = ND_{FN(alt)}$

Total Negative Agreement: $TNA = NA + PD_{FP(alt)}$

Unpaired evaluation: Total Negative Deviation: $TND = ND + ND_{FN(alt)} + PA_{FP(alt)}$

Total Negative Agreement: $TNA = NA + NA_{FN(alt)} + PD_{FP(alt)}$

Table 11 — Summary of results for all collaborators obtained with the reference and alternative methods (after confirmation) for level L_1 or L_2

	Reference-method positive (R+)	**Reference-method negative** (R-)
Alternative-method positive (A+)	+/+ Positive Agreement (PA)	-/+ Positive Deviation (PD)
Alternative-method negative (A-)	+/− Total Negative Deviation (TND)	−/− Total Negative Agreement (TNA)

Based on data summarized in Table 11, calculate the values for sensitivity of the alternative method (see Formula (8)) and of the reference method (see Formula (9)), as well as the relative trueness (see Formula (10)) and false positive ratio and false negative ratio for the alternative method after the additional confirmation of the results (see Formula (11)).

Sensitivity for the alternative method:

$$SE_{alt} = \frac{(PA + PD)}{(PA + TND + PD)} \times 100\,\% \quad (8)$$

Sensitivity for the reference method:

$$SE_{ref} = \frac{(PA + TND)}{(PA + TND + PD)} \times 100\,\% \quad (9)$$

Relative trueness:

$$RT = \frac{(PA + TNA)}{N} \times 100\,\% \quad (10)$$

False positive ratio (FPR) and false negative ratio (FNR) for the alternative method:

$$\text{Paired evaluation: } FPR = \frac{PD_{FP(alt)}}{TNA} \times 100\,\%$$

$$\text{Unpaired evaluation: } FPR = \frac{PA_{FP(alt)} + PD_{FP(alt)}}{TNA} \times 100\,\% \quad (11)$$

$$\text{False negative ratio: } FNR = \frac{NA_{FN(alt)} + ND_{FN(alt)}}{PA + TND + PD}$$

where

N is the total number of samples (TNA + PA + PD + TND);

FP is the false positive results;

FN is the false negative results.

For explanation of the abbreviated terms used, see Tables 9 to 11.

The confirmed alternative-method results shall be used to determine whether the alternative method produces comparable results to the reference method.

5.2.4

Replace the text with the following:

5.2.4 Interpretation of trueness data

5.2.4.1 Paired study

For a paired study, calculate the difference between (TND – PD) and the sum of (TND + PD) for the level(s) where fractional recovery was obtained (so L_1 and possibly L_2). The values found for (TND – PD) and (TND + PD) shall not be higher than the Acceptability Limits (ALs) given in Table 12 with respect to the number of participating laboratories (N_{lab}).

Table 12 — Acceptability limits for a paired study design in relation to the number of collaborating laboratories

N_{lab}	(TND – PD)	(TND + PD)
10	3	4
11	4	4
12 to 13	4	5
14 to 16	4	6
17	4	7
18	5	7
19 to 20	5	8

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

5.2.4.2 Unpaired study

For an unpaired study, calculate the difference between (TND – PD) for the level(s) where fractional recovery was obtained (so L_1 and possibly L_2). The observed value found for (TND – PD) shall not be higher than the AL. The AL is defined as $[(TND - PD)_{max}]$ and calculated per level where fractional recovery was obtained as described below using the following three parameters:

$$(p+)_{ref} = \frac{P_{x(ref)}}{N_{x(ref)}} \quad (12)$$

where

$P_{x(ref)}$ is number of samples with a positive result obtained with the reference method at level x (L_1 or L_2) for all laboratories;

$N_{x(ref)}$ is number of samples tested at level x (L_1 or L_2) with the reference method by all laboratories.

$$(p+)_{alt} = \frac{CP_{x(alt)}}{N_{x(alt)}} \quad (13)$$

where

$CP_{x(alt)}$ is number of samples with a confirmed positive result obtained with the alternative method at level x (L_1 or L_2) for all laboratories;

$N_{x(alt)}$ is number of samples tested at level x (L_1 or L_2) with the alternative method by all laboratories.

$$(TND - PD)_{max} = \sqrt{3N_{x(ref)} \times \left((p+)_{ref} + (p+)_{alt} - 2\left((p+)_{ref} \times (p+)_{alt}\right)\right)} \quad (14)$$

where $N_{x(ref)}$ is number of samples tested at level x (L_1 or L_2) with the reference method by all laboratories.

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

5.2.4.3 Calculation of the relative level of detection

For both a paired and unpaired study, the variation of the relative levels of detection (RLOD) between laboratories shall be assessed. Conduct this evaluation in accordance with Annex F. The ALs for the RLOD of paired and unpaired studies are found in 5.1.4.2.

In addition, the data can be evaluated using the probability of detection (POD) model described in Reference [14] and included in the AOAC validation guidelines.[6] The evaluation using the POD model can give additional information on the equivalence of the methods.

5.2.4.4 Calculation of the LOD_{50}

LOD_{50} shall be calculated for each category used in the interlaboratory study for the alternative method and optionally for the reference method. The LOD_{50} is used in method verification (see ISO 16140-3). For method verification, it is only necessary to calculate the LOD_{50} for the alternative method. This shall be done by the expert laboratory only at the time the interlaboratory study starts. The MPN and 95 % confidence interval shall be determined for the fractional level only. Determine for the L_1 level the contamination level by performing a 3-level MPN for the particular (food) item tested in the interlaboratory study using the reference method. For the low (or fractional) contamination level, analyse 20 test portions plus 5 test portions at approximately 2 times the test portion size and 5 test portions at approximately half of the test portion size evaluated in the validation study (e.g. if the reference method test portions were 25 g, evaluate 5 test portions at 50 g and 5 test portions at 10 g). To each test portion, add a proportionate amount of enrichment broth as described in the reference method to maintain the enrichment volume to mass ratio (e.g. a reference method with a 1:10 enrichment ratio, add 450 ml to the 50 g test portions and 90 ml to the 10 g test portions). Analyse the test portions following the reference method from enrichment to confirmation. Use the number of positive results per test portion size to calculate the MPN value. An Excel®-based program for calculating MPN values is freely available for download at https://standards.iso.org/iso/7218 (download the file "MPN calculation Excel program").

Use the number of positive results per test portion size from the alternative method and the MPN value obtained using the reference method to calculate the LOD_{50} of the alternative method and 95 % confidence interval. An Excel®-based program for calculating LOD_{50} values is freely available for download at https://standards.iso.org/iso/16140/-2/ed-1/en/amd/1/ (download the file "PODLOD-interlab"). The LOD_{50} value shall be expressed as cfu/test portion.

6.1.1

Add the following text at the end of the subclause:

The organizing laboratory shall be competent to perform both the reference method as well as the alternative method.

NOTE Competence can be demonstrated in different ways (e.g. for the reference method, a documented proof of meeting the requirements of ISO/IEC 17025, and for the alternative method, a documented training).

6.1.2.3, first paragraph after Figure 1

Replace the text with the following:

Determine the average of each pair of data values and the difference between the values as in Table 13 and plot these differences against the corresponding averages per category and for all categories to illustrate the degree of agreement between the reference method and the alternative method. Figure 2 shows the line of identity (zero difference), the line of bias (average difference) as well as the upper and lower 95 % prediction limits of the individual sample-specific bias values. These limits of the prediction range are called "limits of agreement".

6.1.2.3, text after Table 13

Replace the text with the following:

Compute per category and for all categories the average difference $\bar{D}$, the standard deviation of differences s_D and the limits of agreement using Formula (15):

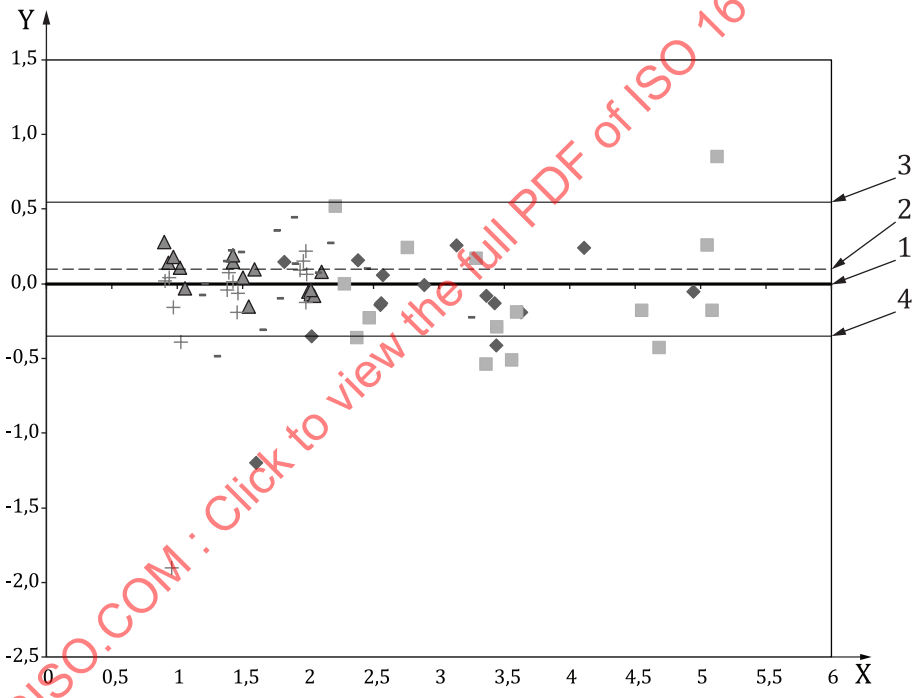
$$\left[\bar{D} \pm T \cdot \sqrt{\frac{s_D^2}{n}}\right] \tag{15}$$

where

n is the number of data pairs;

T is the percentile of a Student's t distribution for the prediction probability β (β = 95 % is used) and for $(n-1)$ degrees of freedom, that is: $T_{\left(\frac{1-\beta}{2}\right);(n-1)}$.

Plot as in Figure 2 the individual sample differences against the mean values on a graph that shows the line of identity (zero difference), the line of bias (average difference) as well as and the upper and lower 95 % prediction limits (limits of agreement) of the individual sample-specific bias values (both separately per category and across all categories). This illustrates the degree of bias and the (lack of) agreement of the data.



Key

◆ cat. 1
■ cat. 2
▲ cat. 3
+ cat. 4
- cat. 5

X mean of $\log_{10}$ cfu/g
Y difference (alternative - reference)
1 line of identity
2 overall bias
3 95 % upper limit of agreement
4 95 % lower limit of agreement

Figure 2 — Bland-Altman difference plot for all categories

The results of the difference and scatter plot shall be interpreted based on a visual observation of the overall bias, the spread of the individual sample-specific bias values, and any bias values lying outside the limits of agreement. If the individual bias values are normally distributed, it can be expected that 1 in 20 values will lie outside the limits of agreement. Discrepancies can be seen as an indication that the individual bias values do not follow a normal distribution, e.g. due to the presence of outliers. Any such departures from expectations should be documented.

6.1.3.3

Replace Formula (19) with the following:

$$s_{\text{ref}} = \sqrt{\frac{1}{q}\sum_{i=1}^{q} s_{\text{ref},i}^2} \qquad (19)$$

Replace the second sentence below Figure 3 with the following text:

If any of the upper or lower limits exceeds the Acceptability Limits and the standard deviation, S_{ref} > 0,125 but ≤ 0,25, the following additional evaluation procedure (step 9) is followed:

Add the following text below the second sentence:

In cases of S_{ref} > 0,25, no recalculation of the AL is allowed. In this case, investigations should be made (e.g. root cause analysis) in order to provide an explanation of the high S_{ref}, correct possible issues and, as an option, repeat the experiment.

6.2.1

Add the following text at the end of the first paragraph:

The interlaboratory study shall be conducted with collaborators belonging to more than one country.

The collaborators shall be competent to perform both the reference method as well as the alternative method.

NOTE Competence can be demonstrated in different ways (e.g. for the reference method, a documented proof of meeting the requirements of ISO/IEC 17025, and for the alternative method, a documented training).

6.2.3, title and header of Table 18

Replace the text with the following:

Table 18 — Summary of the results of the interlaboratory study per each analyte level (k_i)

Collaborators (k)	**Level** (k_i)	**Reference method** x_{ijk}	**Alternative method** y_{ijk}
		Result	**Result**

Annex F

Replace the text with the following:

Annex F
(normative)

Calculation of the relative level of detection (RLOD) using data from the interlaboratory study

F.1 General

The relative level of detection (RLOD) is estimated using data from the method comparison study in Annex D. To further characterize the performance of a qualitative method in terms of detection capability, it is necessary to estimate the magnitude of variation of RLOD between laboratories using data from the interlaboratory study.

RLOD is estimated in the interlaboratory study using similar statistical models as in the method comparison study (see Annex D). In the case of the interlaboratory study, it is investigated whether the RLOD values differ between laboratories. A random effect model is used for the laboratory effect to estimate the RLOD from the interlaboratory study. In this random effect model, it is assumed that the laboratories taking part to the interlaboratory study are chosen at random from the population of laboratories using the method, thus the results of the interlaboratory study are valid for all laboratories using the method. The statistical approach is detailed in References [27] and [28].

Given it is assumed that the contamination levels of the samples in the interlaboratory study are known, the LODs of the alternative method, LOD_{alt}, and of the reference method, LOD_{ref}, are estimated separately and RLOD is derived as their ratio.

F.2 Estimation of LOD

Calculate the LOD_{50} (contamination level giving a 50 % probability of detection or an expectation of 50 % positive results with the method) of the reference method and of the alternative method as follows.

For a given method (reference or alternative method), the number of positive samples, y, at a contamination level d in laboratory i is binomial distributed with expectation p, see Formula (F.1):

$$p = p_i(\mathrm{d}) = 1 - \exp(-F_0 L_i \mathrm{d}) = 1 - \exp(-\exp(\mu + l_i + \ln d)) \qquad \text{(F.1)}$$

where

d is the contamination level of the sample, in cfu/test portion;

F_0 is the fixed effect of the method;

L_i is the random effect of the laboratory i;

l_i $= \ln L_i$;

μ $= \ln F_0$.

The laboratory effects l_i are assumed to be normally distributed with mean 0 and laboratory standard deviation σ.

p according to Formula (F.1) is transformed into η, see Formula (F.2):

$$\eta = \ln(-\ln(1-p)) = \mu + l_i + \ln d \quad \text{(F.2)}$$

η is a complementary log-log (CLL) model, a special case of the general linear mixed model. ln$d$ are known; μ and σ are estimated with the maximum likelihood method. The estimates $\hat{\mu}$, $\hat{\sigma}$ and the approximate standard error of $\hat{\mu}$, $s_{\hat{\mu}}$, are used to calculate the level of detection LOD of the method, see Formula (F.3), i.e. the estimated LOD of the population of laboratories, for a specified probability of detection, p, see Formula (F.1).

$$\mathrm{LOD}_p = \frac{-\ln(1-p)}{e^{\hat{\mu}}} \quad \text{(F.3)}$$

An approximate confidence interval of the LOD of the method for a specified probability of detection, p and the confidence level $1 - \alpha$ is given in Formula (F.4):

$$\left(\frac{-\ln(1-p)}{e^{\hat{\mu}+t_{\nu;1-\alpha/2}\cdot s_{\hat{\mu}}}}, \frac{-\ln(1-p)}{e^{\hat{\mu}-t_{\nu;1-\alpha/2}\cdot s_{\hat{\mu}}}}\right) \quad \text{(F.4)}$$

where $t_{\nu;1-\alpha/2}$ is the $(1-\alpha/2)$-quantile of the t-distribution with ν degrees of freedom, i.e. the value $t_{\nu;1-\alpha/2}$ for which the cumulative distribution function of the t-distribution is equal to $1-\alpha/2$: $P(t_\nu \leq t_{\nu;1-\alpha/2}) = 1-\alpha/2$, and $\nu = k - 1$ where k is the number of participating laboratories.

F.3 Relative level of detection (RLOD) of the alternative method as compared to that of the reference method

The RLOD is the ratio of LODs of the alternative method and of the reference methods calculated in Clause F.2, see Formula (F.5).

$$\mathrm{RLOD} = \frac{LOD_{\mathrm{p;alt}}}{LOD_{\mathrm{p;ref}}} = \exp\left(\hat{\mu}_{\mathrm{ref}} - \hat{\mu}_{\mathrm{alt}}\right) \quad \text{(F.5)}$$

An Excel®-based program for the calculation of the LOD according to this annex and Reference [27] is freely available for download at https://standards.iso.org/iso/16140/-2/ed-1/en/amd/1/ (download the file "PODLOD-interlab").

An acceptability limit (AL) of 2,5 is defined for RLOD for unpaired studies and of 1,5 for paired studies. Any RLOD ≤ 2,5 for unpaired studies (1,5 for paired studies) is interpreted as the LOD of the alternative method does not differ significantly from the LOD of the reference method. A RLOD > 2,5 for unpaired studies (>1,5 for paired studies) is interpreted as the alternative method has a LOD significantly larger than the LOD of the reference method.

F.4 Example

F.4.1 General

The example data set (see Table F.1) for the RLOD calculations concerns an interlaboratory unpaired study of an alternative method for the detection of *Listeria monocytogenes* in milk at three levels of contamination, with 10 participating laboratories. Each laboratory used the reference method (see ISO 11290-1) and the alternative method (alt). The number of repeated measurements is $n = n_{\mathrm{ref}} = n_{\mathrm{alt}} = 8$ for each contamination level in each laboratory and $y = y_{\mathrm{ref}} = y_{\mathrm{alt}}$ = the number of positive results obtained. Given the unpaired study design, RLOD has an acceptability limit (AL) of 2,5.

Table F.1 — Example of a data set from an interlaboratory study of an alternative method for the detection of *Listeria monocytogenes* in milk

Laboratory	d[cfu/25 g]	n_{ref}	y_{ref}	n_{alt}	y_{alt}
A	0,000	8	0	8	0
B	0,000	8	0	8	0
D	0,000	8	0	8	0
F	0,000	8	0	8	0
G	0,000	8	0	8	0
H	0,000	8	0	8	0
J	0,000	8	0	8	0
L	0,000	8	0	8	0
M	0,000	8	0	8	0
O	0,000	8	0	8	0
A	0,096	8	8	8	8
B	0,096	8	6	8	6
D	0,096	8	7	8	7
F	0,096	8	8	8	8
G	0,096	8	7	8	7
H	0,096	8	5	8	5
J	0,096	8	6	8	5
L	0,096	8	7	8	7
M	0,096	8	6	8	6
O	0,096	8	7	8	7
A	1,012	8	8	8	8
B	1,012	8	8	8	8
D	1,012	8	8	8	8
F	1,012	8	8	8	8
G	1,012	8	8	8	8
H	1,012	8	8	8	8
J	1,012	8	8	8	8
L	1,012	8	8	8	8
M	1,012	8	8	8	8
O	1,012	8	8	8	8

F.4.2 Reference method

The estimates of the parameters defined in Clause F.2 are $\hat{\mu}_{\text{ref}} = -0{,}278$, $\hat{\sigma}_{\text{ref}} = 0$, $s_{\hat{\mu}_{\text{ref}}} = 0{,}140$. $\text{LOD}_{\text{ref}} = 0{,}037$ with a 95 % confidence interval 0,027 – 0,050. Since $\hat{\sigma}_{\text{ref}} = 0$, there is no difference in the performance of the laboratories.

F.4.3 Alternative method

The estimates of the parameters defined in Clause F.2 are $\hat{\mu}_{\text{alt}} = -0{,}320$, $\hat{\sigma}_{\text{alt}} = 0$, $s_{\hat{\mu}_{\text{alt}}} = 0{,}139$. The $\text{LOD}_{\text{alt}} = 0{,}038$ with a 95 % confidence interval 0,028 – 0,052. Since $\hat{\sigma}_{\text{alt}} = 0$, there is no difference in the performance of the laboratories.

F.4.4 RLOD of the alternative method as compared to the reference method

The RLOD is the ratio of the LOD of the alternative method by the LOD of the reference method, as in Formula (F.6):

$$\text{RLOD} = \frac{LOD_{\text{alt}}}{LOD_{\text{ref}}} = \exp\left(\hat{\mu}_{\text{ref}} - \hat{\mu}_{\text{alt}}\right) = \exp\left(-0,278 + 0,320\right) = 1,04 \qquad \text{(F.6)}$$

Thus, the alternative method is only very slightly less sensitive (4 % higher LOD) than the reference method, due to only one less positive result for a sample at the intermediate contamination level in laboratory J. Given that the RLOD of 1,04 is less than AL (2,5), the LOD of the alternative method is satisfactory in comparison to the LOD of the reference method.

Annex H, Table H.1

Replace the row "Step 1" with the following:

Step 1	X_i	**1,74**	**2,11**	**2,68**	**2,72**	**3,65**	**3,77**
Alternative	1	2,00	1,95	2,95	2,63	3,96	3,89
	2	1,78	1,40	2,91	2,71	3,38	3,96
	3	1,85	1,78	2,66	2,68	3,57	3,78
	4	1,93	1,70	2,72	2,72	3,53	3,79
	5	1,65	1,81	2,76	2,76	3,89	3,78

Replace the row "Step 2" with the following:

Step 2	Y_i	**1,85**	**1,78**	**2,76**	**2,71**	**3,57**	**3,78**

Annex H, Table H.2

Replace the table with the following:

Sample	**Central value (Ref)**	**Central value (Alt)**	**Bias**	**Upper** β-ETI	**Lower** β-ETI	**Upper AL**	**Lower AL**
Sample 1	1,74	1,85	0,105	0,330	−0,120	0,5	−0,5
Sample 2	2,11	1,78	−0,336	−0,111	−0,561	0,5	−0,5
Sample 3	2,68	2,76	0,082	0,307	−0,143	0,5	−0,5
Sample 4	2,72	2,71	−0,008	0,217	−0,234	0,5	−0,5
Sample 5	3,65	3,57	−0,085	0,140	−0,310	0,5	−0,5
Sample 6	3,77	3,78	0,014	0,240	−0,211	0,5	−0,5