



BSI Standards Publication

Microbiology of the food chain — Method validation

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

National foreword

This British Standard is the UK implementation of EN ISO 16140-2:2016+A1:2024. It is identical to ISO 16140-2:2016, incorporating amendment 1:2024. It supersedes BS EN ISO 16140-2:2016, which is withdrawn.

The start and finish of text introduced or altered by amendment is indicated in the text by tags. Tags indicating changes to ISO text carry the number of the ISO amendment. For example, text altered by ISO amendment 1 is indicated by A1 A1.

The UK participation in its preparation was entrusted to Technical Committee AW/9, Microbiology.

A list of organizations represented on this committee can be obtained on request to its committee manager.

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English Version

**Microbiology of the food chain - Method validation - Part 2:
Protocol for the validation of alternative (proprietary)
methods against a reference method (ISO 16140-2:2016)**

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 (ISO 16140-2:2016)

Mikrobiologie der Lebensmittelkette -
Verfahrensvalidierung - Teil 2: Arbeitsvorschrift für die
Validierung von alternativen (urheberrechtlich
geschützten) Verfahren anhand eines
Referenzverfahrens (ISO 16140-2:2016)

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European foreword

This document (EN ISO 16140-2:2016) has been prepared by Technical Committee ISO/TC 34 "Food products" in collaboration with Technical Committee CEN/TC 275 "Food analysis - Horizontal methods" the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by January 2017, and conflicting national standards shall be withdrawn at the latest by January 2017.

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Endorsement notice

The text of ISO 16140-2:2016 has been approved by CEN as EN ISO 16140-2:2016 without any modification.

European foreword to Amendment 1

This document (EN ISO 16140-2:2016/A1:2024) has been prepared by Technical Committee ISO/TC 34 "Food products" in collaboration with Technical Committee CEN/TC 463 "Microbiology of the food chain" the secretariat of which is held by AFNOR.

This Amendment to the European Standard EN ISO 16140-2:2016 shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by March 2025, and conflicting national standards shall be withdrawn at the latest by March 2025.

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Endorsement notice

The text of ISO 16140-2:2016/Amd 1:2024 has been approved by CEN as EN ISO 16140-2:2016/A1:2024 without any modification.

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Foreword

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

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

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: [Foreword - Supplementary Information](#)

The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This first edition of ISO 16140-2, together with ISO 16140-1, cancels and replaces ISO 16140:2003, which has been technically revised. It also incorporates the Amendment ISO 16140:2003:Amd.1:2011.

ISO 16140 consists of the following parts, under 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*

The following parts are under preparation:

- *Part 3: Protocol for the verification of reference and validated alternative methods implemented in a single laboratory*
- *Part 4: Protocol for single-laboratory (in-house) method validation*
- *Part 5: Protocol for factorial interlaboratory validation of non-proprietary methods*
- *Part 6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing*

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.

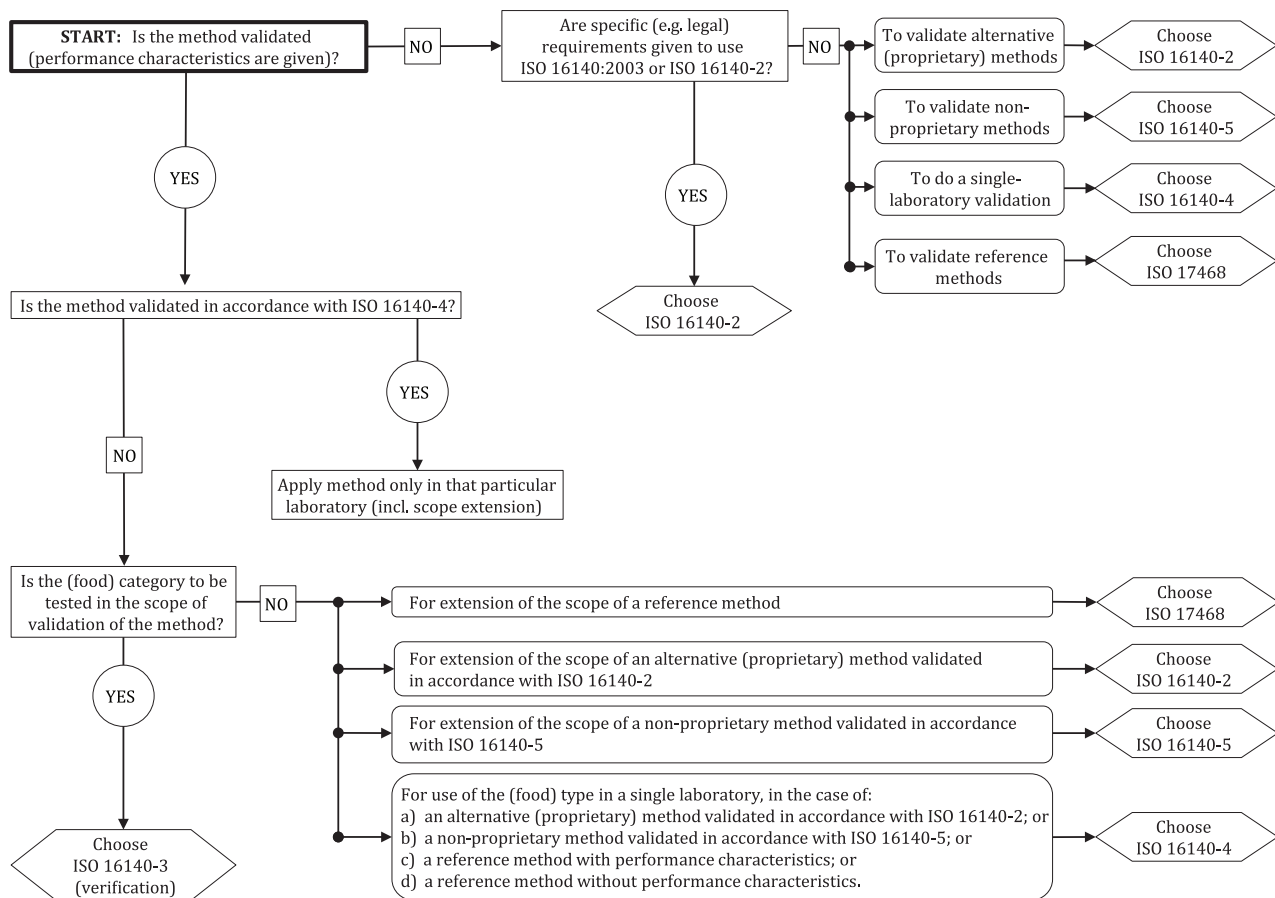
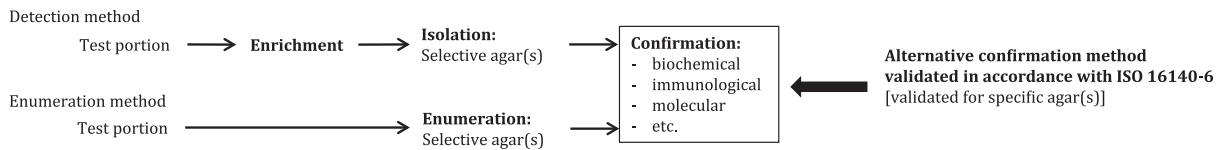


Figure 0.1 — Flow chart for application of the ISO 16140 series

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).

Reference method



Alternative method validated in accordance with ISO 16140-2

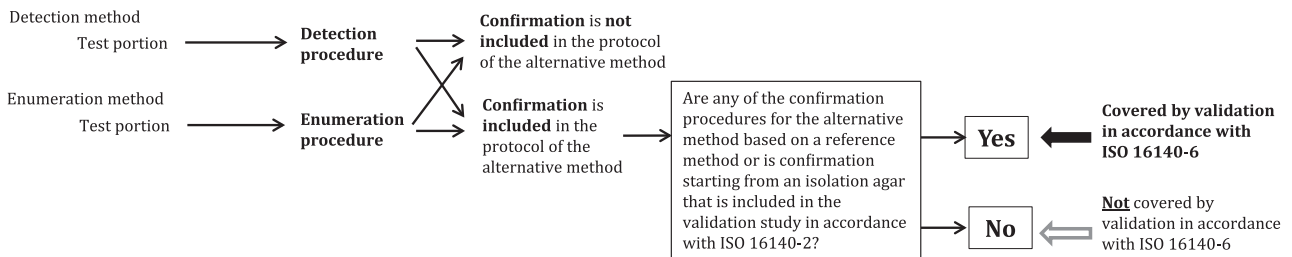


Figure 0.2 — Use of validated alternative confirmation methods (see ISO 16140-6)

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. ^(A1)

Microbiology of the food chain — Method validation —

Part 2:

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

1 Scope

This part of ISO 16140 specifies the general principle and the technical protocol for the validation of alternative, mostly proprietary, methods for microbiology in the food chain. Validation studies according to this part of ISO 16140 are intended to be performed by organizations involved in method validation.

This part of ISO 16140 is applicable to the validation of methods 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, and
- samples from the primary production stage.

This part of ISO 16140 is in particular applicable to bacteria and fungi. Some clauses of this part of ISO 16140 could be applicable to other (micro) organisms or their metabolites on a case-by-case-basis. In the future, guidance for other organisms (e.g. viruses and parasites) will be included in either this part or a separate part of ISO 16140.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 16140-1, *Microbiology of the food chain— Method validation — Part 1: Vocabulary*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 16140-1 apply.

4 General principles for the validation of alternative methods

The validation protocol comprises two phases:

- a method comparison study of the alternative (proprietary) method against the reference method carried out in the organizing laboratory;
- an interlaboratory study of the alternative (proprietary) method against the reference method carried out in different laboratories.

The technical rules for performing the method comparison study and the interlaboratory study are given in [Clause 5](#) and [Clause 6](#), depending upon whether the alternative (proprietary) method is

qualitative or quantitative in nature. The data generated in some parts of the validation study are evaluated using the so-called Acceptability Limits (AL) and no statistical evaluation of the data are conducted. These AL are based on experts' opinion and data generated in existing validation studies.

[A₁] 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. **[A₁]**

5 Qualitative methods — Technical protocol for validation

5.1 Method comparison study

5.1.1 General considerations

The method comparison study is the part of the validation process that is performed in the organizing laboratory. It consists of three parts namely the following:

- a comparative study of the results of the reference method to the results of the alternative method in (naturally and/or artificially) contaminated samples (so-called sensitivity study);
- a comparative study to determine the relative level of detection (RLOD) in artificially contaminated samples (so-called RLOD study);
- an inclusivity/exclusivity study of the alternative method.

The results (tables and calculations) of the different parts and the interpretation of the results, including discrepant results, shall be given in a study report.

Test portions size shall be used as written in the reference method.

A1 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). **A1**

5.1.2 Paired or unpaired study

The reference and alternative methods shall be performed with, as far as possible, exactly the same sample (same test portion). However, a distinction is made between studies where the same test portion can be used for both the reference and the alternative method due to both methods having exactly the same first step in the (enrichment) procedure and those where different test portions need to be used for the reference and the alternative method (e.g. due to different enrichment broths). In the case where the same test portion is used for both methods, the results from both methods are highly related to each other. For example, when the sample is not contaminated, both methods should find the result of that sample negative. Due to this relationship, the data produced by the reference and the alternative method are named **paired** or matched. In this part of ISO 16140, the wording “paired study” will be used for this type of study.

The opposite situation where there is no shared initial (enrichment) step for both the reference and the alternative method is also possible. In this case, different test portions coming from the same batch or lot of product have to be used for the two methods and the resulting data are named **unpaired** or unmatched. In this part of ISO 16140, the word “unpaired study” will be used for this type of study. The choice of having a **paired** study or an **unpaired** study depends on the protocols of the reference and alternative method. If there is a common initial step in the (enrichment) procedures, a **paired** study design is mandatory.

This clause describes the method comparison study if the reference and alternative method have a joint initial step in the (enrichment) procedures (**paired** study) and if the reference and alternative method do not have a joint initial (enrichment) step (**unpaired** study). Differences between both types of studies are indicated in the text where appropriate.

A1 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. **A1**

5.1.3 Sensitivity study

The sensitivity study aims to determine the difference in sensitivity between the reference and the alternative method. This study is conducted using naturally and/or artificially contaminated samples. Different categories and types shall be tested for this. Acceptability Limits have been defined for the maximum acceptable difference depending on the type of study (paired/unpaired) and the number of categories tested.

5.1.3.1 Selection of categories to be used

The selection of categories and types used within the validation will depend on the type or group of microorganism and the scope of the validation.

If the method is to be applied for a broad range of foods, then at least five categories of food shall be studied. The validation study report shall state the food categories used in the study. If the method is to be validated for a restricted number of food categories, e.g. “ready-to-eat, ready-to-reheat meat products”, and “heat-processed milk and dairy products”, then only these categories need to be studied. In addition to food, feed samples, environmental samples, and primary production stage samples can be included as additional categories. This will broaden the application of the use of the alternative method for these additional categories.

For all selected categories (food and others), at least three different types per category shall be included in the study. [Annex A](#) presents an overview of the relevant types and categories for specific microorganisms that might be relevant for the validation. [Annex A](#) should be used to facilitate the selection of categories, types, and items for the specific microorganism involved. It should not be regarded as a mandatory choice.

When selecting samples for the study, it is of the highest priority to find those that are naturally contaminated. If it is not possible to acquire a sufficient number of naturally contaminated samples, artificial contamination of samples is permissible (see [Annex B](#) and [Annex C](#)). Details on the preparation of the artificially inoculated samples should be given in the validation study report. It is desirable that food samples come from as wide a distribution as possible in order to reduce any bias from local food specialities and to broaden the range of validation.

It shall be ensured that with the selection of the different types, both high and low (natural) background microflora, different types of stresses due to processing, and raw (unprocessed) items are included in the study.

EXAMPLE For the validation of a method for detection of *Listeria monocytogenes* and the category “ready-to-eat, ready-to-reheat meat products”, the types can be (1) cooked meat products (lower background flora, heat stress), (2) fermented or dried meat products (high background flora, pH stress), and (3) raw cured (smoked) ($a_w < 0,92$) (intermediate background flora, a_w stress).

In some cases, for example, for an alternative method that is applicable for a broad range of foods, it is possible to combine the “ready-to-eat” and “raw” categories from the same product group. For example, the categories raw and ready-to-eat meat (products) can be combined into one category having three types divided over relevant raw and ready-to-eat food types. The selection of (combined) food categories should be based on risk analysis.

5.1.3.2 Number of samples

For each category being examined, a minimum of 60 individual samples shall be tested made up of at least three types with at least 20 samples representative for each type (three types $\times$ 20 samples for each type = 60 samples). Fractional positive results by either the reference or alternative method (i.e. samples should not be all positive or all negative) shall be obtained for each type tested. In the ideal situation, 10 samples (50 %) tested per type should be positive and 10 negative, but should range between 25 % and 75 %. For each category, at least 30 samples shall have a positive result by the reference and/or the alternative method.

5.1.3.3 Alternative-method result and confirmation

Many alternative-method protocols contain two steps, the first being the enrichment and detection step and the second being the confirmation of the detection result from step one. The end result of the alternative method is the result after step two. The end result will be the same as the result after enrichment and detection in case there is no confirmation step included in the protocol of the alternative method.

The (end) result of the alternative method shall be confirmed for the sensitivity study part. All results obtained with the alternative method in an unpaired study shall be confirmed. In a **paired** study, only the positive results obtained with the alternative method, for which the corresponding result with the reference method was negative, shall be confirmed. This confirmation is needed to determine whether the result is a true-positive or false-positive result. The confirmation test or tests shall be able to recover and confirm the identity of the isolate as being the target of the method. These test(s) can be based on the confirmation procedure of the reference method, the confirmation step of the alternative method in case this procedure is able to isolate and confirm the identity of the target analyte, a combination of both, or by any other means that is able to isolate and confirm the identity of the target analyte.

If the enrichments of the reference and alternative methods differ in terms of the number of enrichments (i.e. primary/non-selective and secondary/selective) or total duration of incubation, an additional confirmation pathway is necessary for the validation study. The first pathway shall be that to be used with the alternative method according to its procedure/instructions (regular testing conditions by the alternative method according to the kit insert procedure; this does not include the complementary tests which can be performed during the validation study). The second pathway shall divert a portion of the alternative method's incubated enrichment to that of the reference method such that at minimum, the total duration of incubation of the reference method enrichment(s) is/are respected. The results of the two confirmation pathways are to be reported separately.

[A1] 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). **[A1]**

5.1.3.4 Calculation and interpretation for sensitivity

In general, the data shall be presented in a report in order to have an overview of the raw data obtained. Information shall be given on the type of contamination (naturally contaminated or artificially contaminated) of the samples used, the type of study design that was used (e.g. **paired** study or **unpaired** study), and the confirmation test(s) used to confirm the alternative-method result. For artificially contaminated samples, the (reference to the) procedure used for preparation shall be specified (see also [Annex C](#)).

The results obtained for the reference and alternative methods originating from the same sample, meaning from one test portion in case of a **paired** study or two test portions in case of an **unpaired** study, shall be described for a **paired** study according to [Table 1](#) and for an **unpaired** study according to [Table 2](#). **[A1]** 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). **[A1]** [Table 3](#) is prepared for the summarized sample results for all categories per category (≥ 60 samples) and per type (≥ 20 samples) for both a **paired** and **unpaired** study.

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:

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

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

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

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. [A1](#)

5.1.4 Relative level of detection study

A comparative study is conducted to evaluate the level of detection (LOD) of the alternative method against the reference method. The evaluation is based on the calculation of the relative level of detection (RLOD). In the study, replicates of artificially contaminated samples are used at three or more levels of contamination. Preferably, the levels are known as it allows calculation of the LOD. [A1](#) The level of contamination shall be determined. This allows calculation of the LOD₅₀ 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. [A1](#)

5.1.4.1 Selection of categories, number of samples, and replicates tested

For the selection of categories and types, see [5.1.3.1](#). The same categories will be used as selected for the sensitivity study (see [5.1.3](#)). For each category, one relevant type is selected. In order to have a better representation of the evaluated category, this type should be different from those used in the sensitivity study (if possible). The samples shall be artificially inoculated. Procedures for the preparation of artificially inoculated samples are presented in [Annex C](#). Each type will be inoculated with a different strain.

[A1](#) 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. ^(A1)

Positive deviating test results obtained with the alternative method shall be additionally confirmed (see 5.1.3.3). The RLOD shall be evaluated after confirmation.

NOTE 1 In order to have a better assurance that fractional recovery will be obtained, more levels of contamination can be produced and tested.

NOTE 2 The level of contamination needed targets the LOD of the reference method if the alternative method has a lower LOD than the reference method.

5.1.4.2 Calculation and interpretation of the RLOD

The RLOD is defined as the ratio of the LODs of the alternative method and the reference method:

$$RLOD = \frac{LOD_{alt}}{LOD_{ref}}$$

(5)

For each category, at least the RLODs shall be estimated by fitting a complementary-log-log (CLL) model to the combined absence/presence data of both methods as a function of method. The contamination levels are not needed for the calculations of the RLOD since they are included in the model resulting in curves in a graph of probability of detection versus log dose (contamination level). The statistical model and the calculations are worked out in Annex D. Calculations can be performed with the Excel^{®1)} spreadsheet of this part of ISO 16140. The Excel[®] spreadsheet for calculating RLOD values is freely available for download at <http://standards.iso.org/iso/16140> and then select the RLOD file. For calculations using this Excel[®] spreadsheet, the option of “unknown concentration” shall be used. Calculate for each item *i* the RLOD_{*i*}. Tabulate the results as indicated in Table 5.

Table 5 — Presentation of RLOD before and after confirmation of the alternative-method results

	RLOD using the alternative-method results	RLOD using the confirmed alternative-method results
Item (category) (<i>i</i>)	RLOD _{<i>i</i>}	RLOD _{<i>i</i>}
1		
2		
...		
k		
Combined		

An Acceptability Limit (AL) for the RLOD based on the confirmed alternative-method results specifies the maximum increase in LOD of the alternative versus the reference method that would not be considered as relevant in consideration of the fitness for purpose of the method. Consequently, AL will be a value >1. The interpretation should be made for each item.

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.

The AL for **paired** study data are set at 1,5, meaning that the LOD for the alternative method shall not be higher than 1,5 times the LOD of the reference method. An LOD value for the alternative method smaller than the LOD value for the reference method is always accepted as this means that the alternative method is likely to detect lower levels of contamination than the reference method.

The AL for **unpaired** study data are set at 2,5, meaning that the LOD for the alternative method shall not be higher than 2,5 times the LOD of the reference method. An LOD value for the alternative method smaller than the LOD value for the reference method is always accepted as this means that the alternative method is likely to detect lower levels of contamination than the reference method.

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 for the item or category involved. The reasons for acceptance of the alternative method in case the AL is not met shall be stated in the study report.

In addition to the calculation of the RLOD, the data may be evaluated using the AOAC 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.

A1 5.1.4.3 Calculation of the LOD₅₀

LOD₅₀ shall be calculated for each category for the alternative method and optionally for the reference method. The LOD₅₀ 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 program²⁾ 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 LOD₅₀ and 95 % confidence interval. An Excel®-based program for calculating LOD₅₀ 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 LOD₅₀ 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. **A1**

A1 2) 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. **A1**

5.1.5 Inclusivity and exclusivity study

5.1.5.1 Selection and number of strains

A range of strains shall be used. Criteria for selecting test strains are given in [Annex E](#). The strains used should take into account the measurement principle of the alternative method (e.g. culture-based, immunoassay-based, and molecular). Different measurement principles may require the use of different test panels of strains.

Each strain used shall be characterized biochemically and/or serologically and/or genetically in sufficient detail for its identity to be known. Strains used should preferentially have been isolated from foods, feeds, the food-processing environment, or primary production taking into account the scope of the validation. However, clinical, environmental, and culture collection strains can be used. The original source of all isolates should be known and they should be held in a local (e.g. expert laboratory), national, or international culture collection to enable them to be used in future testing, if required.

For inclusivity testing, at least 50 pure cultures of (target) microorganisms shall be tested. For testing the inclusivity for *Salmonella* methods, at least 100 pure cultures of different serotypes of *Salmonella* shall be tested.

For exclusivity testing, at least 30 pure cultures of (non-target) microorganisms shall be tested.

Some microorganisms will be difficult or impossible to culture like viruses or protozan parasites. Where the target organism cannot be cultured, pure suspensions of the test strains should be used for spiking at the earliest appropriate step of the method.

NOTE 1 For some microorganisms, it will be difficult to obtain the required number of strains for inclusivity and exclusivity. In these cases, an agreed set of test strains should be selected by the parties involved in the validation study.

NOTE 2 Guidelines for the preservation and maintenance of strains in (local) collections can be found in ISO 11133.^[2]

5.1.5.2 Inoculation of target strains (inclusivity)

Each test is performed once and only with the alternative method (including a confirmation step if prescribed in the alternative-method protocol). Inoculation of a suitable growth medium is carried out with a dilution of a pure culture of each test strain. This culture is used for testing the inclusivity. No sample is added.

The pure culture should be grown in a non-selective broth under optimal conditions of growth to provide high cell populations in a stationary phase. The inoculum level shall be 10 times to 100 times greater than the minimum detection level of the alternative method being validated and the protocol of the alternative method shall be used including all (enrichments) detailed in the instructions of the alternative method. If the alternative method includes more than one (enrichment) protocol (e.g. for different sample types), then use the most challenging one with the complete panel of test strains. When negative or doubtful results are obtained, the test should be repeated and with the reference method included, checking that the strain could be detected with the appropriate reference method. If results are negative, consideration could be given to repeat the test with the addition of a food item. If the alternative protocol includes a confirmation step, the confirmation tests shall be included in testing the selected strains.

5.1.5.3 Inoculation of non-target strains (exclusivity)

Each test is performed once and only with the alternative method (including a confirmation step if prescribed in the alternative-method protocol). Inoculation of a suitable growth medium is carried out with a dilution of a pure culture of each test strain. This culture is used for testing the exclusivity. No sample is added.

The pure culture should be grown in a non-selective broth under optimal conditions of growth to provide high cell populations in a stationary phase. If the method involves a growth in a selective medium before a detection step, then for the purposes of exclusivity testing, the selective medium should be replaced by a non-selective medium. If the alternative method gives a positive or doubtful result, then the test should be repeated using the complete (enrichment) protocol recommended in the instructions of the alternative method using selective enrichments if these are noted in the instructions. If the alternative method includes more than one type of enrichment (e.g. for different sample types), then each of these should be used with the complete panel of test strains. Additionally, the reference method should be used to check that the strain could not be detected with the reference method.

5.1.5.4 Expression and interpretation of the results

Tabulate the results as in [Table 6](#). The interpretation shall be done by the laboratory in charge of the method comparison study. The report should state any anomalies from the expected results.

Table 6 — Presentation of the results for inclusivity and exclusivity

Microorganisms	Alternative method	
	Test result	Confirmed result
Inclusivity (target strains)		
1		
2		
Etc.		
Exclusivity (non-target strains)		
1		
2		
Etc.		

5.2 Interlaboratory study

5.2.1 General considerations

The aim of the interlaboratory study is to determine the difference in sensitivity between the reference and the alternative method when tested by different collaborators using identical samples (reproducibility conditions). The conditions for conducting the interlaboratory study should reflect, as much as possible, the normal conditions used by the individual collaborators in order to fulfil reproducibility conditions as far as possible.

[A1] 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). **[A1]**

The distinction between **paired** and **unpaired** studies is indicated in the text. However, no separate sections are given as the effect for the measurement protocol and analysis of data are limited. The interlaboratory study is managed by the organizing laboratory.

The results (tables and calculations) of the different parts and the interpretation of the results, including discrepant results, shall be given in a study report.

5.2.2 Measurement protocol

The interlaboratory study shall produce at least 10 valid data sets from at least 10 collaborators. The collaborators shall come from a minimum of five different organizations, but preferably 10 organizations excluding the organizing laboratory.

A maximum of three data sets can be produced by one organization. Technicians involved in the preparation of the samples used in the interlaboratory study shall not take part in the testing of those samples within the interlaboratory study.

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

The protocol is as follows

- in cases where different (enrichment) protocols for the alternative method exist, a challenging (enrichment) protocol shall be selected, e.g. the protocol having the shortest incubation time or the most selective conditions. The selected item shall be relevant for the chosen protocol. This relevant item (for selection see [Annex A](#)) which is used to prepare the test samples should contain a natural background microflora;
- the item shall be inoculated with the target organism. The protocol for inoculation of the samples shall be appropriate for the selected item. Samples shall be prepared by the organizing laboratory to ensure homogeneity between samples using preparation protocols contained in [Annex B](#) and [Annex C](#);
- A_1 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. A_1
- at least eight blind replicates at each level of contamination are analysed by each collaborator by both methods, so in total, a minimum of 48 results (eight replicates $\times$ three levels $\times$ two methods) per collaborator;
- for tests which give **paired** results [a **paired** result occurs when the initial (enrichment) step is the same for the alternative and reference method], one sample is required to obtain a result for the alternative and the reference method. For tests which give **unpaired** results [an **unpaired** result occurs when the alternative and reference methods start from different initial (enrichment) steps], different test portions from the same sample 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, the first giving the results from the reference method and the second giving the results 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), then the confirmation of the reference method also confirms the alternative method. In cases when the reference method gives a negative result and the alternative method gives a positive result, then confirmation of the positive result is required. If the results for alternative and reference methods have been obtained from different (enrichment) steps (**unpaired** data), then all results obtained with the alternative method shall be taken forward for confirmation. The procedure for confirmation should be included in the protocol of the study and shall be able to recover and confirm the identity of the isolate as being the target of the method;
- the organizing laboratory can indicate that broths, plates, and/or isolates shall be retained for a certain period of time to be able to confirm results obtained by a collaborator if needed;
- the analysis of samples shall be performed by each collaborator at the stipulated date;
- in either case, the combination “number of levels of contamination/number of replicates/number of non-outlier collaborators” shall be selected so that at least 480 results (240 by each method) are generated for use in the calculations for each method.

The organizing laboratory using all recorded data shall determine which results are suitable for use in analysing the data. The organizing laboratory shall examine the raw data and other information requested in the data sheet to ascertain that all collaborators have performed the analyses according

to both the alternative and reference methods as written. When there is evidence that results might be obtained under inappropriate conditions and/or the methods have not been followed strictly, these or all results from the collaborator are excluded for further analysis.

When the interlaboratory study is completed, all the information on data sheets and the results shall be submitted to the organizing laboratory and examined as follows:

- disregard data from collaborators if transit conditions and times fall outside the specified acceptable tolerances (the limits for transport time and temperature have to be set before the samples are shipped);
- disregard data from collaborators that received samples/test kits, etc. that were damaged during transportation;
- disregard data from collaborators using media formulation that are not in accordance with the (reference) method;
- disregard data from collaborators if the questionnaire suggests that the laboratory has deviated from either the standard protocol or the critical operating conditions.

5.2.3 A1 Summary of data and trueness calculations A1

The results obtained by the individual collaborators in the interlaboratory study are summarized in [Table 7](#) and in [Table 8](#).

Table 7 — Positive results by the reference method

Collaborators	Contamination level		
	L_0	L_1	L_2
Collaborator 1	/8 ^a	/8 ^b	/8 ^c
Collaborator 2	/8	/8	/8
Collaborator 3	/8	/8	/8
Etc.	/8	/8	/8
Total	P_0	P_1	P_2
a	Number of positive reference-method results at level 0.		
b	Number of positive reference-method results at level 1.		
c	Number of positive reference-method results at level 2.		

Table 8 — Positive results (before and after confirmation) by the alternative method

Collaborators	Contamination level					
	L_0		L_1		L_2	
Collaborator 1	/8 ^a	/8 ^b	/8 ^c	/8 ^d	/8 ^e	/8 ^f
Collaborator 2	/8	/8	/8	/8	/8	/8
Collaborator 3	/8	/8	/8	/8	/8	/8
Etc.	/8	/8	/8	/8	/8	/8
Total	P_0	CP_0	P_1	CP_1	P_2	CP_2
a	Number of positive alternative-method results at level 0.					
b	Number of confirmed alternative-method results at level 0.					
c	Number of positive alternative-method results at level 1.					
d	Number of confirmed alternative-method results at level 1.					
e	Number of positive alternative-method results at level 2.					
f	Number of confirmed alternative-method results at level 2.					