BS EN ISO 16140-2:2016

BSI Standards Publication

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)

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National foreword

This British Standard is the UK implementation of EN ISO 16140-2:2016. Together with BS EN ISO 16140-1:2016, it supersedes BS EN ISO 16140:2003+A1:2011 which is withdrawn.

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

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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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of an identical text or by endorsement, at the latest by January 2017, and conflicting national standards shall be withdrawn at the latest by January 2017.

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This document supersedes EN ISO 16140:2003.

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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.

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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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Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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

Today, many alternative, mostly proprietary, methods exist that are used to assess the microbiological quality of raw materials and finished products and the microbiological status of manufacturing procedures. These methods are often faster and easier to perform than the corresponding standardized method. The developers, end users, and authorities need a reliable common protocol for the validation of such alternative methods. The data generated will also provide potential end users with performance data for a given method, thus, enabling them to make an informed choice on the adoption of a particular method. The data generated can also be the basis for the certification of a method by an independent organization.

This part of ISO 16140

— is intended to provide a specific protocol and guidelines for the validation of proprietary methods intended to be used as a rapid and/or easier method to perform than the corresponding reference method,

— can also be used for the validation of other non-proprietary methods that are used instead of the reference method,

— is intended as the successor of the validation protocol published in the first version of ISO 16140 (ISO 16140:2003), and

— is mainly written for the validation of methods that are capable of culturing the target microorganism, but can also be applied to methods for microorganisms that cannot be cultured such as viruses (e.g. Norovirus) and protozan parasites (e.g. *Cryptosporidium* or *Giardia*). In these cases, some wordings are to be interpreted so as to fit the situation for non-culturable organisms.

The use of this part of ISO 16140 involves expertise on relevant areas such as microbiology, statistical design, and analysis as indicated in the respective sections. The statistical expertise encompasses overview of sampling theory and design of experiments, statistical analysis of (qualitative and quantitative) microbiological data, and overview of statistical concepts on random sampling, sample heterogeneity, sample stability, design of experiments, and variance components.

When this part of ISO 16140 is next reviewed, account will be taken of all information then available regarding the extent to which the guidelines have been followed and the reasons for deviation from them in the case of particular products.

The harmonization of validation methods cannot be immediate and for certain groups of products, International Standards and/or national standards may already exist that do not comply with this part of ISO 16140. It is hoped that when such standards are reviewed, they will be changed to comply with ISO 16140 so that eventually, the only remaining departures from this part of ISO 16140 will be those necessary for well-established technical reasons. For example, ISO 16297[3] deals with a very specific validation for a specific subject (the hygienic status of raw milk samples) and will remain as a vertical standard besides ISO 16140. If such a validation is needed, the vertical standard is more important.

INTERNATIONAL STANDARD ISO 16140-2:2016(E)

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.

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.

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.

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 × 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.

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. 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)
-	+	+	Positive Deviation (PD)
-	+	-	Negative Agreement due to false positive alternative-method result (NA)[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]	**Interpretation** (based on the confirmed alternative-method result)
+	+	+	Positive Agreement (PA)
+	+	-	Negative Deviation due to false positive alternative-method result (ND)[b]
-	-	-	Negative Agreement (NA)
-	-	+	Negative Agreement due to false negative alternative-method result (NA)
+	-	-	Negative Deviation (ND)
+	-	+	Negative Deviation due to false negative alternative-method result (ND)
-	+	+	Positive Deviation (PD)
-	+	-	Negative Agreement due to false positive alternative-method result (NA)[b]

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

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

Table 3 — Summary of results obtained with the reference and alternative methods 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-)	+/- Negative Deviation (ND)	−/− Negative Agreement (NA)

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 (1) and of the reference method (2), as well as the relative trueness (3) and false positive ratio for the alternative method after the additional confirmation of the results (4) as follows:

$$\text{Sensitivity for the alternative method: } SE_{alt} = \frac{(PA + PD)}{(PA + ND + PD)} \times 100\ \% \quad (1)$$

$$\text{Sensitivity for the reference method: } SE_{ref} = \frac{(PA + ND)}{(PA + ND + PD)} \times 100\ \% \quad (2)$$

$$\text{Relative trueness: } RT = \frac{(PA + NA)}{N} \times 100\ \% \quad (3)$$

$$\text{False positive ratio for the alternative method: } FPR = \frac{FP}{NA} \times 100\ \% \quad (4)$$

where *N* is the total number of samples (NA + PA + PD + ND) and FP is the false-positive results. For explanation of the abbreviations used, see Table 1 to Table 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 (ND – PD) for both **paired** and **unpaired** studies and the sum of (ND + PD) for **paired** studies. Check whether the difference and/or sum of PD and ND 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 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. 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 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 categories used

Number of categories	Paired study		Unpaired study
	(ND[a] - PD[b])	(ND + PD)	(ND - PD)
1	3	6	3
2	4	8	4
3	5	10	5
4	5	12	5
5	5	14	5
6	6	16	6
7	6	18	7
8	6	20	7
[a] ND = number of samples with Negative Deviation results.			
[b] PD = number of samples with Positive Deviation results.			

NOTE 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 should be presented in the validation report as additional information, but is not used in the overall assessment of the alternative-method performance.

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. However, this is not required.

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.

A minimum of three levels per type will 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). At least the low level should have fractional recovery by the reference method (fractional recovery at the low level should be between 25 % and 75 % of the number of samples tested). An estimate for the level of contamination (except for the negative control) should be made. 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 replicates samples should be tested by both methods. The negative control level shall not produce positive results. When positive results are obtained, the experiments have to be repeated for all levels.

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:

$$RLOD = \frac{LOD_{alt}}{LOD_{ref}} \quad (5)$$

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*)	$RLOD_i$	$RLOD_i$
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.

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.

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.

5.1.5 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. 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;

— 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 should 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;

— 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 × three levels × 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 Calculations and summary of data

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.

Calculate the percentage specificity (SP) of the reference method and the alternative method, using the data after confirmation, based on the results of level L_0 as follows:

$$\text{Specificity for the reference method: } \mathrm{SP_{ref}} = \left[1 - \left(\frac{P_0}{N_-}\right)\right] \times 100\,\% \tag{6}$$

$$\text{Specificity for the alternative method: } \mathrm{SP_{alt}} = \left[1 - \left(\frac{CP_0}{N_-}\right)\right] \times 100\,\% \tag{7}$$

where N_- is the number of all L_0 tests;

P_0 is the total number of false-positive results obtained with the blank samples before confirmation;

CP_0 is the total number of false-positive results obtained with blank samples.

For each of the levels L_1 and L_2, the results for all collaborators are combined in Table 9 for a **paired** study design and in Table 10 for an **unpaired** study design. Table 11 is prepared for the summarized data from all collaborators for both a **paired** and **unpaired** study.

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)
-	+	+	Positive Deviation (PD)
-	+	-	Negative Agreement due to false positive alternative-method result (NA)[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 result[a]**	**Confirmed alternative method[b]**	**Interpretation** (based on the confirmed alternative-method result)
+	+	+	Positive Agreement (PA)
+	+	-	Negative Deviation due to false positive alternative-method result (ND)[c]
-	-	-	Negative Agreement (NA)

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

Table 10 *(continued)*

Result of the (reference or alternative) method per sample			
Reference method	**Alternative-method result[a]**	**Confirmed alternative method[b]**	**Interpretation** (based on the confirmed alternative-method result)
-	-	+	Negative Agreement due to false negative alternative-method result (NA)
+	-	-	Negative Deviation (ND)
+	-	+	Negative Deviation due to false negative alternative-method result (ND)
-	+	+	Positive Deviation (PD)
-	+	-	Negative Agreement due to false positive alternative-method result (NA)[c]

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

Table 11 — Summary of results for all collaborators obtained with the reference and alternative methods 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-)	+/- Negative Deviation (ND)	−/− Negative Agreement (NA)

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

$$\text{Sensitivity for the alternative method: } \mathrm{SE_{alt}} = \frac{(\mathrm{PA}+\mathrm{PD})}{(\mathrm{PA}+\mathrm{ND}+\mathrm{PD})} \times 100\,\% \tag{8}$$

$$\text{Sensitivity for the reference method: } \mathrm{SE_{ref}} = \frac{(\mathrm{PA}+\mathrm{ND})}{(\mathrm{PA}+\mathrm{ND}+\mathrm{PD})} \times 100\,\% \tag{9}$$

$$\text{Relative trueness: } \mathrm{RT} = \frac{(\mathrm{PA}+\mathrm{NA})}{\mathrm{N}} \times 100\ \% \tag{10}$$

$$\text{False positive ratio for the alternative method: } \mathrm{FPR} = \frac{\mathrm{FP}}{\mathrm{NA}} \times 100\ \% \tag{11}$$

where N is the total number of samples (NA + PA + PD + ND) and FP is false-positive results. For explanation of the abbreviations used, see Table 9, Table 10, and Table 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 Interpretation of data

5.2.4.1 Paired study

For a **paired** study, calculate the difference between (ND – PD) and the sum of (ND + PD) for the level(s) where fractional recovery was obtained (so L_1 and possibly L_2). The values found for (ND – PD) and (ND + 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}	(ND – PD)	(ND + PD)
10	3	4
11	4	4
12 - 13	4	5
14 - 16	4	6
17	4	7
18	5	7
19 - 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 (ND – PD) for the level(s) where fractional recovery was obtained (so L_1 and possibly L_2). The observed value found for (ND – PD) shall not be higher than the AL. The AL is defined as $[(ND - PD)_{max}]$ and calculated per level where fractional recovery was obtained as described below using the following three parameters:

$$(p+)_{ref} = \frac{P_x}{N_x} \tag{12}$$

where

P_x = 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 = number of samples tested at level x (L_1 or L_2) with the reference method by all laboratories.

$$(p+)_{alt} = \frac{CP_x}{N_x} \tag{13}$$

where

CP_x = 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 = number of samples tested at level x (L_1 or L_2) with the alternative method by all laboratories.

$$(\text{ND-PD})_{\max} = \sqrt{3N_x \times \left((p+)_{\text{ref}} + (p+)_{\text{alt}} - 2\left((p+)_{\text{ref}} \times (p+)_{\text{alt}}\right)\right)} \quad (14)$$

where

N_x = 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 Use of relative level of detection

Additionally, for both a **paired** and **unpaired** study, an evaluation should be made for the difference between the relative levels of detection (RLOD) between laboratories. Conduct this evaluation according to Annex F. As there is limited experience with the interpretation of this approach, the results are used only for information.

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.

6 Quantitative methods — Technical protocol for validation

6.1 Method comparison study

6.1.1 General considerations

The method comparison study is the part of the validation process that is performed in the organizing laboratory. It consists of four parts.

— A comparative study of the results of the reference method to the results of the alternative method in a variety of different items (naturally and/or artificially) contaminated samples (so-called relative trueness study).

— A comparative study of the results of the reference method to the results of the alternative method in artificially contaminated samples using replicates of a single item per category. The data are analysed using the accuracy profile (AP) approach (so-called AP study).

— A limit of quantification (LOQ) study of the results of the alternative method in artificially contaminated samples using replicates of a single item per category. The data are used to calculate the LOQ of the alternative method. This study is only done for instrumentally-based methods (i.e. methods that are not based on the counting of individual colonies).

— 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.

6.1.2 Relative trueness study

The relative trueness study is a comparative study between the results obtained by the reference method and the results of the alternative method. This study is conducted using naturally and/or artificially contaminated samples. Different categories, types, and items will be tested for this.

6.1.2.1 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 can be studied. In addition to food categories, 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 per type of microorganism that might be relevant for the validation. This annex should be used to facilitate the selection of types and items for the type of 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). It is desirable that food samples come from as wide a range of contamination as possible in order to reduce any bias from local food specialities and broaden the range of validation.

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

EXAMPLE For the validation of a method for enumeration of *Listeria monocytogenes* and the food category "ready-to-eat, ready-to-reheat meat products", the food 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) 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.

6.1.2.2 Number of samples

For each category being examined, a minimum of 15 samples shall be tested and at least three types within that category should be used. For each type, at least five samples representative for this type shall be tested. This results in a minimum of 15 samples per category being tested using the minimum of three different types. The samples should be contaminated at a level that is representative for the natural variation in level of contamination. All samples combined should cover the range of concentration normally observed for the type of microorganism used.

Some naturally contaminated samples may contain high numbers of target analyte and this can result in difficulty in achieving the required range of contamination. In such cases, the naturally contaminated sample can be "diluted" with uncontaminated material of the same item.

The reference and alternative methods shall be performed with, as far as possible, exactly the same sample.

6.1.2.3 Calculation and interpretation of relative trueness study

The results obtained are analysed using the Bland-Altman method.[7] Plot the data for each sample per category and for each sample in all categories and draw the line of identity on which all points would

lie if the two methods gave identical results for each sample analysed. Figure 1 presents an example for a single category. The plot for a single category should show the results of each type tested with a distinct symbol. The plot for all categories should show the results of each category tested with a distinct symbol. This provides a rapid visual assessment of the extent to which the two methods (do not) agree. If any result (either reference or alternative method) is below the quantification limit, the data should be plotted using a substituted value of 1 $\log_{10}$ unit less than the observed value in case of a lower than value (e.g. <2 $\log_{10}$ units will be amended to 1 $\log_{10}$ unit). Similarly, any value greater than the upper limit should be amended by adding 1 $\log_{10}$ unit more (e.g. >6 $\log_{10}$ units will be amended to 7 $\log_{10}$ units). For example, a reference method result of 2,54 $\log_{10}$ cfu/g and an alternative-method result <2 $\log_{10}$ cfu/g will be plotted in the graph as 2,54 for the reference method and 1,0 for the alternative method. Information (based on investigation, e.g. root cause analysis) can be given in the study report in order to provide an explanation of the findings when one of the methods gave a result below the quantification limit.

These amended values shall not be used for the calculation of the data presented in Table 13, but shall be included in the difference plot (see Figure 2).

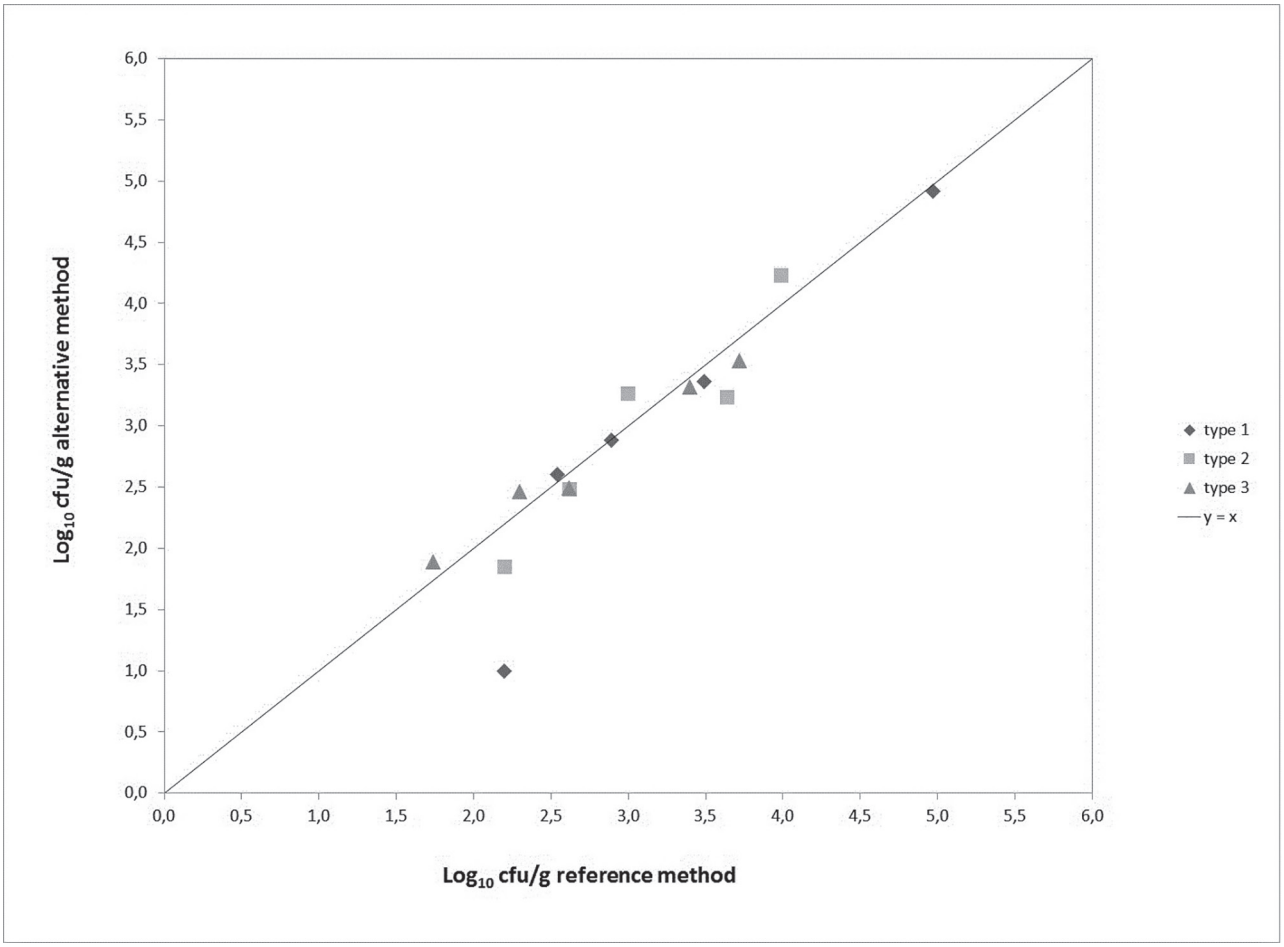

Figure 1 — Scatter plot of reference-method versus alternative-method results for a single category

Determine the average of each pair of data values and the difference between the values as in Table 13 and plot these derivatives (see Figure 2) per category and for all categories to illustrate the degree of bias and the (lack of) agreement of the data. Figure 2 shows the line of identity (zero difference), the line of bias, and the upper and lower 95 % confidence limits (CL) of the bias.

Table 13 — Summarized results for all categories

Category	Type	Sample	Log_{10} cfu		Mean	Difference
			Reference-method result	Alternative-method result		
1	1	1	R1	A1	(R1 + A1)/2	D1 = A1 – R1
		2	R2	A2	(R2 + A2)/2	D2 = A2 – R2
		3	R3	A3	(R3 + A3)/2	D3 = A3 – R3
		4	R4	A4	(R4 + A4)/2	D4 = A4 – R4
		5	R5	A5	(R5 + A5)/2	D5 = A5 – R5
1	2	6	R6	A6	(R6 + A6)/2	D6 = A6 – R6
		7	R7	A7	(R7 + A7)/2	D7 = A7 – R7
		8	R8	A8	(R8 + A8)/2	D8 = A8 – R8
		9	R9	A9	(R9 + A9)/2	D9 = A9 – R9
		10	R10	A10	(R10 + A10)/2	D10 = A10 – R10
1	3	…	…	…	…	…
Average category 1				$\overline{D}_1$		
Standard deviation category 1				s_{D1}		
…	…	…	…	…	…	…
x4			Rx	Ax	(Rx + Ax)/2	Dx = Ax - Rx
Average category x				$\overline{D}_x$		
Standard deviation category x				s_{Dx}		
Average all categories				$\overline{D}_{all}$		
Standard deviation all categories				s_{Dall}		

Compute per category and for all categories the average difference $\overline{D}$, the standard deviation of differences s_D and the limits of agreement using the following formula:

$$\left[\overline{D} \pm T \cdot s_D \sqrt{1+\frac{1}{n}}\right] \tag{15}$$

where n is the number of data pairs, T is the percentile of a Student-t distribution for β the chosen probability of the interval and $(n-1)$ 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, and the upper and lower 95 % confidence limits of agreement (CLs) of the bias for all categories. This illustrates the degree of bias and the (lack of) agreement of the data. Figure 2 shows a plot for each value together with the line of identity (zero difference), the line of bias, and the upper and lower 95 % confidence limits of the bias.

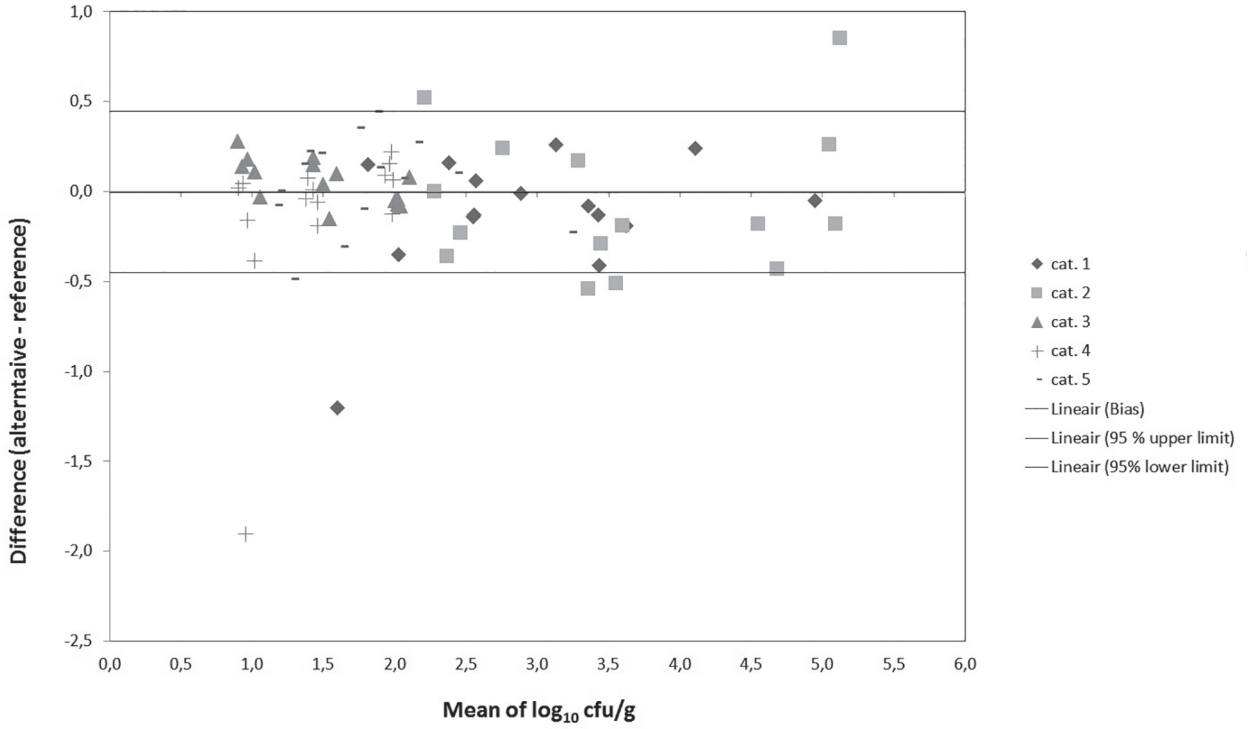

Figure 2 — Bland-Altman difference plot for all categories

The results of the difference and scatter plot will be interpreted based on a visual observation on the amount of bias and extreme results. It will be expected that not more than one in 20 data values will lie outside the CLs. Any disagreements with the expectation should be recorded.

6.1.3 Accuracy profile study

The accuracy profile study is a comparative study between the results obtained by the reference method and the results of the alternative method. This study is conducted using artificially contaminated samples. One type per category will be tested.

6.1.3.1 Selection of categories to be used

See 6.1.2.1.

6.1.3.2 Number of samples

For each category being examined, at least one type shall be tested using six samples per type. Of the six samples, there should be two at a low level, two at an intermediate level, and two at a high level of contamination. These levels should cover the whole range of contamination of the selected type. For each sample, five replicates representing five different test portions from the same sample shall be used.

NOTE The six samples might be different belonging to the same type, but not necessarily the same item. For example, one sample might be full fat milk powder, another is infant formula belonging to the same food type (dried milk products), but not the same food item (see Annex A).

6.1.3.3 Calculation and interpretation of accuracy profile study

Tabulate the results as in Table 14 based on log-transformed counts.

Table 14 — Results of the accuracy study (in $\log_{10}$ cfu/g)

Category	Type	Item (level)	Reference method					Alternative method				
			t.p.[a] 1 (x_1)[b]	t.p. 2 (x_2)	t.p. 3 (x_3)	t.p. 4 (x_4)	t.p. 5 (x_5)	t.p. 1 (y_1)[c]	t.p. 2 (y_2)	t.p. 3 (y_3)	t.p. 4 (y_4)	t.p. 5 (y_5)
Category 1	Type 1	Sample 1 (low)										
		Sample 2 (low)										
		Sample 3 (intermediate)										
		Sample 4 (intermediate)										
		Sample 5 (high)										
		Sample 6 (high)										
…	…	…										
Category x	Type x	Sample 1 - 6										

[a] t.p. = test portion.

[b] (x_a) = log10 test result for the reference method (x) for test portions 1 to 5.

[c] (y_a) = log10 test result for the alternative method (y) for test portions 1 to 5.

The accuracy profile is used to check the requirement that the alternative method produces a result for a sample that differs from the value produced by the reference method by less than a certain acceptability criterion. The principle of the accuracy profile is explained in more detail in Annex G.

The following notation is used: i refers to the sample and q is the number of samples ($1 \le i \le q$); j refers to the test portions and n is the number of test portions ($1 \le j \le n$). Calculations are performed per category/type as the following sequence of operations:

— x_{ij}, the $\log_{10}$ transformed test result of sample i for replicate j with $1 \le i \le q$ and $1 \le j \le n$ using the reference method;

— y_{ij}, the $\log_{10}$ transformed test result of sample i for replicate j with $1 \le i \le q$ and $1 \le j \le n$ using the alternative method.

For each item sample or just sample, measurements are made under repeatability conditions for both methods. y_i values are assumed to be normally distributed. The β-expectation tolerance interval (β-ETI) of the y_i values is computed according to Reference [8]. It is assumed that a combined standard deviation can be calculated that holds for all item samples.

The Acceptability Limit is set at: AL = ±0,5 $\log_{10}$ units. It is expressed as a difference between the reference and the alternative method.

Step 1: For each sample i, calculate the central value X_i as the median of the $\log_{10}$ transformed counts obtained with the reference method, x_{ij}. These values are the reference values of the validation samples:

$$X_i = \text{median}(x_{ij}) \tag{16}$$

Step 2: For each sample i, calculate the central value Y_i as the median of the $\log_{10}$ transformed counts obtained with the alternative method y_{ij}. These values are the alternative values of the validation samples:

$$Y_i = \text{median}(y_{ij}) \tag{17}$$

Step 3: For each sample *i*, calculate the standard deviation $s_{\text{alt},i}$ as follows: $s_{\text{alt},i} = \sqrt{\frac{1}{n-1}\sum_{j=1}^{n}(y_{ij} - \bar{y}_i)^2}$

Step 4: Calculate the combined standard deviation s_{alt} as follows: $s_{\text{alt}} = \sqrt{\frac{1}{q}\sum_{i=1}^{q} s_{\text{alt},i}^2}$

Step 5: Calculate the combined standard deviation of the reference method s_{ref} (analogous to step 3 and step 4) as follows:

$$s_{\text{ref},i} = \sqrt{\frac{1}{n-1}\sum_{j=1}^{n}\left(x_{ij} - \bar{x}_i\right)^2} \tag{18}$$

and

$$s_{\text{ref}} = \sqrt{\frac{1}{n}\sum_{j=1}^{n} s_{\text{ref},i}^2} \tag{19}$$

Step 6: For each sample *i*, compute the absolute bias as the difference of the medians calculated for both methods $B_i = Y_i - X_i$. This is an estimate of the lack of trueness of the alternative method in comparison to the reference method.

Step 7: For each sample *i*, compute the limits of the β-ETI. This is the interval where the expected proportion of future results will fall is β. For each sample, β-ETI is expressed as:

$$\left[B_i \pm T \cdot s_{\text{alt}}\sqrt{1+\frac{1}{n}}\right] \tag{20}$$

where *T* is the percentile of a Student-*t* distribution for β the chosen probability and $q\cdot(n-1)$ degrees of freedom (24 in de-requested setup), that is: $T_{\left(\frac{1-\beta}{2}\right);q(n-1)}$. For the purpose of this part of ISO 16140, β is set at 80 %. *T* is the coverage factor of the β-ETI of the validation sample. It defines the upper limit U_i and the lower limit L_i:

$$U_i = B_i + T \cdot s_{\text{alt}}\sqrt{1+\frac{1}{n}} \text{ and } L_i = B_i - T \cdot s_{\text{alt}}\sqrt{1+\frac{1}{n}} \tag{21}$$

Step 8: For each category, tabulate the different values calculated for the samples as in Table 15.

Table 15 — Presentation of the statistical results of the comparison study

Category	Sample	Central value (Ref)	Central value (Alt)	Bias	Upper β-ETI	Lower β-ETI	Upper AL	Lower AL
Category 1	Sample 1	X_i	Y_i	B_i	U_i	L_i	+AL	-AL
	Sample 2							
	Sample 3							
	Sample 4							
	Sample 5							
	Sample 6							

Make a graphical representation of computed results as follows:

— the horizontal axis is for reference values X_i in $\log_{10}$ units;

— the vertical axis is for the bias, the Acceptability Limits, and the tolerance interval limits $U_i - X_i$ and $L_i - X_i$ all expressed in $\log_{10}$ units as differences to the corresponding reference value of the sample.

Make a graphic representation like the example given in Figure 3. The upper and lower tolerance-interval limits are connected by straight lines to interpolate the behaviour of the limits between the different levels of the validation samples. The horizontal line represents the reference values obtained with the reference method. The differences between reference values and average levels of contamination are represented by black dots. Whenever no biases exist, these recovered values are located on the horizontal reference line. In addition, Acceptability Limits are represented by two dashed horizontal lines and β-ETI limits as broken full lines.

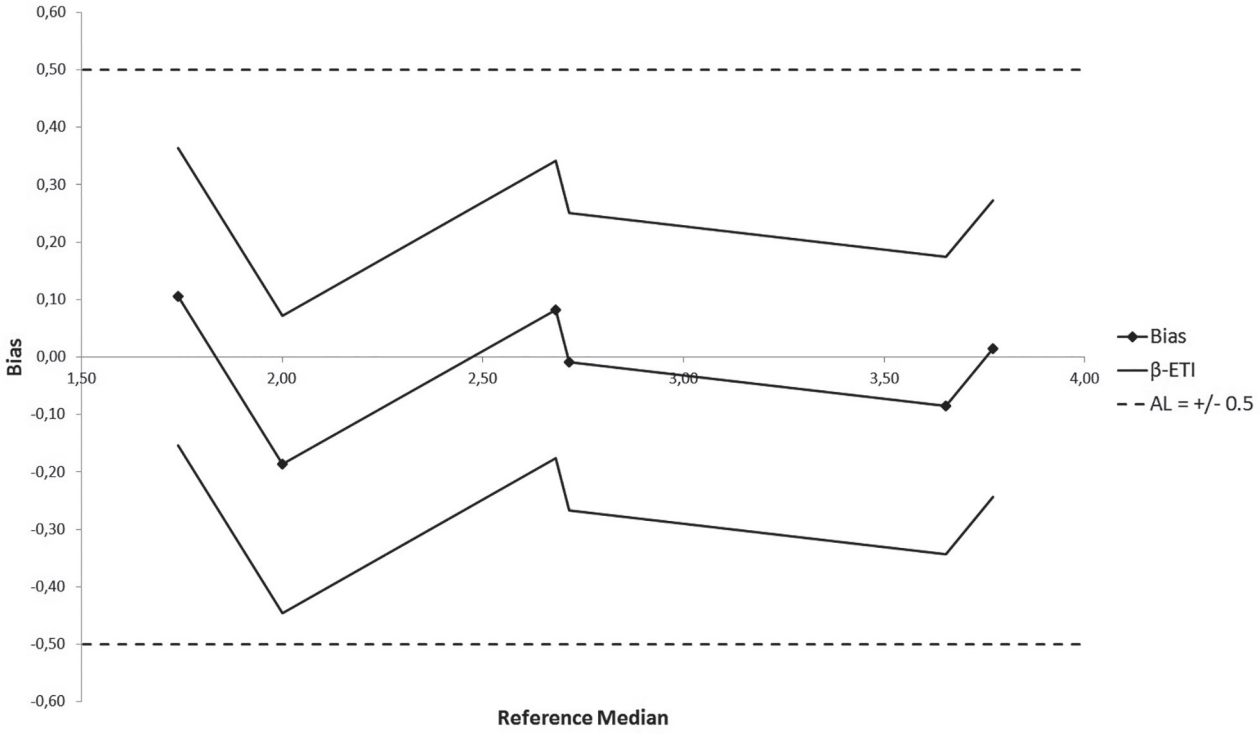

Figure 3 — Example of accuracy profile for a category in the method comparison study

If for all i in the accuracy profile $U_i \leq \text{AL}$ and $L_i \geq -\text{AL}$, the alternative method is accepted as being equivalent to the reference method for the individual categories and the combined categories.

If any of the upper or lower limits exceeds the Acceptability Limits and the standard deviation, $S_{\text{ref}} > 0{,}125$, the following additional evaluation procedure is followed:

Step 9: Calculate new Acceptability Limits as a function of the standard deviation: $\text{AL}_s = 4 \cdot s_{\text{ref}}$. If for all i in the accuracy profile $U_i \leq \text{AL}_s$ and $L_i \geq -\text{AL}_s$, the alternative method is accepted as being equivalent to the reference method for the given combination category and type.

NOTE If $S_{\text{ref}} \leq 0{,}125$, the new Acceptability Limits would be smaller than or equal to 0,5. The second evaluation would yield the same results in that case.

The alternative method is accepted as being equivalent to the reference method if it is equivalent for all individual and combined categories. An example on the use of the accuracy profile is presented in Annex H. Investigations should be made (e.g. root cause analysis) in order to provide an explanation of the findings when the methods are not equivalent for individual or combined categories. Based on the

investigations, it is decided whether the methods are regarded as equivalent or not for the category or categories involved. The results of the evaluation shall be stated in the study report.

For the Accuracy Profile calculations, an Excel® spreadsheet is freely available for download at: http://standards.iso.org/iso/16140 and then select the AP file for the method comparison study.

6.1.4 Limit of quantification study

6.1.4.1 General considerations

For some alternative methods, it is of interest to determine the limit of quantification (LOQ). The LOQ is only relevant when the measurement principle of the alternative method is not based on counting visible colonies of the target microorganism and shall therefore be determined in these cases. Examples of methods for which the LOQ needs to be determined are the instrumental measurement of conductivity or fluorescence which is related to the growth of the microorganism.

6.1.4.2 Selection of categories to be used

Select the same categories and types as used for the accuracy profile study (see 6.1.2.1 and 6.1.3.2).

6.1.4.3 Number of samples

If the LOQ needs to be determined, blank samples are tested per type/category used. These blank samples are used to verify the limit of quantification of the alternative method. A minimum of 10 test portions from the same sample shall be used. Examine the test portions with the alternative method.

6.1.4.4 Calculation and interpretation of limit of quantification study

The 10 results per type/category are used to estimate the baseline or threshold standard deviation s_0. Calculate the standard deviation s_0 of the n results as follows:

$$s_0 = \sqrt{\frac{1}{n-1}\sum_{j=1}^{n}\left(y_j - \bar{y}\right)^2} \tag{22}$$

where

n is the total number of test portions used;

y_j is the $\log_{10}$ transformed result of test portion j;

$\bar{y}$ is the average $\log_{10}$ transformed result of all test portions.

The limit of quantification is calculated as $LOQ = 10\ s_0$.

6.1.5 Inclusivity and exclusivity study

Inclusivity and exclusivity testing is not required for general enumeration methods such as total plate count (TPC) and yeast and mould (Y&M) methods. It is required for enumeration methods designed for specific microorganisms (e.g. *Listeria*).

6.1.5.1 Selection and number of test 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 or the food-processing environment, or primary production taking into account the scope of the validation. However, clinical, environmental, and culture collection strains can also be used. The original source of all isolates should be known and they should be held in a local (e.g. an 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 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.

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

6.1.5.2 Target microorganisms (inclusivity)

Each test is performed once and with the alternative method, the reference method, and a non-selective agar. The inoculum level should be at least 100 times greater than the minimum level for quantification of the alternative method being validated. When using a plate method as the alternative method, the inoculum level shall obtain a countable number on the plate. If results are negative, consideration could be given to repeat the test with the addition of a food item.

6.1.5.3 Non-target microorganisms (exclusivity)

Each test is performed once and with the alternative and the reference method. The inoculum level should be similar to the greatest level of contamination expected to occur in any of the categories being used. No sample is added. The pure culture should be grown in a suitable non-selective broth under optimal conditions of growth for at least 24 h and diluted to an appropriate level before testing begins.

If the organism cannot be cultured, a stock suspension should be diluted to an appropriate level before use.

6.1.5.4 Expression and interpretation of results

Tabulate the results as in Table 16 for the inclusivity tests and Table 17 for the exclusivity tests. 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 16 — Presentation of results for inclusivity

Microorganisms	Reference method	Alternative method	Non-selective agar
1			
2			
Etc.			

The interpretation of the inclusivity data for an alternative method using a plating medium is made on a qualitative basis. However, quantitative data should facilitate the interpretation of the data.

Table 17 — Presentation of results for exclusivity

Microorganisms	Reference method	Alternative method
1		
2		
Etc.		

6.2 Interlaboratory study

6.2.1 General considerations

The aim of the interlaboratory study is to compare the performance of the alternative method to the reference method by different collaborators using identical samples examined under reproducibility conditions and to compare these results with pre-set criteria for the acceptable difference between the reference method and the alternative method. Wherever possible, the study conditions should reflect the normal variation between laboratories. The interlaboratory study is organized 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.

6.2.2 Measurement protocol

The interlaboratory study shall produce at least eight valid data sets from at least eight collaborators. The collaborators shall come from a minimum of four different organizations, but preferably eight 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 accuracy and precision estimates should be calculated from a large number of duplicate test results. This figure should be a minimum of 96 results for the one item chosen consisting of eight collaborators, three levels of contamination, two methods of enumeration (reference and alternative), and duplicate measurements, i.e. 8 × 3 × 2 × 2 = 96.

General guidelines for conducting the interlaboratory studies are described in ISO 5725-2.[1] The organizer is responsible for the preparation of the test protocol and a data sheet for the recording of all experimental data and critical experimental conditions used by each laboratory. It is necessary for each collaborator to demonstrate his competence in the use of the alternative and the reference method prior to participating in the study proper.

The protocol is as follows

— A relevant item (for selection see Annex A) is used to prepare the test samples. The item should contain a natural background microflora.

— The selected item can be inoculated with the target organism. The protocol for inoculation of the samples shall be appropriate for the selected item. Samples shall be prepared to ensure homogeneity between samples using preparation protocols contained in Annex B and Annex C. In general, liquid samples (compared to solid samples) give greater assurance to obtain homogeneity. The samples shall be shown to be homogeneous by the organizing laboratory. Homogeneity tests and criteria for acceptance are described in ISO/TS 22117.[4]

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

— Duplicate samples are tested by each collaborating laboratory at the three levels of contamination. All samples should be blind coded to ensure that the analysts are not aware of their level of contamination.

— The analysis of samples shall be performed in each laboratory at the stipulated date.

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. No outlier tests are performed on the selected data.

6.2.3 Calculations, summary, and interpretation of data

The $\log_{10}$ transformed test results of the different collaborators for both the reference and alternative method are presented in Table 18. Note the data as follows:

— x_{ijk}, the $\log_{10}$ transformed test result on level i for replicate j of collaborator k with $1 \le i \le q$, $1 \le j \le n$ and $1 \le k \le p$ using the reference method;

— y_{ijk}, the $\log_{10}$ transformed test result on level i for replicate j of collaborator k with $1 \le i \le q$, $1 \le j \le n$ and $1 \le k \le p$ using the alternative method.

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

		Reference method x_{ijk}		**Alternative method y_{ijk}**	
Collaborators (i)	Level (k)	Result		Result	
1	Blank				
2	Blank				
Etc.	Blank				
(l)	Blank				
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
1	Low				
2	Low				
Etc.	Low				
(l)	Low				
1	Medium				
2	Medium				
Etc.	Medium				
(l)	Medium				
1	High				
2	High				
Etc.	High				
(l)	High				

Calculations are performed as a sequence of operations starting with the $\log_{10}$ transformation of all test results. The following notation is used: i refers to the level and q is the number of levels ($1 \le i \le q$); j refers to the replicate and n is the number of replicates ($1 \le j \le n$); k refers to the collaborator and p is the number of collaborators ($1 \le k \le p$). Detailed calculations can be obtained in Reference [9] or Reference [13].

Step 1: For each level of contamination, calculate X_i as the global average of the $\log_{10}$ transformed replicate counts obtained with the reference method x_{ijk} for a given sample. These averages are assigned **reference values** of the samples used in the validation study:

$$X_i = \frac{\sum_{j=1}^{n}\sum_{k=1}^{p} x_{jk}}{np} \tag{23}$$

Step 2: Calculate, for each level i (using y_{ijk}), the reproducibility standard deviation s_{Ri} as follows:

$$s_{Ri} = \sqrt{s_{Li}^2 + s_{ri}^2} \tag{24}$$

This procedure is described in detail in ISO 5725-2.[1]

Step 3: For each level, compute $\bar{y}_i$, the global average of measurements made with the alternative method.

Step 4: For each level, compute the absolute bias as $\bar{y}_i - X_i$. This is an estimate of the lack of trueness of the alternative method when compared to the reference.

Step 5: For each level, compute the limits of the β-ETI according to Reference [12]. The β-ETI is the interval where the expected proportion of future results will fall is β.

a) For each level, β-ETI can be expressed as: $\bar{y}_i \pm k_{Mi} \cdot s_{TIi}$

where k_{Mi} represents a coverage factor and s_{TIi} the standard deviation of the tolerance interval of the given level *i.*

b) The standard deviation of the tolerance interval is equal to

$$s_{TIi} = s_{Ri}\sqrt{1 + \frac{1}{p \cdot n \cdot G^2}} \tag{25}$$

c) The coverage factor for the level i is equal to $k_{Mi} = T$

where $T = T(\frac{1}{2}(1-\beta), \nu)$, i.e. the percentile of the Student-*t* distribution, β the chosen probability, p the number of collaborators, n the number of replicates, and ν the number of degrees of freedom. For the purpose of this part of ISO 16140, β is set at 80 %. The intermediate parameters are: $G = \sqrt{\frac{H+1}{n \cdot H + 1}}$ and $H = \frac{s_{Li}^2}{s_{ri}^2}$

The number of degrees of freedom is calculated as follows: $\nu = \frac{(H+1)^2}{\frac{\left(H + \frac{1}{n}\right)^2}{p-1} + \frac{1 - \frac{1}{n}}{p \cdot n}}$

NOTE 1 *G*, *H,* and ν will have different values for each level *i* and should be indexed. For reasons of readability, the index *i* is not used here.

NOTE 2 The number of degrees of freedom *(*dof*)* ν is not an integer and it is necessary to get the *T* value for the non-integer (dof). This can be done by using extended statistical table or by interpolation between the two upper and lower rounded values of ν.

NOTE 3 It is necessary to use another method for the calculation of the β-ETI than for the method comparison study (see 6.1.3.3), because measurements are made under reproducibility conditions in the interlaboratory study and under repeatability conditions in the method comparison study.

Step 6: For each level *i*, compute the limits of the β-ETI expressed as: $\left[\bar{y}_i \pm k_{Mi} \cdot s_{Ri}\right]$

It defines the upper limit U_i and the lower limit L_i: $U_i = \bar{y}_i + k_{Mi} \cdot s_{Ri}$ and $L_i = \bar{y}_i - k_{Mi} \cdot s_{Ri}$.

Step 7: Make a graphical representation of computed results as follows:

— the horizontal axis is for target values X_i in $\log_{10}$ units;

— the vertical axis is for the bias $\bar{y}_i - X_i$, the Acceptability Limits AL (±0,50 log units), and the tolerance interval limits $U_i - X_i$ and $L_i - X_i$ all expressed in $\log_{10}$ units as differences to the corresponding reference value of the sample.

Results are illustrated in an accuracy profile graph like the example given in Annex I (Figure I.2). This graph is used as a graphical decision-support tool. The upper and lower tolerance-interval limits are connected by straight lines to interpolate the behaviour of the limits between the different levels of the validation samples. The horizontal line represents the reference values obtained with the reference method. The differences between reference values and average levels of contamination determined by the alternative method $\bar{y}_i$ are represented by black dots. Whenever no biases exist, these recovered values are located on the horizontal reference line. In addition, Acceptability Limits are represented by two dashed horizontal lines and β-ETI limits as broken full lines. The Acceptability Limit is set at ±0,5 $\log_{10}$ units.

The alternative method is regarded as being equivalent to the reference method when the values for the β-ETI fall within the Acceptability Limits for all levels of contamination.

Step 8: If any of the values for the β-ETI fall outside the Acceptability Limits, the following additional evaluation procedure is followed. Calculate the pooled average reproducibility standard deviation of the reference method $s_{R,\text{ref}} = \sqrt{\frac{1}{q}\sum_{i=1}^{q} s_{Ri}^2}$.

Step 9: Calculate new Acceptability Limits as a function of the standard deviation: $\text{AL}_s = 3{,}3 \cdot s_{R,\text{ref}}$.

If for all *i* in the accuracy profile $U_i - X_i \leq \text{AL}_s$ and $L_i - X_i \geq -\text{AL}_s$, the alternative method is accepted as being equivalent to 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 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.

For the Accuracy Profile calculations, an Excel® spreadsheet is freely available for download at http://standards.iso.org/iso/16140 and then select the AP file for the interlaboratory study.

An example of the application of the accuracy profile to interlaboratory studies is presented in Annex I.

Annex A
(informative)

Classification of sample types and suggested target combinations for validation studies

Table A.1 outlines the classification of mainly foods intended to guide method developers for the relative validation of alternative methods. The intrinsic properties of foods such as levels of indigenous microflora, fat content, pH, salt content, water activity, and the presence of antimicrobial compounds can have a substantial influence on the outcome of a method. Therefore, the intrinsic properties of foods have been considered to the extent possible in the classification of foods, but the wide variety of foods available makes this consideration difficult to apply past the level of food type.

Regulatory authorities in different jurisdictions often have slightly different requirements as to the classification of foods. These differences have been included in the notes of Table A.1 as much as possible.

Table A.1 — Classification of samples and their relevance for testing for various microorganisms

Categories	**Types**	**Items (some examples)**	Total viable count	Lactic Acid Bacteria	Yeasts and moulds	*Enterobacteriaceae*	*Escherichia coli*	Coagulase positive staphylococci	*Salmonella* spp.	*Listeria* spp.	*L. monocytogenes*	Shiga toxin-producing *E. coli (STEC)*	*Cronobacter* spp.	*Campylobacter*	(Pathogenic) *Yersinia enterolitica*	*Vibrio* spp.	*Bacillus cereus* (vegetative cells or spores)	*Clostridium perfringens* (vegetative cells or spores)	*Clostridium botulinum* (vegetative cells or spores)
Raw milk and dairy products	Raw milks and/or fermented/acidified milks (not heat treated)	Raw milk	Y				Y	Y	Y	Y	Y	Y		Y	Y				
		Raw fermented/ acidified, raw milk yoghurts, raw dairy-based drinks					Y	Y	Y	Y	Y	Y			Y				
	Raw milk-based products, with high fat content and/or high background microflora	Raw butters					Y	Y	Y	Y	Y	Y			Y				
		Raw creams					Y	Y	Y	Y	Y	Y			Y				
		Hard and semi-hard cheeses (e.g. Comté, Beaufort)					Y	Y	Y	Y	Y	Y			Y				
		Blue cheeses (Roquefort)					Y	Y	Y	Y	Y	Y			Y				
		Soft cheeses (e.g. Brie, Munster)					Y	Y	Y	Y	Y	Y			Y				

Table A.1 *(continued)*

Categories	**Types**	**Items (some examples)**	Total viable count	Lactic Acid Bacteria	Yeasts and moulds	*Enterobacteriaceae*	*Escherichia coli*	Coagulase positive staphylococci	*Salmonella* spp.	*Listeria* spp.	*L. monocytogenes*	Shiga toxin-producing *E. coli (STEC)*	*Cronobacter* spp.	*Campylobacter*	(Pathogenic) *Yersinia enterolitica*	*Vibrio* spp.	*Bacillus cereus* (vegetative cells or spores)	*Clostridium perfringens* (vegetative cells or spores)	*Clostridium botulinum* (vegetative cells or spores)
Heat-processed milk and dairy products	Pasteurized dairy products	Milk-based desserts, ice creams, drinks, creams	Y	Y	Y	Y	Y	Y	Y	Y	Y						Y		
	Sterilized or UHT dairy products	UHT milks, canned milks or creams															Y		
	Pasteurized milk-based products	Fermented/ acidified pasteurized milk, yoghurts, dairy-based products			Y	Y	Y	Y	Y	Y	Y						Y		
		Pasteurized milks	Y			Y	Y	Y	Y	Y	Y						Y		
		Butters			Y	Y	Y	Y	Y	Y	Y								
		Creams			Y	Y	Y	Y	Y	Y	Y								
		Hard and semi-hard cheeses (heat processed) (e.g. Comté, Emmental, Gouda)			Y		Y	Y	Y	Y	Y								
		Blue cheeses (Bleu de Bresse)					Y	Y	Y	Y	Y								
		Soft cheeses (e.g. Brie, Munster)					Y	Y	Y	Y	Y								Y
	Dry	Milk powders	Y			Y	Y	Y	Y	Y	Y						Y		
		Powder for milk-based desserts	Y			Y	Y	Y	Y	Y	Y						Y		

Table A.1 *(continued)*

Categories	**Types**	**Items (some examples)**	Total viable count	Lactic Acid Bacteria	Yeasts and moulds	*Enterobacteriaceae*	*Escherichia coli*	Coagulase positive staphylococci	*Salmonella* spp.	*Listeria* spp.	*L. monocytogenes*	Shiga toxin-producing *E. coli (STEC)*	*Cronobacter* spp.	*Campylobacter*	(Pathogenic) *Yersinia enterolitica*	*Vibrio* spp.	*Bacillus cereus* (vegetative cells or spores)	*Clostridium perfringens* (vegetative cells or spores)	*Clostridium botulinum* (vegetative cells or spores)
Raw meat and ready-to-cook meat products (except poultry)	Fresh meats (unprocessed)	Carcasses, meat cuts, Carpaccio's	Y			Y	Y	Y	Y	Y	Y	Y		Y	Y				
		Minced meat, meat preparations, Carpaccio's	Y			Y	Y	Y	Y	Y	Y	Y		Y	Y				
		Carcasses, swabs, rinsates	Y			Y	Y	Y	Y	Y	Y	Y		Y	Y				
	Ready-to-cook (processed)	Frozen burger patties, marinated beef shish-kabobs	Y			Y	Y	Y	Y	Y	Y	Y		Y	Y				
Ready-to-eat, ready-to-reheat meat products	Cooked meat products	Cooked ham, pâté	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y		Y	Y		Y	Y	Y
	Fermented or dried meat products	Salami	Y			Y	Y	Y	Y	Y	Y	Y		Y	Y		Y	Y	Y
	Raw cured (smoked) (a_w >0,92)	Filet de sax, lard	Y			Y	Y	Y	Y	Y	Y	Y		Y	Y		Y	Y	Y
	Raw cured (smoked) (a_w <0,92)	Cobourg ham, dry cured ham	Y			Y	Y	Y	Y	Y	Y	Y							
	Canned meat (ambient stable)	Corned beef															Y	Y	Y

Table A.1 *(continued)*

Categories	**Types**	**Items (some examples)**	Total viable count	Lactic Acid Bacteria	Yeasts and moulds	*Enterobacteriaceae*	*Escherichia coli*	Coagulase positive staphylococci	*Salmonella* spp.	*Listeria* spp.	*L. monocytogenes*	Shiga toxin-producing *E. coli (STEC)*	*Cronobacter* spp.	*Campylobacter*	(Pathogenic) *Yersinia enterolitica*	*Vibrio* spp.	*Bacillus cereus* (vegetative cells or spores)	*Clostridium perfringens* (vegetative cells or spores)	*Clostridium botulinum* (vegetative cells or spores)
Raw poultry and ready-to-cook poultry products	Fresh meats (unprocessed)	Carcasses, meats, cuts	Y			Y	Y	Y	Y	Y	Y			Y					
		Carcasses, swabs, rinsates	Y			Y	Y	Y	Y	Y	Y			Y					
		Minced meat, meat preparations	Y			Y	Y	Y	Y	Y	Y			Y					
	Ready-to-cook products (processed)	Seasoned chicken breasts	Y			Y	Y	Y	Y	Y	Y			Y					
Ready-to-eat, ready-to-reheat meat poultry products	Cooked meat products	Cooked turkey filet	Y	Y	Y	Y	Y	Y	Y	Y	Y			Y			Y	Y	Y
	Fermented or dried meat products	Chicken sausage	Y			Y	Y	Y	Y	Y	Y			Y			Y	Y	Y
	Raw cured (smoked) (a_w >0,92)	Smoked turkey filet	Y			Y	Y	Y	Y	Y	Y			Y			Y	Y	Y
	Canned (ambient stable)	Canned poultry meat, canned duck pâté															Y	Y	Y

Table A.1 *(continued)*

Categories	**Types**	**Items (some examples)**	Total viable count	Lactic Acid Bacteria	Yeasts and moulds	*Enterobacteriaceae*	*Escherichia coli*	Coagulase positive staphylococci	*Salmonella* spp.	*Listeria* spp.	*L. monocytogenes*	Shiga toxin-producing *E. coli (STEC)*	*Cronobacter* spp.	*Campylobacter*	(Pathogenic) *Yersinia enterolitica*	*Vibrio* spp.	*Bacillus cereus* (vegetative cells or spores)	*Clostridium perfringens* (vegetative cells or spores)	*Clostridium botulinum* (vegetative cells or spores)
Eggs and egg products (derivates)	Eggs (unprocessed)	Shell eggs	Y			Y	Y	Y	Y	Y	Y			Y					
	Egg products (heat processed) with additives (salt or sugar >2 %)	Egg yolk, egg white, whole liquid egg	Y	Y	Y	Y	Y	Y	Y	Y	Y						Y	Y	Y
	Egg products (heat processed) without additives	Egg yolk, egg white, whole liquid egg	Y	Y	Y	Y	Y	Y	Y	Y	Y						Y	Y	Y
	Dry	Egg powder	Y			Y	Y	Y	Y	Y	Y						Y	Y	Y
Raw and ready-to-cook fish and seafoods (unprocessed)	Fish (unprocessed)	Fish	Y			Y	Y	Y	Y	Y	Y			Y	Y	Y			
	Shellfish (unprocessed)	Oyster, clam, scallop, mussel	Y			Y	Y	Y	Y	Y	Y			Y	Y	Y			
	Crustaceans (unprocessed)	Shrimp, crab and crab meat, lobster	Y			Y	Y	Y	Y	Y	Y			Y	Y	Y			
	Ready-to-cook fish and seafoods (processed)	Frozen fish sticks													Y	Y			

Table A.1 *(continued)*

Categories	**Types**	**Items (some examples)**	Total viable count	Lactic Acid Bacteria	Yeasts and moulds	*Enterobacteriaceae*	*Escherichia coli*	Coagulase positive staphylococci	*Salmonella* spp.	*Listeria* spp.	*L. monocytogenes*	Shiga toxin-producing *E. coli (STEC)*	*Cronobacter* spp.	*Campylobacter*	(Pathogenic) *Yersinia enterolitica*	*Vibrio* spp.	*Bacillus cereus* (vegetative cells or spores)	*Clostridium perfringens* (vegetative cells or spores)	*Clostridium botulinum* (vegetative cells or spores)
Ready-to-eat, ready-to-reheat fishery products	Cooked fishery products	Shelled and shucked products of cooked crustaceans, fish and seafood terrines	Y	Y	Y	Y	Y	Y	Y	Y	Y					Y	Y	Y	Y
	Acidified and marinated fishery products	Roll herring, anchovy	Y	Y	Y	Y	Y	Y	Y	Y	Y						Y		
	Smoked or cured, and other processed products (a_w >0,92)	Smoked fish	Y	Y	Y	Y	Y	Y	Y	Y	Y					Y	Y		Y
	Smoked or cured, and other processed products (a_w <0,92)	Smoked fish, dried (salted) fish	Y	Y	Y	Y	Y	Y	Y	Y	Y					Y	Y		
	Canned (ambient stable fish)	Canned fish, canned crab															Y	Y	Y

Table A.1 *(continued)*

Categories	**Types**	**Items (some examples)**	Total viable count	Lactic Acid Bacteria	Yeasts and moulds	*Enterobacteriaceae*	*Escherichia coli*	Coagulase positive staphylococci	*Salmonella* spp.	*Listeria* spp.	*L. monocytogenes*	Shiga toxin-producing *E. coli (STEC)*	*Cronobacter* spp.	*Campylobacter*	(Pathogenic) *Yersinia enterolitica*	*Vibrio* spp.	*Bacillus cereus* (vegetative cells or spores)	*Clostridium perfringens* (vegetative cells or spores)	*Clostridium botulinum* (vegetative cells or spores)
Fresh produce and fruits	Cut ready-to-eat fruits	Fruit mixes	Y			Y	Y	Y	Y	Y	Y	Y		Y		Y			
	Cut ready-to-eat vegetables	Bagged pre-cut leafy vegetables, salads, shredded carrot	Y			Y	Y	Y	Y	Y	Y	Y		Y	Y	Y			
	Produce grown in or in contact with the ground	Potatoes, yams, sweet potatoes, cassava, dahlia, carrots, cruciferous vegetables					Y		Y	Y	Y	Y		Y	Y	Y			
	Sprouts	Soy, fenugreek, alfalfa, mung	Y			Y	Y	Y	Y	Y	Y	Y		Y	Y	Y			
	Raw fruit/ vegetable juices (unpasteurized)	Freshly squeezed strawberry juice, smoothies, carrot juice	Y			Y	Y	Y	Y	Y	Y	Y		Y	Y	Y			Y
	Leafy greens	Basil, cilantro, green onions, lettuce and parsley				Y	Y	Y	Y	Y	Y	Y		Y	Y	Y			
	Vegetables and fruits (unprocessed) not described above	Crops				Y	Y	Y	Y	Y	Y	Y		Y	Y	Y			

Table A.1 *(continued)*

Categories	**Types**	**Items (some examples)**	Total viable count	Lactic Acid Bacteria	Yeasts and moulds	*Enterobacteriaceae*	*Escherichia coli*	Coagulase positive staphylococci	*Salmonella* spp.	*Listeria* spp.	*L. monocytogenes*	Shiga toxin-producing *E. coli (STEC)*	*Cronobacter* spp.	*Campylobacter*	(Pathogenic) *Yersinia enterolitica*	*Vibrio* spp.	*Bacillus cereus* (vegetative cells or spores)	*Clostridium perfringens* (vegetative cells or spores)	*Clostridium botulinum* (vegetative cells or spores)
Processed fruits and vegetables	Heat-processed fruit/ vegetables juices	Pasteurized apple juice	Y						Y	Y	Y						Y	Y	Y
	Canned fruits and vegetables (ambient stable)	Canned pineapples															Y	Y	Y
	Heat-processed vegetables and fruits	Blanched spinach, frozen vegetables blanched	Y			Y	Y	Y	Y	Y	Y						Y	Y	Y
	Fermented/ acidified vegetables	Fermented cabbage, pickle	Y			Y	Y	Y	Y	Y	Y						Y	Y	Y
Dried cereals, fruits, nuts, seeds and vegetables	Low and IMF fruits (a_w <0,85)	Syrups, concentrates, jams, semi-dried prunes	Y		Y			Y	Y								Y		
	Seasonings	Spices, herbs, peppers	Y		Y			Y	Y								Y		
	Nuts and seeds	Nuts, nut meats, nut butters, seeds	Y		Y			Y	Y	Y	Y						Y		
	Dried fruits and vegetables (a_w <0,60)	Freeze-dried vegetables	Y		Y				Y	Y	Y						Y		
	Dried cereals	Corn, oat, breakfast cereals	Y		Y				Y	Y	Y						Y		
	Flours	Wheat, buckwheat, oat	Y		Y				Y	Y	Y						Y		

Table A.1 *(continued)*

Categories	**Types**	**Items (some examples)**	Total viable count	Lactic Acid Bacteria	Yeasts and moulds	*Enterobacteriaceae*	*Escherichia coli*	Coagulase positive staphylococci	*Salmonella* spp.	*Listeria* spp.	*L. monocytogenes*	Shiga toxin-producing *E. coli (STEC)*	*Cronobacter* spp.	*Campylobacter*	(Pathogenic) *Yersinia enterolitica*	*Vibrio* spp.	*Bacillus cereus* (vegetative cells or spores)	*Clostridium perfringens* (vegetative cells or spores)	*Clostridium botulinum* (vegetative cells or spores)
Infant formula and infant cereals	Probiotic ingredients	Pre-blend, spray dried, culture powders	Y			Y	Y	Y	Y				Y				Y	Y	
	Non-probiotic ingredients	Dehydrated milk, dehydrated yoghurt, dehydrated berries	Y			Y	Y	Y	Y				Y				Y	Y	
	Non-probiotic infant formula	Whey-based (dairy), soy-based (vegetables) fortification formulation	Y			Y	Y	Y	Y				Y				Y	Y	
	Probiotic infant formula	Whey-based (dairy), soy-based (vegetables) fortification formulation	Y			Y	Y	Y	Y				Y				Y	Y	
	Non-probiotic infant cereals	Infant cereals	Y		Y	Y	Y	Y	Y								Y	Y	
	Probiotic infant cereals	Probiotic infant cereals	Y		Y	Y		Y	Y								Y		

Table A.1 *(continued)*

Categories	**Types**	**Items (some examples)**	Total viable count	Lactic Acid Bacteria	Yeasts and moulds	*Enterobacteriaceae*	*Escherichia coli*	Coagulase positive staphylococci	*Salmonella* spp.	*Listeria* spp.	*L. monocytogenes*	Shiga toxin-producing *E. coli (STEC)*	*Cronobacter* spp.	*Campylobacter*	(Pathogenic) *Yersinia enterolitica*	*Vibrio* spp.	*Bacillus cereus* (vegetative cells or spores)	*Clostridium perfringens* (vegetative cells or spores)	*Clostridium botulinum* (vegetative cells or spores)
Chocolate, bakery products and confectionary	Pastries	Bakery products with custard, confectionaries	Y	Y	Y	Y	Y	Y	Y	Y	Y						Y		
	Dry powdered	Cake mixes	Y		Y			Y	Y	Y	Y						Y		
	Low moisture	Crackers, breads, cookies	Y		Y			Y	Y								Y		
	Dry and sugared low moisture (a_w <0,85)	Cake, pralines, marzipan	Y		Y			Y	Y								Y		
	Dry and sugared low moisture (a_w <0,65)	Biscuits, chocolate, confectionary, honey, sugar, candy syrups	Y		Y			Y	Y								Y		

Table A.1 *(continued)*

Categories	**Types**	**Items (some examples)**	Total viable count	Lactic Acid Bacteria	Yeasts and moulds	*Enterobacteriaceae*	*Escherichia coli*	Coagulase positive staphylococci	*Salmonella* spp.	*Listeria* spp.	*L. monocytogenes*	Shiga toxin-producing *E. coli (STEC)*	*Cronobacter* spp.	*Campylobacter*	(Pathogenic) *Yersinia enterolitica*	*Vibrio* spp.	*Bacillus cereus* (vegetative cells or spores)	*Clostridium perfringens* (vegetative cells or spores)	*Clostridium botulinum* (vegetative cells or spores)
Multi-component foods or meal components	Composite foods with substantial raw ingredients (excluding patisserie)	Refrigerated pasta salads, sandwiches, chocolate mousse, bavarois	Y	Y	Y	Y	Y	Y	Y	Y	Y						Y	Y	Y
	Composite processed foods (cooked)	Hot meals	Y			Y	Y	Y	Y	Y	Y			Y			Y	Y	Y
	Ready to (re)heat food: refrigerated	Cooked chilled foods, boiled rice or pasta, vol-au-vent in vacuum	Y		Y	Y	Y	Y	Y	Y	Y			Y			Y	Y	Y
	Ready to (re) heat food: frozen	Frozen fries, pizza, stuffed croissants	Y		Y	Y	Y	Y	Y	Y	Y						Y	Y	Y

Table A.1 *(continued)*

Categories	**Types**	**Items (some examples)**	Total viable count	Lactic Acid Bacteria	Yeasts and moulds	*Enterobacteriaceae*	*Escherichia coli*	Coagulase positive staphylococci	*Salmonella* spp.	*Listeria* spp.	*L. monocytogenes*	Shiga toxin-producing *E. coli (STEC)*	*Cronobacter* spp.	*Campylobacter*	(Pathogenic) *Yersinia enterolitica*	*Vibrio* spp.	*Bacillus cereus* (vegetative cells or spores)	*Clostridium perfringens* (vegetative cells or spores)	*Clostridium botulinum* (vegetative cells or spores)
Multi-component foods or meal components	Ready to (re)heat food: ambient stable (canned)	Vol-au-vent in glass bottles															Y	Y	Y
	Ready to (re)heat food: dry	Dehydrated (instant) soups	Y						Y	Y	Y						Y	Y	Y
	Mayonnaise-based delisalads (acid) with raw ingredients	Raw vegetable salads with dressing	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y					Y		Y
	Mayonnaise-based delisalads (acid) with processed ingredients	Sandwich spreads	Y	Y	Y	Y	Y	Y	Y	Y	Y						Y		Y
	Ambient stable acid foods (pH <4,8)	Ketchup, sauces, dressings, mayonnaises, mustard	Y	Y	Y	Y	Y	Y	Y	Y	Y						Y		Y

Table A.1 *(continued)*

Categories	**Types**	**Items (some examples)**	Total viable count	Lactic Acid Bacteria	Yeasts and moulds	*Enterobacteriaceae*	*Escherichia coli*	Coagulase positive staphylococci	*Salmonella* spp.	*Listeria* spp.	*L. monocytogenes*	Shiga toxin-producing *E. coli (STEC)*	*Cronobacter* spp.	*Campylobacter*	(Pathogenic) *Yersinia enterolitica*	*Vibrio* spp.	*Bacillus cereus* (vegetative cells or spores)	*Clostridium perfringens* (vegetative cells or spores)	*Clostridium botulinum* (vegetative cells or spores)
Pet food and animal feed	Animal origin ingredients	Meat and bone meal, chicken and feather meal, fish meal, animal digest	Y		Y	Y	Y	Y	Y						Y		Y		
	Plant origin ingredients	Com meal, soybean meal, vegetables	Y		Y	Y	Y	Y	Y								Y		
	Other ingredients	Microbial products such as yeast extracts, probiotics	Y		Y	Y	Y	Y	Y								Y		
	Dry food ($a_w \leq 0,7$)	Pellets, treats	Y		Y	Y	Y	Y	Y								Y		
	Wet food ($a_w > 0,7$)	Fresh meat, sausages, croquettes	Y		Y	Y	Y	Y	Y						Y		Y	Y	Y
	Canned	Meat, fish															Y		
	Animal feeds (bovine, ovine, pig)	Cereals, flours	Y		Y	Y	Y	Y	Y								Y		
	Animal feeds (poultry)	Cereals, flours	Y		Y	Y	Y	Y	Y								Y		
	Animal feeds (fish)	Cereals, flours	Y		Y	Y	Y	Y	Y								Y		

Table A.1 *(continued)*

Categories	**Types**	**Items (some examples)**	Total viable count	Lactic Acid Bacteria	Yeasts and moulds	*Enterobacteriaceae*	*Escherichia coli*	Coagulase positive staphylococci	*Salmonella* spp.	*Listeria* spp.	*L. monocytogenes*	Shiga toxin-producing *E. coli (STEC)*	*Cronobacter* spp.	*Campylobacter*	(Pathogenic) *Yersinia enterolitica*	*Vibrio* spp.	*Bacillus cereus* (vegetative cells or spores)	*Clostridium perfringens* (vegetative cells or spores)	*Clostridium botulinum* (vegetative cells or spores)
Environmental samples (food or feed production)	Equipment or production environment	Swabs, dusts	Y			Y	Y	Y	Y	Y	Y	Y	Y	Y	Y		Y	Y	Y
	Waters used in the manufacturing process	(Recycled) washing water, process water	Y			Y	Y	Y	Y	Y	Y	Y	Y	Y	Y		Y	Y	Y
Primary production samples (PPS)	Animal faeces	Swab samples (boot socks), faeces rectal							Y			Y		Y	Y				
	Environmental samples and non-faeces	Dust samples, hygiene swabs, water from drinkers, litters, hatchery samples							Y			Y		Y	Y				

NOTE 1 If relevant, some categories or items can be gathered or split.

NOTE 2 Some regulation bodies have specific requirements to get a regulatory approval on the validation study claim.[2)]

NOTE 3 Unprocessed products, according to the REGULATION (EC) No 852/2004, are described as "Foodstuffs that have not undergone processing and includes products that have been divided, parted, severed, sliced, boned, minced, skinned, ground, cut, cleaned, trimmed, husked, milled, chilled, frozen, deep-frozen, or thawed". This does not include sanitation processes allowed by certain jurisdictions. Therefore, a distinction between raw products not submitted and products submitted to sanitation processes is needed. Different jurisdictions have different definitions for processed and unprocessed products. Check with the appropriate authority in your jurisdiction.

EXAMPLE Fresh meat (REGULATION (EC) No 853/2004) means meat that has not undergone any preserving process other than chilling, freezing, or quick-freezing including meat that is vacuum-wrapped or wrapped in a controlled atmosphere.

NOTE 4 Processing according to the REGULATION (EC) No 852/2004 is described as "Any action that substantially alters the initial product including heating, smoking, curing, maturing, drying, marinating, extraction, extrusion, or a combination of those processes". Processed products may contain ingredients that are necessary for their manufacture or to give them specific characteristics. Different jurisdictions have different definitions for processed and unprocessed products. Check with the appropriate authority in your jurisdiction.

NOTE 5 Minced meat preparations include portioned, cut, or minced meat (<1 % NaCl or spices) intended to undergo a heat treatment before consumption presented as seasoned, marinated, coated, with herbs and spices, or other ingredients are added to improve sensory properties or texture.

NOTE 6 Poultry meat preparations include marinated and spiced meat cuts, chicken fillets, chicken wing, i.e. intact structure either with or without skin.

NOTE 7 Seafoods include live bivalve molluscs and by analogy marine gastropods, echinoderms, and tunicates.

NOTE 8 Ready-to-eat (RTE) food: Food intended by the producer or the manufacturer for direct human consumption without the need for cooking or other processing effective to eliminate or reduce to an acceptable level of microorganisms of concern.

NOTE 9 Ready-to-cook (RTC) food: Food designed by the producer or the manufacturer as requiring cooking or other processing effective to eliminate or reduce to an acceptable level microorganisms of concern.

NOTE 10 Ready-to-reheat (RTRH) food: Food designed by the producer or the manufacturer as suitable for direct human consumption without the need for cooking, but which may benefit in organoleptic quality from some warming prior to consumption.

NOTE 11 For definitions of feeding stuff, refer to REGULATION (EC) No 79/373/EEC.[5]

NOTE 12 Water mentioned in Table A.1 is water used in the manufacturing process or for PPS. In these cases, filtration of samples is not needed.

NOTE 13 If specific sample sizes of a considered item are to be tested in a food category, for instance, 375 g ground beef, it is needed to test a complete technical protocol in the method comparison study for this specific case.

NOTE 14 When a method is to be validated for infant formula and/or infant cereals containing probiotica, the items containing probiotics need to be selected and validated as a full category.

NOTE 15 If the study targets spore-formers, both vegetative cells and spores are included.

2) http://www.fda.gov/; http://www.hc-sc.gc.ca/; http://www.fsis.usda.gov/

Annex B
(normative)

Order of preference for use of naturally and artificially contaminated samples in validation studies

This annex gives the order of preference and information on the use of different kinds of samples in both method comparison studies and interlaboratory studies.

1st option: Naturally contaminated samples

The ideal naturally contaminated sample has a level of contamination for the target organism that is close to the (expected) level of detection.

2nd option: Contamination by mixture

If naturally contaminated samples are found to contain a level that is too high, then the concentration can be reduced by "dilution" of the naturally contaminated sample with a similar item containing a normal background microflora. The mixed sample should be made to have a homogeneous distribution of the target microorganism.

3rd option: Artificially contaminated samples

Strains used for the artificial inoculation should, by preference, have been isolated from the same item and take into account the natural diversity of the target organism e.g. serotype, genotype, and phenotype.

The level of target microflora should be representative of the contamination which occurs in that product naturally. The item should contain a "normal" background microflora.

4th option: Reference materials

Reference materials such as certified reference material containing appropriate, but well-defined levels of target analyte (microorganisms) in a stable, but stressed state can be used to spike samples for analysis by both qualitative and quantitative methods. For qualitative studies, their use should be limited when only a few strains or serotypes of food origin of the target analyte are available as reference materials.

Annex C
(informative)

General protocols for contamination by mixture and artificial contamination of foods

C.1 General

This annex provides examples for artificial contamination of matrices. Methods used by organizing laboratories are not limited to the methods shown here. For the artificial contamination of samples, two possibilities are given: the first named seeding, the other named spiking. The seeding protocol is based on the contamination of natural samples by a diluted culture and subsequent storage of the sample for an extended period in order for the microorganism to adapt to the environmental conditions of the food. The spiking protocol is based on the application of relevant stress conditions to a diluted culture and subsequently inoculation of the stressed culture into the food item. More information on the spiking methodology is described in Reference [10].

Guidelines for environmental samples (e.g. surfaces) are published by AOAC[6] (see sections 4.1.3.11, 4.1.3.3 and 4.1.3.8.2 of the guidelines).

C.2 Contamination by mixture

X grams of naturally contaminated sample are mixed up with *y* grams of non-contaminated sample in order to reach the desired level of contamination.

Store the food sample contaminated by mixture at the appropriate storage temperature for that food item. Allow the microbial population to equilibrate in the food item for a minimum of 1 d before any analysis.

C.3 Artificial contamination of foods using seeding protocol

NOTE The focus of the seeding protocol is on achieving fractional recovery.

C.3.1 Artificial contamination of high moisture foods with a liquid (broth) culture

Preparation of foods with microorganism(s) by single sample inoculation.

a) Culture target strain: Inoculate a tube of non-selective enrichment broth with the designated strain. Incubate the broth under optimal growth conditions for the strain. Enumerate the level of growth of the starting culture.

b) Adjust level by dilution: After incubation, dilute the starting culture in a suitable diluent to achieve the desired food contamination level(s). The level of dilution required is dependent on the food to be inoculated, the strain chosen, the intended level of contamination, the expected background microflora level, and the storage conditions of the food item.

c) Thermal stress: Strains used to inoculate foods that are exposed to thermal processing during manufacturing should be heat stressed, e.g. at 50 °C for 15 min, prior to inoculation of the food.

d) Inoculation of the food: Inoculate the food by pipetting a known volume or spraying a known volume of the culture at the chosen dilution into the food. Inoculation of individual samples is allowable. Alternatively, the entire preparation of the food can be inoculated and mixed thoroughly

to achieve homogeneity. The volume of the inoculum should be as low as possible as it should not influence the a_w significantly. Generally, 0,25 ml per 25 g of sample is used (1 %).

e) Mix to ensure homogeneity: After inoculation, the food is mixed thoroughly to ensure homogeneity. If the inoculum is added in steps. Mixing should be done after each step.

f) Sample stabilization/stress: Store the food at the temperature that is the normal storage temperature for that food. Consider the potential growth or survival of the organism during the storage period. Regarding the different storage temperatures, store the inoculated foods for the minimum period indicated below.

   1) Frozen food: At least 2 weeks at –20 °C;

   2) Refrigerated storage: At least 48 h at 2 - 8 °C;

   3) Room temperature storage: At least 2 weeks at 20 - 25 °C.

   EXAMPLE Nutmeat would be stored at room temperature, orange juice would be stored at 2 - 8 °C, and ice cream would be stored at –20 °C.

g) Verification of contamination level: If needed, a check of the inoculation level can be done after the samples have been stored for the appropriate stabilization time. If necessary, adjust contamination levels to be certain that the desired contamination is achieved.

C.3.2 Artificial contamination of low moisture foods with a lyophilized culture

a) Prepare a lyophilized culture: Inoculate a tube of non-selective enrichment broth with the designated strain. Incubate the broth at optimal conditions for the strain. After incubation, collect the bacterial cells by centrifugation. Wash cells twice with a sterile buffered diluent. Repeat centrifugation and decant the supernatant. Resuspend the pellet into sterile 10 % NFDM (non-fat dried milk). Transfer resuspended cells into appropriate containers for lyophilization.

b) Assess level of target organism: Collect the lyophilized cell suspensions in a sterile container. Manually crush the lyophilized culture to create a homogenous fine powder before assessment of the level of contamination. Use a non-selective method for the determination of the level of contamination and incubate the plates under optimal growing conditions.

c) Inoculate the lyophilized culture into the food to attain the required level: Mix 0,1 g of the lyophilized culture with 10 g of the uninoculated food item in, for example, a sterile plastic bag. The bag is shaken until the inoculum appears to be evenly distributed throughout the food item. Perform serial 10-fold dilutions with the item (e.g. 1 g from first step with 9 g item, etc.) to dilute the lyophilized culture to the appropriate level. Ensure that proper mixing occurs at each dilution level.

d) Store the food at the appropriate storage temperature (preferably the normal storage temperature) for that food. Allow the microbial population to equilibrate in the food for a minimum of two weeks before any analysis.

e) Verification of the contamination level: If needed, a check of the inoculation level can be done after the samples have been stored for the appropriate time.

C.4 Artificial contamination of foods using spiking protocol

The spiking protocol exists of different steps as presented below.

a) Culture target strain: Inoculate a tube of non-selective enrichment broth with the designated strain. Incubate the broth at optimal conditions for the strain.

b) Adjust level by dilution: After incubation, dilute the culture in a suitable diluent until the desired level(s). Injury protocols are usually done on pure cultures with 10^4 to 10^5 cells/ml.

c) Apply an injury protocol: Apply an appropriated culture treatment as in the examples below.

EXAMPLE 1 Heat treatment (e.g. 15 min at 50 °C) by immersion in a given temperature bath.

EXAMPLE 2 Freezing treatment (e.g. 72 h at –20 °C).

EXAMPLE 3 Chemical treatment (e.g. treatment at high salt concentration or at low pH).

EXAMPLE 4 Storage at 4 °C (e.g. for one week minimum).

The conditions for applying stress strongly depend on the type of microorganism and even the selected strain. The selected stress protocol should also resemble the stress of the microorganisms found in the food sample to be used for spiking.

d) Injury measurement: Injury efficiency is usually evaluated by enumerating the pure culture on selective and non-selective agars. More than 0,5 $\log_{10}$ cfu/ml difference is expected for a sufficient stress application.

e) Adjust level by dilution: Dilute the culture, if necessary, in a suitable diluent until the desired level in order to inoculate the food item. For qualitative methods, this level should be at the level of detection of the method.

f) Inoculate into food by pipetting a known volume or spraying a known volume: The diluted culture is inoculated into the item by spraying or pipetting. Inoculation of individual samples is preferred. The volume of the inoculum should be as low as possible as it should not influence the water activity significantly. Generally, 0,25 ml per 25 g of sample is used.

g) Mix to ensure homogeneity: After inoculation, the item is mixed thoroughly to ensure homogeneity. If the inoculum is added in steps, mixing should be done after each step.

Annex D
(informative)

Models for RLOD calculations using data from the method comparison study

D.1 General

The level of detection (LOD) is the lowest level of contamination where reliable detection is expected. In this part of ISO 16140, the LOD_{50} is used, the level of contamination with an expectation of 50 % positive test results. The relative level of detection (RLOD) is the ratio of the LOD of the alternative method and the LOD of the reference method. As such, this measure is not dependent of the chosen level of positive test results.

Table D.1 gives an example of data used for the calculation of LOD and RLOD: levels of contamination, numbers of tests performed, numbers of positive results for the reference method, and the alternative method. The numbers of positive results are described by a binomial distribution. The numbers of CFU present in the test portions that are actually being analysed are (theoretically) described by a Poisson distribution.

Table D.1 — Example of data used for the calculation of LOD and RLOD

Category	Level	x (cfu/g)	n_{tot}	$n_{pos\ ref}$	$n_{pos\ alt}$
Milk and dairy products	1	0	5	0	0
Milk and dairy products	2	0,022 4	20	12	10
Milk and dairy products	3	0,037 33	5	5	5

The LOD and RLOD are calculated from the data by linking the binomially distributed observations to the Poisson distributed number of CFU in the test portions by application of a Generalized Linear Model (GML). For general information on GLMs, see Reference [11]. More specifically, the model used is a complementary log-log model (CLL).

d is the level of contamination in CFU per unit of weight or volume (e.g. CFU/g) and p is the probability of obtaining a positive result: $p = \mathrm{E}\left(\frac{n_{pos}}{n_{tot}}\right)$

By application of following nonlinear link function, p is transformed into a parameter η that can be linearly predicted from $\ln d$ (logarithm of the contamination): $\eta = \ln(-\ln(1-p))$.

In addition to the link function, a linear predictor function is used modelling η as a function of $\ln d$. For the calculation of LOD and RLOD, different models are used. These models can be fitted in the statistical software packages. For the purpose of this part of ISO 16140, specific Excel®-based programs are made available to calculate LOD and RLOD.

D.2 Contamination levels not known

When the contamination levels are not known, only a direct estimation of RLOD is possible. This option is prescribed in 5.1.4.2. No information about LOD can be obtained. In this case, the flowing predictor function is used: $\eta = a_{0i} + L_i + D$.

Parameters L_i are fitted estimates of the contamination level of the samples. RLOD is calculated in the same way: $\mathrm{RLOD} = \exp(-D)$.

The details of the model are worked out in Reference [15] and Reference [16]. In this part of ISO 16140, a very limited test setup is used for the estimation of RLOD. The additional fitting of parameters in this approach goes at the expense of the reliability of the estimate of RLOD.

D.3 Contamination levels known, estimation of RLOD through LOD

When the contamination levels are (approximately) known, RLOD can be estimated either directly or through the LODs of the alternative method and the reference method. In the latter case, for each category the LODs are estimated followed by the calculation of RLOD: $\text{RLOD}=\frac{\text{LOD}_{\text{alt}}}{\text{LOD}_{\text{ref}}}$

The model or predictor function used for the estimation of LOD is: $\eta = a_0 + f_i + \ln d$

where

$a_0 = \ln A_0$ (A_0 being the sample size in g or ml);

$f_i = \ln F_i$ (F_i designating the category effect of category i);

d = level of contamination (cfu/g or cfu/ml).

The details of the model are worked out in Reference [15] and Reference [16].

The LOD is estimated as: $\text{LOD} = \frac{\ln(1-p)}{A_o \hat{F}}$

with p = 0,5 (because LOD_{50} is determined). $\hat{F}$ is obtained from solving the following formula:

$$\sum_{j=1}^{q}\left(\frac{y_j d_j}{\exp(A_0 \hat{F} d_j) - 1} - (n_j - y_j) d_j\right) = 0 \qquad \text{(D.1)}$$

The approach of calculation RLOD through LODs allows the assessment of the performance characteristic LOD of the alternative method.

Annex E
(normative)

Points to be considered when selecting strains for testing inclusivity and exclusivity

E.1 General

This annex outlines the minimum test requirements for general use. In the selection of test strains, the majority shall originate from the categories tested in the study and cover the recognized range of the target analyte with respect of the following: diversity in identification characteristics, e.g. biochemical, serotype, phage type, geographical distribution, incidence, and any other claims made by the producers of the alternative method.

E.2 Target group categories

a) Undefined group, for example, total count, coliform, yeast, and lactic-acid bacteria.

b) Family, for example, *Enterobacteriaceae*.

c) Genus, for example, *Salmonella, Pseudomonas*, and *Listeria*.

d) Species, for example, *Listeria monocytogenes, Staphylococcus aureus*, and *Escherichia coli*.

e) Sero or phage type, for example, *Salmonella enterica* serovar Enteritidis.

E.3 Target group selection in an inclusivity study

a) For undefined groups for which the target group is defined by the reference method, the strains used shall be selected from those capable of typical growth in the reference method.

b) For families: Use strains from a range of genera in that family and if possible, include a representative member of all genera in the family.

c) For genera: Use a range of species from that genus and if possible, test as many species as possible in the genus.

d) For species: A range of strains from that species. For the selection of strains, other more detailed ways for subtyping need to be considered. For example, *Salmonella* and *Listeria* are serotyped and phage typed. In the future, selection according to other (genetic) typing methods may be appropriate. In defining the positive strains to be used, organizing laboratories should use available up to date information to ensure that strains are relevant to the target categories at the time of testing.

e) For sero or phage specific strains: A range of sources of that sero or phage type.

E.4 Non-target groups selection in an exclusivity study

a) The non-target groups (that is those expected to be negative and being used for cross reactivity tests) should be specified according to the target group.

b) When the target group is a family: Non-target strains shall include other closely associated families.

c) When the target group is a genus: Non-target strains shall include other genera considered to be similar biochemically or genetically to the target genus.

d) When the target group is a species: Non-target strains shall include other species within the target genus.

e) When the target group is sero or phage specific: Non-target strains shall include other sero or phage type strains within the same species.

Annex F
(informative)

Considerations for calculations of the relative level of detection (RLOD) between laboratories as obtained in an interlaboratory study

The relative level of detection (RLOD) is estimated in the interlaboratory study using similar statistical models as in the method comparison study (RLOD study, see Annex D). In the case of the interlaboratory study, it is investigated whether there are differences between the laboratories.

The numbers of positive samples are described by a binomial distribution. The expected fraction of positive samples [$P = E(n_{pos}/n_{tot})$] is linked to the number of cfu in the sample by p ($P = 1-e^{-\lambda}$) and additive models for z [$z = ln(\lambda)$] are used. This implies a complementary log-log relation between z and p: $z = ln(\lambda) = ln[-ln(1-p)]$. The options binomial distribution and complementary log-log (CLL) link function can be specified as inputs in a generalized linear model as available in all major statistical packages.

The models fitted are the following:

$$z = \ln(s \cdot x) + D \tag{F.1}$$

$$z = \ln(s \cdot x) + D + \text{Lab}_j \tag{F.2}$$

where

s is the sample quantity (e.g. in g or ml);

x is the contamination level of the sample (cfu/g or cfu/ml);

D is the difference between the alternative and the reference methods on the scale of z (the log level scale), averaged over the laboratories;

Lab j is the systematic deviation for the jth laboratory method on the scale of z (the log level scale), averaged over the reference and the alternative methods. Laboratory effects are modelled most simply as fixed effects in the CLL model (see 5.1.4.2 and Annex D for more explanation of the CLL model).

Parameters D and Lab_j are estimated by fitting the CLL model to the combined data. A statistical test is performed to investigate the laboratory differences. If these are statistically insignificant, the simpler model is used for RLOD estimation; else the model including laboratory effects is used.

For both types of model, RLOD is calculated as e^{-D}, where D is the estimated parameter.

In all cases, an approximate 90 % confidence interval [$RLOD_{low}$, $RLOD_{upp}$] for the resulting RLOD is calculated, for example, as: $\left[e^{-D-t_{df}(0,95)\cdot se(D)}, e^{-D+t_{df}(0,95)\cdot se(D)} \right]$

where

se(D) is the approximate standard error of the estimate *D;*

df is the residual degrees of freedom of the fitted model;

$t_{df}(0,95)$ is the one-sided 95 % critical value of the Student *t* distribution with *df* degrees of freedom.

An example data set for the calculations concerns a validation in 10 laboratories of an alternative method for *Listeria innocua* in milk at three levels of contamination. A hypothetical Acceptability Limit AL = 4 is assumed. For statistical analysis, the data are arranged per level/method/laboratory combination in a data set with 60 rows (three levels × two methods × 10 laboratories) (Table F.1).

Table F.1 — Example of a data set from an interlaboratory study

Level (cfu/25 ml)	Method	Lab	n_{pos}	n_{tested}	Method	Lab	n_{pos}	n_{tested}
0	Reference	A	0	8	Alternative	A	0	8
0	Reference	B	0	8	Alternative	B	0	8
0	Reference	D	0	8	Alternative	D	0	8
0	Reference	F	0	8	Alternative	F	0	8
0	Reference	G	0	8	Alternative	G	0	8
0	Reference	H	0	8	Alternative	H	0	8
0	Reference	J	0	8	Alternative	J	0	8
0	Reference	L	0	8	Alternative	L	0	8
0	Reference	M	0	8	Alternative	M	0	8
0	Reference	O	0	8	Alternative	O	0	8
2,4	Reference	A	8	8	Alternative	A	8	8
2,4	Reference	B	6	8	Alternative	B	8	8
2,4	Reference	D	7	8	Alternative	D	6	8
2,4	Reference	F	8	8	Alternative	F	7	8
2,4	Reference	G	7	8	Alternative	G	8	8
2,4	Reference	H	5	8	Alternative	H	7	8
2,4	Reference	J	6	8	Alternative	J	5	8
2,4	Reference	L	7	8	Alternative	L	5	8
2,4	Reference	M	6	8	Alternative	M	7	8
2,4	Reference	O	7	8	Alternative	O	6	8
25,3	Reference	A	8	8	Alternative	A	8	8
25,3	Reference	B	8	8	Alternative	B	8	8
25,3	Reference	D	8	8	Alternative	D	8	8
25,3	Reference	F	8	8	Alternative	F	8	8
25,3	Reference	G	8	8	Alternative	G	8	8
25,3	Reference	H	8	8	Alternative	H	8	8
25,3	Reference	J	8	8	Alternative	J	8	8
25,3	Reference	L	8	8	Alternative	L	8	8
25,3	Reference	M	8	8	Alternative	M	8	8
25,3	Reference	O	8	8	Alternative	O	8	8

Before the data are fitted, non-informative parts of the data sets are removed. In this case, all results from laboratories A and F which have no fractional results for any level are excluded.

The remaining data have been analysed by fitting the CLL model (see Annex D) as a generalized linear model (GLM). Algorithms to fit GLMs are available in all major statistical software packages. For this example, GenStat has been used. See Reference [11] for general information on GLMs.

Fitting the CLL model including laboratory effects to the combined data from three samples and eight laboratories (A and F excluded), the laboratory effects Lab_j for laboratories D, G, H, J, L, M, and O expressed as a difference with laboratory B were found to be 0,39; 0,39; −0,34; −0,17; 0,39; 0,00; and 0,39 respectively (all standard errors 0,44). These laboratory differences are not statistically significant in an analysis of deviance test (decrease in deviance 6,37 with seven degrees of freedom, asymptotic chi-squared *p* value 0,50). Consequently, the CLL model without laboratory effects was fitted giving an estimate for the method difference *D* of −0,046 5 (standard error 0,22). The RLOD is then estimated as exp[-(−0,046 5)] = 1,05 with a 90 % confidence interval of 0,73 - 1,51.

Thus, the alternative method is only very slightly less sensitive (5 % higher LOD) than the reference method (due to only one less positive result for a sample at the intermediate in lab J). This is not a significant difference as follows both from the fact that the confidence interval includes the value one, and from an analysis of deviance test (decrease in deviance 0,04 with one degree of freedom, asymptotic chi-squared *p* value 0,83). Regarding the validation, the alternative method would be validated with any Acceptability Limit (AL) down to 1,6.

Annex G
(informative)

Principle of the accuracy profile for validation of quantitative models

G.1 Acceptance criterion

The validation strategy for quantitative methods is based on the idea that end users and decision makers actually require that an analytical procedure gives a result Z that differs from the assigned value of the sample X by less than an acceptability criterion noted as λ. This should be understood as a fitness-for-purpose objective. This requirement can be expressed by the following formula:

$$|Z - X| < \lambda \tag{G.1}$$

Criterion λ represents the closeness of agreement between the assigned value and the obtained result that is acceptable. A method that produces a large proportion of acceptable results is valid with regard to decision-maker needs and can be said to be fit-for-purpose. Thus, the acceptability value λ depends on the objective of the analytical procedure. It can be obtained by consensus between analyst and end users or by using regulatory references. In the framework of this part of ISO 16140, Acceptability Limits are obtained by consensus between parties involved and expressed in $\log_{10}$ units.

G.2 Assigned reference value

When analysing samples, their exact values are not known. However, when validating a method, it is assumed that a value can be adequately assigned to the material used for ascertaining method validity. This is usually done by using (certified) reference materials, but these are hardly available in microbiology. In the framework of the accuracy profile approach, the assigned value of a sample is obtained by applying the reference method.

An assigned reference value can be derived from one single measurement or from aggregated replicates (average or median).

When interpreting the validation data in the framework of this part of ISO 16140, the reference value is assumed to be constant, although it is estimated from experimental data.

G.3 Tolerance interval

In order to make a decision, whether the alternative method is valid or not, it is necessary to verify whether the alternative method is able to produce a large proportion of acceptable results on average. Since it is not easy to compute this proportion directly for given Acceptability Limits, an indirect approach is used. This consists of calculating a β-expectation tolerance interval (β-ETI) that contains on average a proportion of β % of results and to verify that the β-ETI is included into the Acceptability Limits. This can be translated into the following formula:

$$\text{Prob}(|Z - X| < \lambda) \geq \beta \tag{G.2}$$

Do not confound the β-expectation tolerance interval with the confidence interval which is used to define an interval where the assigned reference value of a statistical parameter is assumed to be located with a given level of confidence (usually 95 %).

G.4 Principles of accuracy profile

Samples used in the validation studies are selected in order to cover the whole expected domain of application and to have different contamination levels. Different β-ETIs are calculated from experimental measurements performed on each set of samples, the reference values of which are known. Indeed, β-ETIs are the intervals where it is expected that a proportion β of future measurements will fall inside.

Therefore, for a given contamination level, as long as β-ETI is included inside the acceptance limits, conditions for valid analytical method are fulfilled.

Data are interpreted, based on a graphical representation of β-ETI and Acceptability Limits as illustrated in Figure G.1 for one set of samples (e.g. samples from the same category or type).

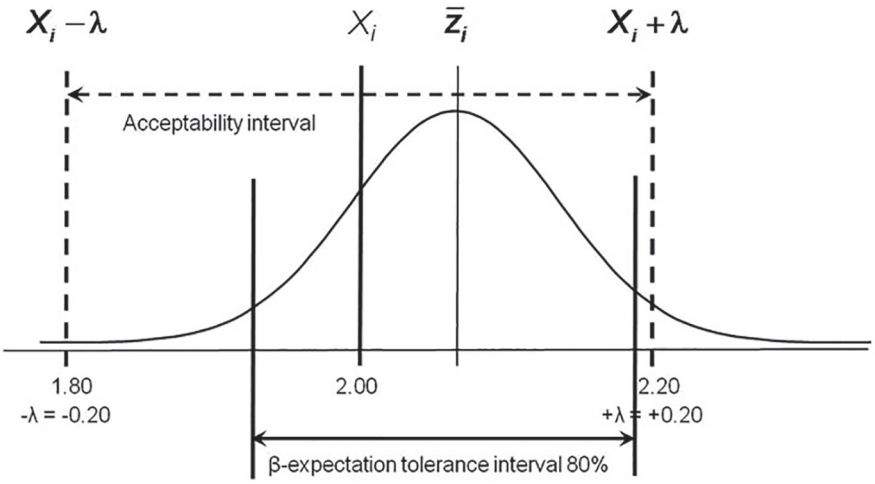

Figure G.1 — Simultaneous representation of acceptability interval and β-ETI for a single validation item sample

Annex H
(informative)

Application of the accuracy profile in the method comparison study

An alternative method for the enumeration of *E-coli* in foods and feed was validated for different categories. In this annex, only the results obtained for the category pet food and animal feed are presented. The other categories require analogous evaluations.

According to the experimental design, six different samples of three different levels of contamination were examined. For each sample, five replicate tests were done with the reference method and the alternative method. Table H.1 summarizes the results obtained and the calculations. The steps and the symbols refer to 6.1.3.3.

Table H.1 — Overview of results and calculations

Counts	repeat	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Reference	1	40	210	350	520	4 500	3 600
	2	55	90	470	410	4 800	7 900
	3	80	320	500	660	7 900	5 500
	4	90	100	480	310	3 800	5 900
	5	50	130	490	590	4 100	8 700
Alternative	1	100	90	890	430	9 100	7 800
	2	60	25	810	510	2 400	9 100
	3	70	60	460	480	3 700	6 000
	4	85	50	520	520	3 400	6 100
	5	45	65	580	580	7 800	6 000
Log_{10} transformed							
Reference	1	1,60	2,32	2,54	2,72	3,65	3,56
	2	1,74	1,95	2,67	2,61	3,68	3,90
	3	1,90	2,51	2,70	2,82	3,90	3,74
	4	1,95	2,00	2,68	2,49	3,58	3,77
	5	1,70	2,11	2,69	2,77	3,61	3,94
Step 1	X_i	**1,740**	**2,114**	**2,681**	**2,716**	**3,653**	**3,771**
Alternative	1	2,00	1,95	2,95	2,63	3,96	3,89
	2	1,78	1,4	2,91	2,71	3,38	3,96
	3	1,85	1,78	2,66	2,68	3,57	3,78
	4	1,93	1,7	2,72	2,72	3,53	3,79
	5	1,65	1,81	2,76	2,76	3,89	3,78
Step 2	Y_i	**1,845**	**1,778**	**2,763**	**2,708**	**3,568**	**3,785**
Step 3	$s_{\text{alt},i}$	0,134	0,207	0,124	0,048	0,248	0,083
	N	5	5	5	5	5	5
Step 4	S_{alt}	**0,156**					
Step 5	$s_{\text{ref},i}$	0,146	0,231	0,064	0,131	0,125	0,151
	S_{ref}	**0,150**					

Table H.1 *(continued)*

Counts	repeat	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Step 6	$Y_i - X_i$	0,105	−0,336	0,082	−0,008	−0,085	0,014
Step 7	$s_{alt} \cdot \sqrt{1+1/n}$	0,171					
	$T(0{,}10;\ 24)$[a]	1,318					
	$T \cdot s \cdot \sqrt{}$	0,225					
	U_i	0,330	−0,111	0,307	0,217	0,140	0,240
	L_i	−0,120	−0,561	−0,143	−0,234	−0,310	−0,211

[a] β-ETI values are calculated for β = 80 %. This results in $T_{\left(\frac{1-\beta}{2}\right);q(n-1)} = T_{(0{,}10;24)} = 1{,}318$. The Excel function *TINV* (inverse Student-*t* distribution), used in the Excel worksheet referenced in this part of ISO 16140, returns a two-tailed *t*-value. Therefore, the *TINV* function is used as follow in the example of Table H.1: $TINV\left((1-\beta);q(n-1)\right) = TINV(0{,}20;24) = 1{,}318$.

Step 8: Tabulate the statistical values calculated for every samples of the category.

Table H.2 — Presentation of the statistical results of the comparison study for the category pet food

Sample	Central value (Ref)	Central value (Alt)	Bias	Upper β-ETI	Lower β-ETI	Upper AL	Lower AL
Sample 1	1,740	1,845	0,105	0,330	−0,120	0,5	−0,5
Sample 2	2,114	1,778	−0,336	−0,111	−0,561	0,5	−0,5
Sample 3	2,681	2,763	0,082	0,307	−0,143	0,5	−0,5
Sample 4	2,716	2,708	−0,008	0,217	−0,234	0,5	−0,5
Sample 5	3,653	3,568	−0,085	0,140	−0,310	0,5	−0,5
Sample 6	3,771	3,785	0,014	0,240	−0,211	0,5	−0,5

It is observed that for sample 2, the β-ETI limit L_i exceeds the Acceptability Limit AL = 0,5 log units.

Since one tolerance interval exceeds the Acceptability Limits, the additional evaluation is carried out according to the steps of 6.1.3.3.

Step 9: The alternative Acceptability Limits are calculated: $AL_s = 4 \cdot s_{ref} = 4 \cdot 0{,}15 = 0{,}6$. Comparison shows that the β-ETI limits U_i and L_i of step 7 do not exceed the new Acceptability Limits $\pm AL_s$. The alternative method is accepted as being equivalent to the reference method for this combination of food category and food type. The final statistical results are presented in Table H.3.

Table H.3 — Presentation of the statistical results of the comparison study for the category pet food

Sample	Central value (Ref)	Central value (Alt)	Bias	Upper β-ETI	Lower β-ETI	Upper AL	Lower AL
Sample 1	1,74	1,85	0,11	0,401	−0,181	0,6	−0,6
Sample 2	2,0	1,78	−0,22	0,071	−0,511	0,6	−0,6
Sample 3	2,68	2,76	0,08	0,371	−0,211	0,6	−0,6

Table H.3 *(continued)*

Sample	Central value (Ref)	Central value (Alt)	Bias	Upper β-ETI	Lower β-ETI	Upper AL	Lower AL
Sample 4	2,72	2,71	−0,01	0,281	−0,301	0,6	−0,6
Sample 5	3,65	3,57	−0,08	0,211	−0,371	0,6	−0,6
Sample 6	3,77	3,79	0,02	0,311	−0,271	0,6	−0,6

Then, make the graphical representation of the final accuracy profile.

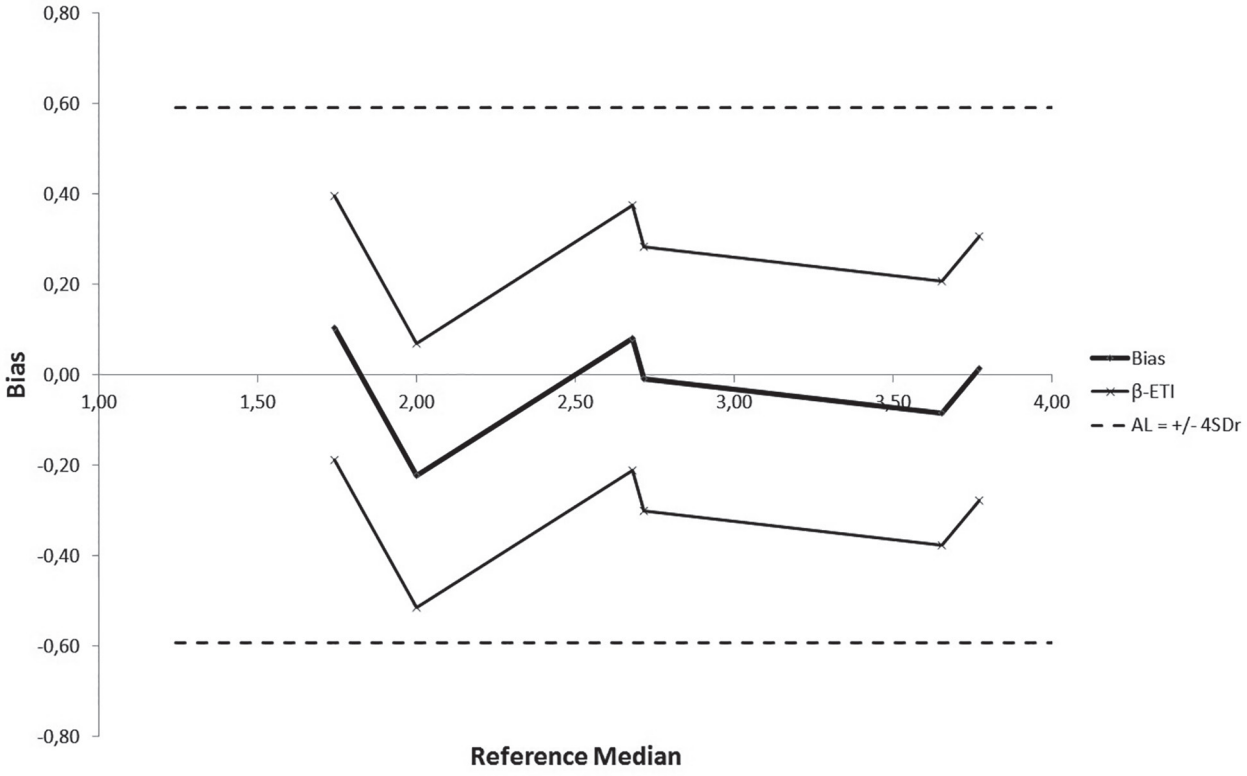

Figure H.1 — Final accuracy profile for the pet food category

Annex I
(informative)

Example of the application of the accuracy profile for an interlaboratory study

As part of the validation of a quantitative alternative method against a reference method, an interlaboratory study was organized. For this study, three samples with nominal levels of contamination were prepared at around 150 cfu/g, 1 500 cfu/g, and 15 000 cfu/g. The samples were coded low, medium, and high respectively. A group of eight laboratories participated in the study. They were coded one to eight. Each participant received samples in order to carry out duplicate analysis with the reference method and the alternative method, i.e. in total four measurements at each level.

The interlaboratory study was organized according to 6.2.2 of this part of ISO 16140. Besides the three levels, also a negative control was included. These results are not shown in this example. After reception of the results from the collaborators, the logistics were checked, e.g. the temperature log, dates of sample reception, and analysis. The test results are presented in Table I.1.

Table I.1 — Raw data

Collaborator	Level	Reference method		Alternative method	
		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
1	Low	300	260	120	220
2	Low	250	210	260	100
3	Low	180	160	110	150
4	Low	150	140	220	210
5	Low	190	164	150	130
6	Low	140	190	160	140
7	Low	180	240	160	160
8	Low	130	150	140	220
1	Medium	1 800	1 900	2 000	2 900
2	Medium	1 200	1 300	2 100	1 300
3	Medium	1 700	1 500	2 100	1 200
4	Medium	1 400	1 300	1 500	2 400
5	Medium	1 700	1 800	1 300	1 600
6	Medium	1 900	1 700	2 100	1 800
7	Medium	2 300	2 600	2 100	2 000
8	Medium	1 200	1 300	2 200	1 400
1	High	21 000	15 000	17 000	27 000
2	High	24 000	16 000	19 000	23 000
3	High	15 000	14 000	21 000	11 000
4	High	13 000	13 000	14 000	14 000
5	High	15 000	22 000	14 000	17 000
6	High	23 000	20 000	21 000	19 000
7	High	12 000	15 000	13 000	12 000
8	High	13 000	12 000	18 000	20 000

The first operation before the steps of the calculations start is the $\log_{10}$ transformation of all test results. Table I.2 contains the $\log_{10}$ transformed test results and the succeeding steps of calculations. The steps and the symbols refer to 6.2.3.

Table I.2 — $\log_{10}$ transformed data and calculations

$\log_{10}$ transformed		**Low**				**Medium**				**High**			
	Collaborator	Reference		Alternative		Reference		Alternative		Reference		Alternative	
	1	2,48	2,41	2,08	2,34	3,26	3,28	3,30	3,46	4,32	4,18	4,23	4,43
	2	2,40	2,32	2,41	2,00	3,08	3,11	3,32	3,11	4,38	4,20	4,28	4,36
	3	2,26	2,20	2,04	2,18	3,23	3,18	3,32	3,08	4,18	4,15	4,32	4,04
	4	2,18	2,15	2,34	2,32	3,15	3,11	3,18	3,38	4,11	4,11	4,15	4,15
	5	2,28	2,21	2,18	2,11	3,23	3,26	3,11	3,20	4,18	4,34	4,15	4,23
	6	2,15	2,28	2,20	2,15	3,28	3,23	3,32	3,26	4,36	4,30	4,32	4,28
	7	2,26	2,38	2,20	2,20	3,36	3,41	3,32	3,30	4,08	4,18	4,11	4,08
	8	2,11	2,18	2,15	2,34	3,08	3,11	3,34	3,15	4,11	4,08	4,26	4,30
Step 1						3,21				4,20			
Step 2	p			8		8		8		8		8	
	s_r	058		0,138		0,028		0,118		0,077		0,093	
	s_L	094		0,000		0,098		0,000		0,071		0,059	
	s_R	111		0,138		0,102		0,118		0,105		0,110	
Step 3	$\bar{y}_i$							3,26				4,23	
Step 4						0,050				0,026			
Step 5	G_i							1,000				0,882	
	H_i	62		0,00		12,11		0,00		0,854		0,400	
	ν	9,21		14,93		7,56		14,93		11,72		13,34	
	T			1,34				1,34				1,34	
	s_{TIi}			0,143				0,121				0,114	
	k_{Mi}							1,382				1,402	
Step 6	$U_i - X_i$							0,213				0,181	
	$L_i - X_i$							−0,112				−0,128	

Figure I.1 shows the data points after $\log_{10}$ transformation. At this stage, a visual inspection shows that the alternative method gives results which are proportional to those of the reference method. Moreover, data are distributed closely to the first bisecting line with slope equal to one and confirm this result. In Figure I.1, also the medians of the measurements obtained with the reference method for each level are shown (vertical lines).

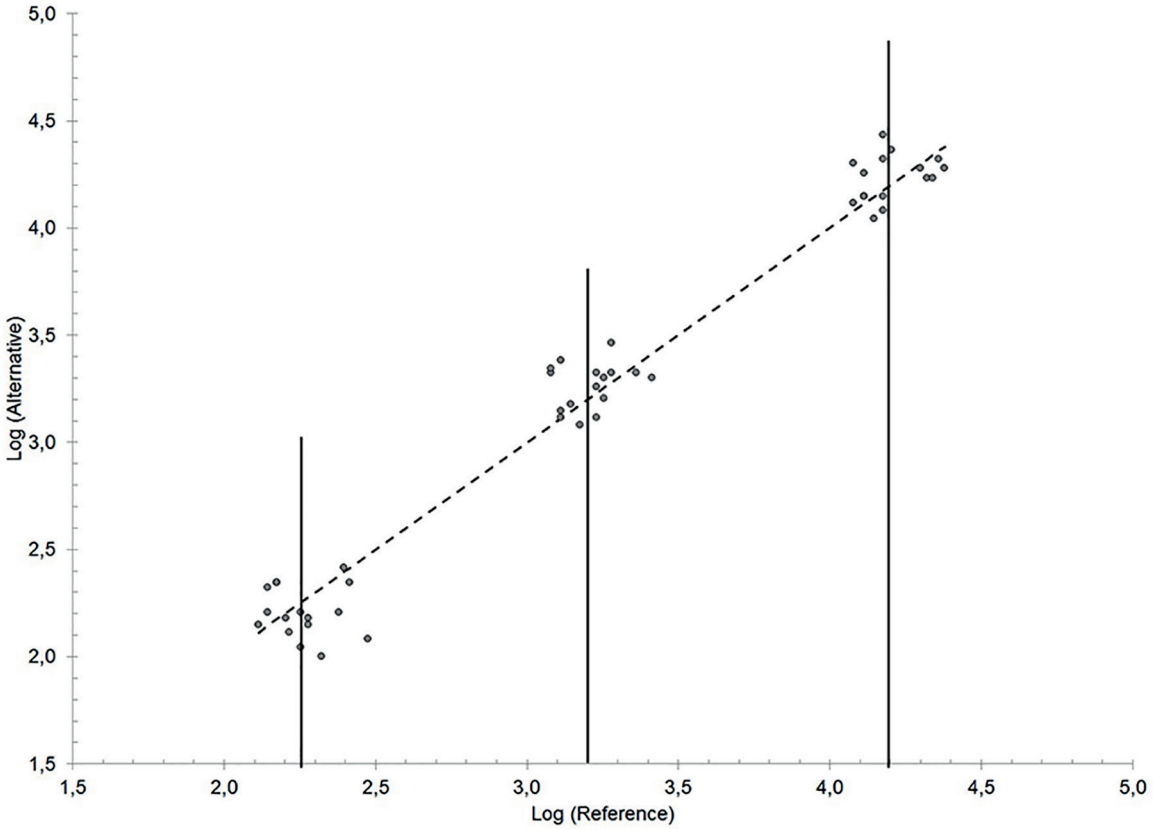

Figure I.1 — Visual linearity checking

In step 6, the limits of the β-ETI are calculated. These values are collected in a graphical representation together with the Acceptability Limits (AL). This graphical representation is shown in Figure I.2. It is observed that for none of the levels, an Acceptability Limit is exceeded. It is concluded that the alternative method is fully validated when compared to the reference method between 185 cfu and 15 000 cfu with and acceptance of 0,5 as stated in step 7.

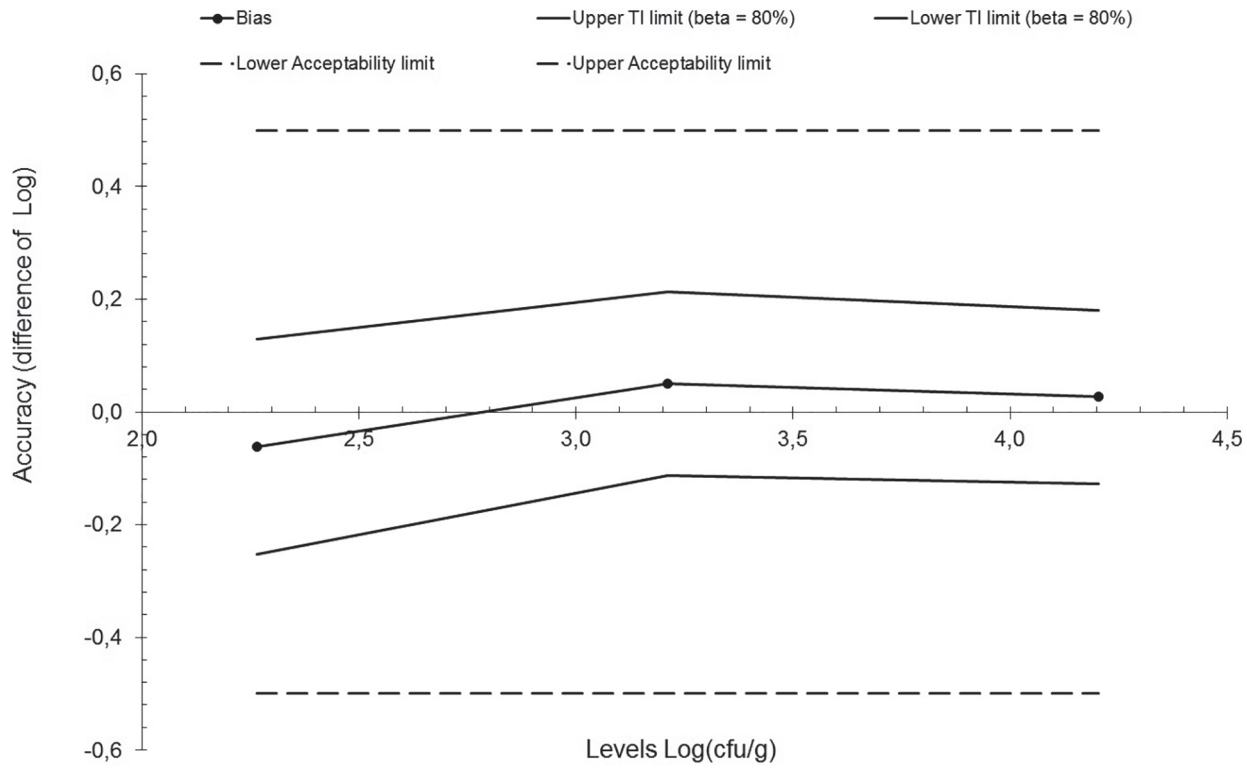

Figure I.2 — Accuracy profile of the alternative method using β = 80 % and λ = 0,5 $\log_{10}$ unit

Note that some of the calculations of Table I.2 have actually not been used because it was not necessary to perform the additional evaluation of step 8 of 6.2.3. These are the calculations used for the determination of the number of degrees of freedom and the pooled reproducibility standard deviation of the reference method (v_{ref} and $s_{R,\text{ref}}$)

However, for illustrative purpose, the pooled interlaboratory reproducibility standard deviation is obtained as

$$s_{R,\text{ref}} = \sqrt{\frac{1}{q}\sum_{i=1}^{q} s_{Ri}^2} = 0{,}106 \qquad \text{(I.1)}$$

This value could be used for the calculation of the new Acceptability Limits, ALs = 0,350, of Step 9.

All calculations can easily be done in a spreadsheet such as Excel®.

Bibliography

[1] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

[2] ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

[3] ISO 16297, *Milk — Bacterial count — Protocol for the evaluation of alternative methods*

[4] ISO/TS 22117, *Microbiology of food and animal feeding stuffs — Specific requirements and guidance for proficiency testing by interlaboratory comparison*

[5] 79/373/EEC, Council Directive of 2 April 1979 on the marketing of compound feeding stuffs, *Official Journal of the European Communities* **L 86**, 6-4-1979, p.30

[6] AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces http://www.eoma.aoac.org/app_j.pdf, 2012

[7] BLAND. J.M., ALTMAN D.G. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet. 1986, **i** pp. 307–310

[8] GUTMAN. I. Statistical Tolerance Regions: Classical and Bayesian. Hafner Publishing, Darien, CT, 1970

[9] HUBERT. Ph. *et al.* Harmonization of strategies for the validation of quantitative analytical procedures: A SFSTP proposal, Part I: *Journal of Pharmaceutical and Biomedical Analysis,* **36**, 2004, pp. 579-586. Part II: *Journal of Pharmaceutical and Biomedical Analysis,* **45**, 2007, pp. 70–81. Part III: *Journal of Pharmaceutical and Biomedical Analysis,* **45**, 2007, pp. 82–96. Part IV. J. Pharm. Biomed. Anal. 2008, **48** pp. 760–771

[10] JASSON. V., UYTTENDAELE, M., RAJKOVIC, A., DEBEVERE, J. Establishment of procedures provoking sub-lethal injury of *Listeria monocytogenes, Campylobacter jejuni* and *Escherichia co*li O157 to serve method performance testing. Int. J. Food Microbiol. 2007, **118** (3) pp. 241–249

[11] MCCULLAGH. P., NELDER, J.A. Generalized linear models. Chapman & Hall, London, Second Edition, 1989

[12] MEE. R.W. β-expectation and β-content tolerance limits for balanced one-way ANOVA random model. Technometrics. 1984, **26** (3) pp. 251–253

[13] ROZET. E. *et al.* Improvement of the decision efficiency of the accuracy profile by means of a desirability function for analytical methods validation: application to a diacetyl-monoxime colorimetric assay used for the determination of urea in transdermal iontophoretic extracts. Anal. Chim. Acta. 2007, **591** pp. 239–247

[14] WEHLING. P. *et al.* Probability of Detection (POD) as a Statistical Model for the Validation of Qualitative Methods. J. AOAC Int. 2011, **94** pp. 335–337

[15] WILRICH. C., WILRICH, P.T. Estimation of the POD function and the LOD of a qualitative microbiological measurement method. J. AOAC Int. 2009, **92** pp. 1763–1772

[16] MĂRGĂRITESCU. I., WILRICH, C. Determination of the Relative Level of Detection of a Qualitative Microbiological Measurement Method with Respect to a Reference Measurement Method. J. AOAC Int. 2013, **96** pp. 1086–1091

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