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खाद्य श्रृंखला की सूक्ष्मजैविकी —  
विधि मान्यकरण

भाग 3 एकल प्रयोगशाला में संदर्भ विधियों और मान्य  
वैकल्पिक तरीकों के सत्यापन के लिए प्रोटोकॉल

**Microbiology of the Food Chain —  
Method Validation**

**Part 3 Protocol for the Verification of Reference  
Methods and Validated Alternative Methods  
in a Single Laboratory**

ICS 07.100.30

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भारतीय मानक ब्यूरो  
BUREAU OF INDIAN STANDARDS  
मानक भवन, 9 बहादुरशाह ज़फर मार्ग, नई दिल्ली – 110002  
MANAK BHAVAN, 9 BAHADUR SHAH ZAFAR MARG  
NEW DELHI-110002  
[www.bis.gov.in](http://www.bis.gov.in) [www.standardsbis.in](http://www.standardsbis.in)

## NATIONAL FOREWORD

This Indian standard (Part 3) which is identical with ISO 16140-3 : 2021 'Microbiology of the food chain — Method validation — Part 3: Protocol for the verification of reference methods and validated alternative methods in a single laboratory' issued by the International Organization for Standardization (ISO) was adopted by the Bureau of Indian Standards on recommendation of the Food Hygiene, Safety Management and Other Systems Sectional Committee and approval of the Food and Agriculture Division Council.

This Indian Standard is published in six parts which are identical adoptions of their corresponding ISO standards. The other parts in this series are:

- Part 1 Vocabulary
- Part 2 Protocol for the validation of alternative (proprietary) methods against a reference method
- 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

The text of ISO Standard has been approved as suitable for publication as an Indian Standard without deviations. Certain terminologies and conventions are, however, not identical to those used in Indian Standards. Attention is particularly drawn to the following:

- a) Wherever the words 'International Standard' appear referring to this standard, they should be read as 'Indian Standard'.
- b) Comma (,) has been used as a decimal marker, while in Indian Standards, the current practice is to use a point (.) as the decimal marker.

In this adopted standard, reference appears to certain International Standards for which Indian Standards also exist. The corresponding Indian Standards, which are to be substituted in their respective places, are listed below along with their degree of equivalence for the editions indicated:

| <i>International Standard</i>   | <i>Corresponding Indian Standard</i>  | <i>Degree of Equivalence</i>     |
|---|---|----------------------------------|
| ISO 6887-1 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions | IS 10232 : 2020 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — General rules for the preparation of initial suspension and decimal dilutions ( <i>second revision</i> ) | Identical with ISO 6887-1 : 2017 |
| ISO 6887-2 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 2: Specific rules for the preparation of meat and meat products                      | IS 15990 : 2012 Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of meat and meat products                             | Identical with ISO 6887-2 : 2003 |
| ISO 6887-3 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products                   | IS 17448 : 2020 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of fish and fishery products  | Identical with ISO 6887-3 : 2017 |

(Continued on third cover)

# Contents

Page

|   |            |
|---|------------|
| <b>Introduction</b> .....   | <b>iii</b> |
| <b>1 Scope</b> .....  | <b>1</b>   |
| <b>2 Normative references</b> .....   | <b>1</b>   |
| <b>3 Terms and definitions</b> .....  | <b>1</b>   |
| <b>4 General principles of verification of qualitative (detection) methods and quantification methods</b> ..... | <b>5</b>   |
| 4.1 General.....  | 5          |
| 4.2 Implementation verification.....  | 5          |
| 4.3 (Food) item verification.....   | 6          |
| 4.4 Requirements for implementation verification and (food) item verification.....                              | 6          |
| 4.5 Performance characteristics.....  | 9          |
| <b>5 Qualitative methods — Technical protocol for verification</b> .....  | <b>9</b>   |
| 5.1 Estimated LOD <sub>50</sub> (eLOD <sub>50</sub> ) determination.....  | 9          |
| 5.2 Experimental design.....  | 9          |
| 5.3 Selection of (food) items.....  | 10         |
| 5.4 Artificial contamination.....   | 10         |
| 5.4.1 Selection of strains.....   | 10         |
| 5.4.2 Inoculation of the test portions.....   | 11         |
| 5.5 Evaluation of results.....  | 13         |
| 5.5.1 Determination of eLOD <sub>50</sub> using protocol 1.....   | 13         |
| 5.5.2 Determination of eLOD <sub>50</sub> using protocol 2.....   | 16         |
| 5.5.3 Use of protocol 3.....  | 17         |
| 5.6 Acceptability limits.....   | 18         |
| 5.7 Root cause analysis.....  | 18         |
| <b>6 Quantitative methods — Technical protocol for verification</b> .....                                       | <b>19</b>  |
| 6.1 Intralaboratory reproducibility standard deviation determination.....                                       | 19         |
| 6.1.1 General.....  | 19         |
| 6.1.2 Experimental design.....  | 19         |
| 6.1.3 Selection of the (food) item.....   | 21         |
| 6.1.4 Natural contamination.....  | 21         |
| 6.1.5 Artificial contamination.....   | 21         |
| 6.1.6 Evaluation of results.....  | 22         |
| 6.1.7 Acceptability limit.....  | 23         |
| 6.1.8 Root cause analysis.....  | 24         |
| 6.2 Estimated bias (eBias) determination.....   | 25         |
| 6.2.1 General.....  | 25         |
| 6.2.2 Experimental design.....  | 25         |
| 6.2.3 Selection of (food) items.....  | 25         |
| 6.2.4 Artificial contamination.....   | 25         |
| 6.2.5 Evaluation of results.....  | 27         |
| 6.2.6 Acceptability limit.....  | 27         |
| 6.2.7 Root cause analysis.....  | 28         |
| <b>7 Validated alternative confirmation and typing methods — Technical protocol for verification</b> .....      | <b>28</b>  |
| 7.1 General.....  | 28         |
| 7.2 Implementation verification.....  | 28         |
| 7.3 Experimental design.....  | 28         |
| 7.3.1 General.....  | 28         |
| 7.3.2 Strain selection.....   | 29         |
| 7.4 Evaluation of results.....  | 29         |
| 7.5 Acceptability limit.....  | 30         |

|                |   |           |
|----------------|---|-----------|
| 7.6            | Root cause analysis .....   | 30        |
| <b>8</b>       | <b>Summary of acceptability limits for the verification of validated methods .....</b>                                    | <b>30</b> |
| <b>Annex A</b> | <b>(informative) Classification of (food) categories and suggested target combinations for verification studies .....</b> | <b>31</b> |
| <b>Annex B</b> | <b>(informative) Guidance on how to choose challenging (food) item(s) for (food) item verification .....</b>              | <b>45</b> |
| <b>Annex C</b> | <b>(informative) Qualitative method verification — Example .....</b>  | <b>47</b> |
| <b>Annex D</b> | <b>(informative) Quantitative method verification — Example .....</b>   | <b>55</b> |
| <b>Annex E</b> | <b>(informative) Validated alternative confirmation or typing method verification — Examples .....</b>                    | <b>60</b> |
| <b>Annex F</b> | <b>(normative) Protocol for the verification of non-validated reference methods in a single laboratory .....</b>          | <b>63</b> |
|                | <b>Bibliography .....</b>   | <b>70</b> |

## Introduction

### 0.1 The ISO 16140 series

The ISO 16140 series has been expanded in response to the need for various ways to validate or verify test methods. It is the successor to ISO 16140:2003. The ISO 16140 series consists of six parts with the general title, *Microbiology of the food chain — Method validation*:

- *Part 1: Vocabulary;*
- *Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method;*
- *Part 3: Protocol for the verification of reference methods and validated alternative methods in a single laboratory;*
- *Part 4: Protocol for method validation in a single laboratory;*
- *Part 5: Protocol for factorial interlaboratory validation for non-proprietary methods;*
- *Part 6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures.*

ISO 17468 is a closely linked International Standard, which establishes technical rules for the development and validation of standardized methods.

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

- The first stage is the validation of the method. Validation is conducted using a study in a single laboratory followed by an interlaboratory study (see ISO 16140-2, ISO 16140-5 and ISO 16140-6). In the case when a method is validated within one laboratory (see ISO 16140-4), no interlaboratory study is conducted.
- The second stage is method verification, where a laboratory demonstrates that it can satisfactorily perform a validated method. This is described in this document (i.e. ISO 16140-3). Verification is only applicable to methods that have been validated using an interlaboratory study.

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

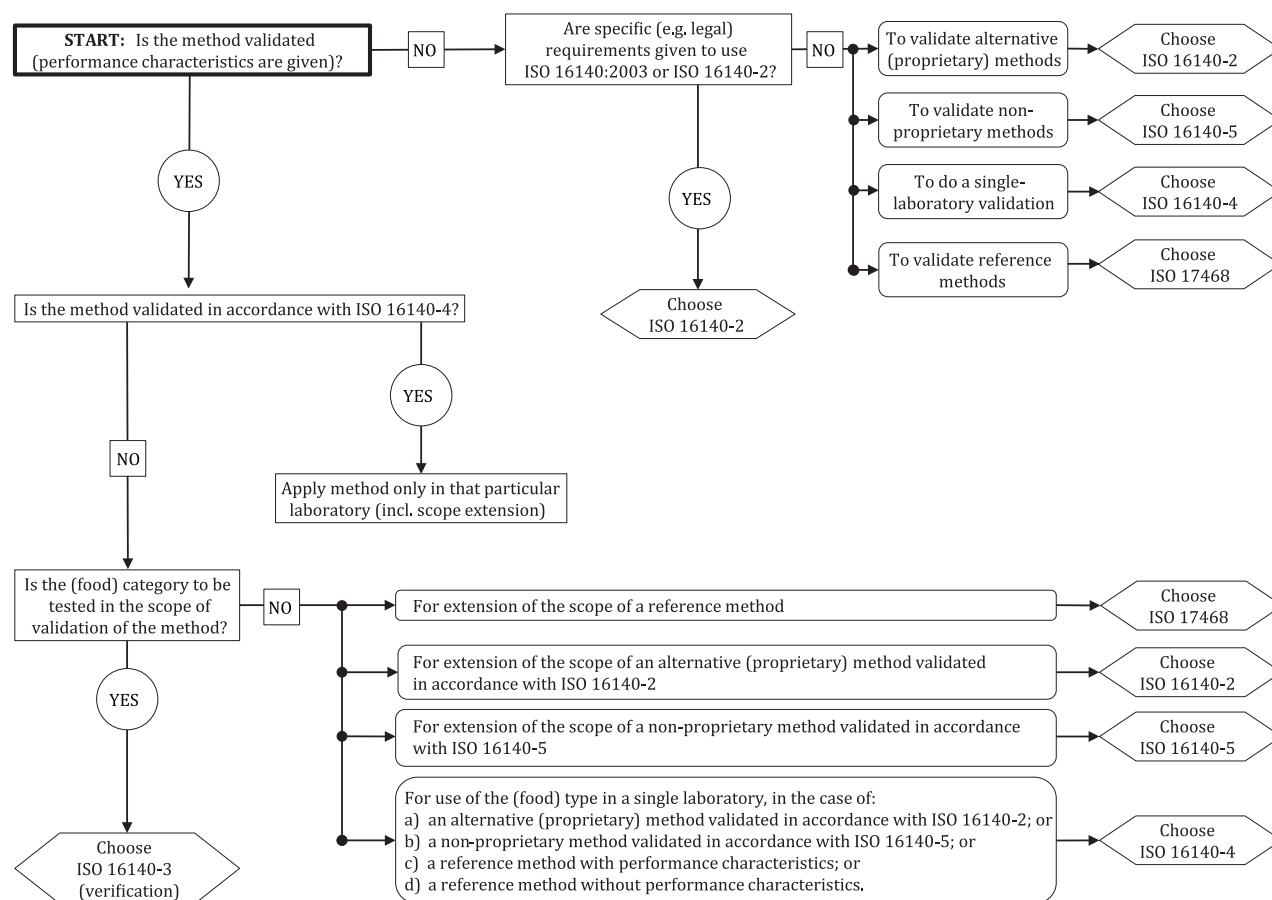
A reference method is defined in ISO 16140-1:2016, 2.59, as an “internationally recognized and widely accepted method”. The note to entry clarifies that “these are ISO standards and standards jointly published by ISO and CEN or other regional/national standards of equivalent standing”.

In the ISO 16140 series, reference methods include standardized reference (ISO and CEN) methods as defined in ISO 17468:2016, 3.5, as a “reference method described in a standard”.

An alternative method (method submitted for validation) is defined in ISO 16140-1:2016, 2.4, as a “method of analysis that detects or quantifies, for a given category of products, the same analyte as is detected or quantified using the corresponding reference method”. The note to entry clarifies that: “The method can be proprietary. The term ‘alternative’ is used to refer to the entire ‘test procedure and reaction system’. This term includes all ingredients, whether material or otherwise, required for implementing the method”.

ISO 16140-4 addresses validation within a single laboratory. The results are therefore only valid for the laboratory that conducted the study. In this case, verification (as described in this document) is not applicable. ISO 16140-5 describes protocols for non-proprietary methods where a more rapid validation is required or when the method to be validated is highly specialized and the number of participating laboratories required by ISO 16140-2 cannot be reached. ISO 16140-4 and ISO 16140-5 can be used for validation against a reference method. ISO 16140-4 (qualitative and quantitative) and ISO 16140-5 (quantitative only) can also be used for validation without a reference method.

The flow chart in [Figure 1](#) gives an overview of the links between the different parts mentioned above. It also guides the user in selecting the right part of the ISO 16140 series, taking into account the purpose of the study and the remarks given above.



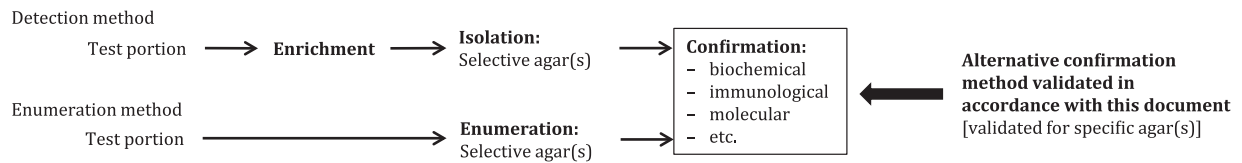
**Figure 1 — Flow chart for application of the ISO 16140 series**

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

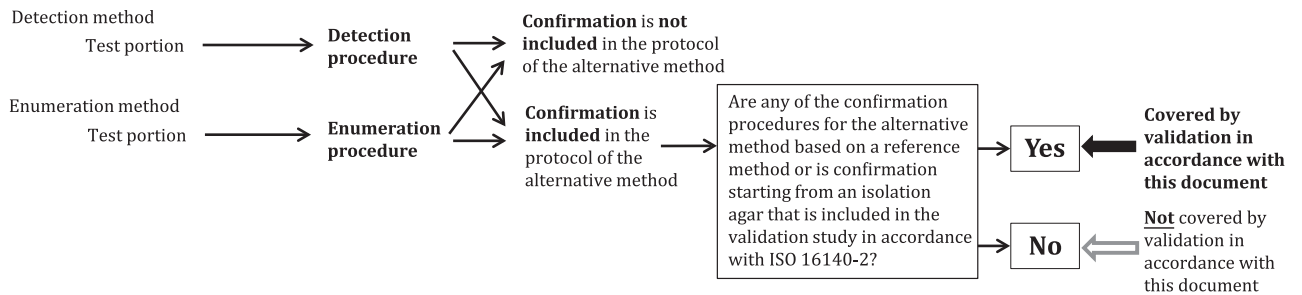
NOTE 2 The general principle for method verification is that the method to be verified (either alternative or reference) has been validated. However, some reference methods (including ISO or CEN standards) are not yet (fully) validated. For verification of these methods, the protocols are described in [Annex F](#).

ISO 16140-6 is somewhat different from the other parts in the ISO 16140 series in that it relates to a very specific situation where only the confirmation procedure of a method is to be validated [e.g. the biochemical confirmation of *Enterobacteriaceae* (see ISO 21528-2)]. The confirmation procedure advances a suspected (presumptive) result to a confirmed positive result. The validation of alternative typing techniques (e.g. serotyping of *Salmonella*) is also covered by ISO 16140-6. The validation study in ISO 16140-6 clearly defines the selective agar(s) from which strains can be confirmed using the alternative confirmation method. If successfully validated, the alternative confirmation method can only be used if strains are recovered on an agar that was used and shown to be acceptable within the validation study. [Figure 2](#) shows the possibilities where an alternative confirmation method validated in accordance with ISO 16140-6 can be applied (see text in the boxes).

### Reference method



### Alternative method validated in accordance with ISO 16140-2



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

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

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

- by laboratories using the ISO 6579-1; or
- by laboratories using an ISO 16140-2 validated alternative method that refers to ISO 6579-1 for confirmation; or
- by laboratories using an ISO 16140-2 validated alternative method that starts the confirmation from XLD and/or BGA agar and/or the specified chromogenic agar.

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

- by laboratories using an ISO 16140-2 validated alternative method that refers only to agars other than those included in the validation to start the confirmation (e.g. Hektoen agar and SS agar only); or
- by laboratories using an ISO 16140-2 validated alternative method that refers only to a confirmation procedure that does not require isolation on agar.

## 0.2 Verification versus validation

ISO 16140-1:2016 defines the terms for validation and verification, as follows:

- **validation:** establishment of the performance characteristics of a method and provision of objective evidence that the performance requirements for a specified intended use are fulfilled;
- **verification:** demonstration that a validated method performs, in the user's hands, according to the method's specifications determined in the validation study and is fit for its intended purpose.

NOTE 1 The user's hand means the user laboratory.

Method verification applies to methods that are:

- reference methods, including ISO or CEN standards, that are validated using at least an interlaboratory study;



NOTE 2 However, some reference methods (including ISO or CEN standards) are not yet (fully) validated. For verification of these methods, the protocols are described in [Annex F](#).

- alternative methods, proprietary or otherwise, when the validation included an interlaboratory study. The method has been validated in accordance with
  - ISO 16140-2 for alternative (proprietary) methods,
  - ISO 16140-5 for non-proprietary methods, or
  - ISO 16140-6 for alternative (proprietary) confirmation and typing methods.

In a validation study, it is not possible to test all existing foods; the diversity and number of samples used in any validation study is limited. In most cases, the validation is based on five different food categories (categories as defined in ISO 16140-1:2016, 2.11, and specified in ISO 16140-2:2016, Annex A). Sometimes the validation is supplemented with additional (other) categories such as pet food and animal feed, environmental samples (food or feed production), and/or primary production samples.

When a minimum of five different food categories are validated, the method is regarded as being validated for a “broad range of foods”. And even though only five food categories are tested during the validation study, the method is expected to work for any type of food samples within the 15 food categories in ISO 16140-2: 2016, Annex A. In other words, the “scope” of validation of the method is a broad range of foods, corresponding to the 15 food categories included in ISO 16140-2:2016, Annex A. The scope of validation is important for selecting categories, types and items for the verification.

Two kinds of verification are distinguished:

- The first one is named **implementation verification**. Its purpose is to demonstrate that the user laboratory is able to perform the method correctly. The user laboratory tests a (food) item that was used in the validation study (for qualitative methods) and any (food) item within the scope of validation (for quantitative methods) and then compares the result obtained from the verification to the result obtained from the validation.
- The second one is named **(food) item verification**. Its purpose is to demonstrate that the user laboratory is capable of testing the (food) items it claims in the scope of laboratory application. The user laboratory tests (food) items included in the scope of validation that are commonly examined by the user. As not all (food) items can be included in the verification, the user laboratory is asked to test challenging (food) items.

The scope specifies the (group of) products – categories or types or items – for which the method can be applied. Different scopes are distinguished:

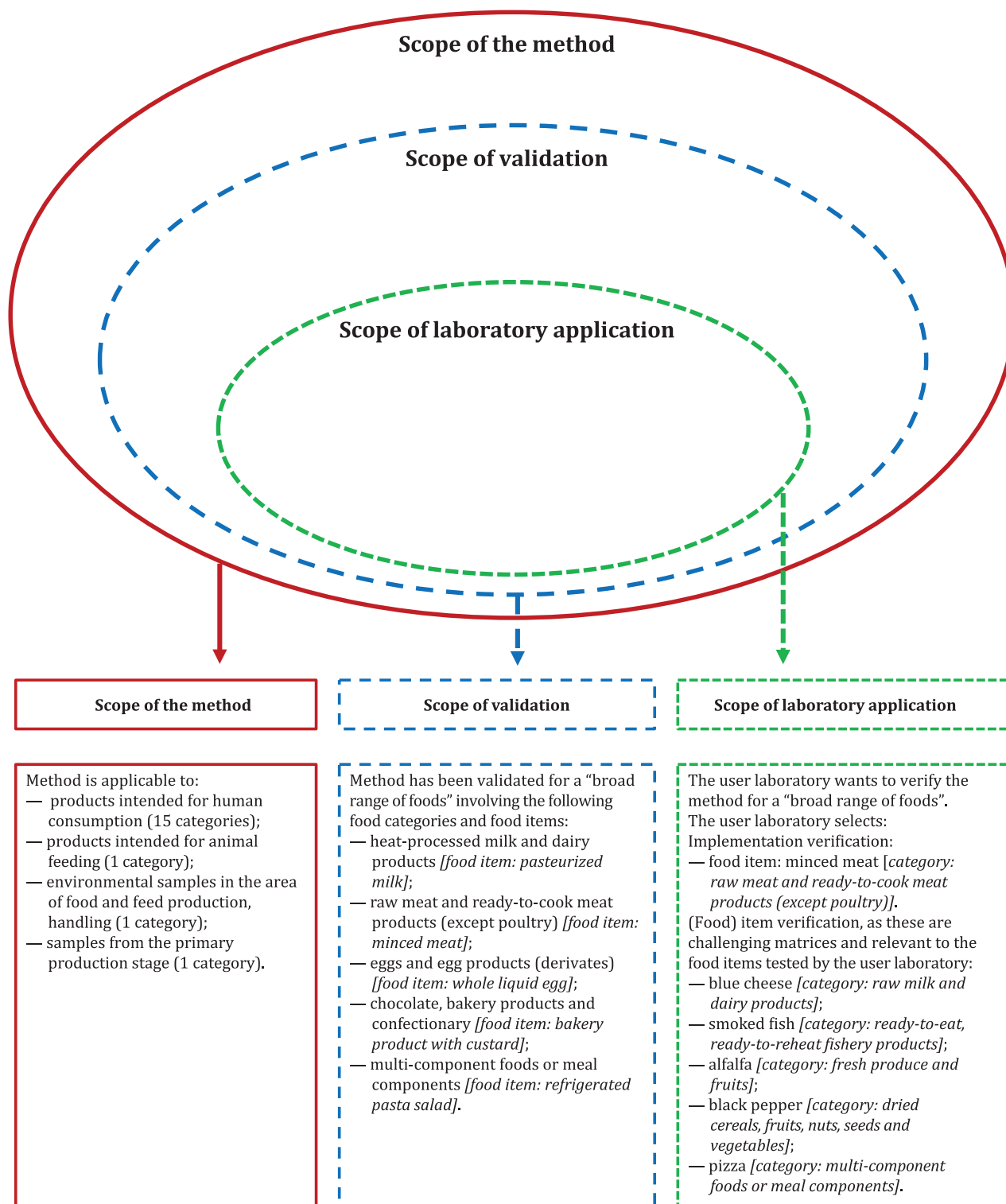
- **scope of the method:** (group of) products – categories or types or items – for which the method is claimed to be applicable.
- **scope of validation:** (group of) products – categories or types or items – for which the applicability of the method is claimed to be validated.

NOTE The claim for the scope of validation is in most cases wider than the products that are included in the validation study itself. For example, in the case of alternative (proprietary) methods validated in accordance with ISO 16140-2:2016: if at least five ( $\geq 5$ ) food categories – by using a minimum of three different food types per category – were tested in the validation study, then the scope of validation is a “broad range of foods” (so all 15 food categories are claimed in the scope of validation). When less than five ( $< 5$ ) food categories were tested, the scope of validation is limited to only those food categories included in the validation.

- **scope of laboratory application:** (group of) products – categories or types or items – for which the method is claimed to be used by the laboratory and are within the scope of validation.

The overlap between the different scopes (including an example) is illustrated in [Figure 3](#).





**Figure 3 — Overlap between the different scopes (including an example)**

At the time of publication of this document (i.e. ISO 16140-3:2021), some reference methods are not yet (fully) validated and would therefore fall outside the scope of this document. It is recognized that standardization organizations (including ISO and CEN committees) will need time to validate their reference methods. Therefore, these non-validated reference methods (including ISO or CEN standards) are verified in a user laboratory according to a specific protocol (see [Annex F](#)). This is seen as a temporary situation until these methods are validated by the ISO and/or CEN committees. For further information, see Reference [\[13\]](#).

In this document:

- “shall” indicates a requirement;
- “should” indicates a recommendation;
- “may” indicates a permission;
- “can” indicates a possibility or a capability.

Information marked “NOTE” is for guidance in understanding or clarifying the associated sentence.

## Indian Standard

# MICROBIOLOGY OF THE FOOD CHAIN — METHOD VALIDATION

## PART 3 PROTOCOL FOR THE VERIFICATION OF REFERENCE METHODS AND VALIDATED ALTERNATIVE METHODS IN A SINGLE LABORATORY

### 1 Scope

This document specifies the protocol for the verification of reference methods and validated alternative methods for implementation in the user laboratory.

This document is applicable to the verification of methods used for the analysis (detection and/or quantification), confirmation and typing of microorganisms in:

- products intended for human consumption;
- products intended for animal feeding;
- environmental samples in the area of food and feed production, handling;
- samples from the primary production stage.

This document is, in particular, applicable to bacteria and fungi. Some clauses can be applicable to other (micro)organisms or their metabolites, to be determined on a case-by-case basis.

The technical protocols for the verification of validated qualitative methods and validated quantitative methods are described in [Clauses 5](#) and [6](#). The technical protocol for the verification of validated alternative confirmation and typing methods is described in [Clause 7](#). The protocols for the verification of non-validated reference methods are described in [Annex F](#).

### 2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6887 (all parts), *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 16140-1:2016, *Microbiology of the food chain — Method validation — Part 1: Vocabulary*

### 3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

### 3.1 alternative confirmation or typing method

confirmation or typing method submitted for validation  
method of analysis that confirms or types the same analyte as is confirmed or typed using the corresponding reference method

Note 1 to entry: The method can be proprietary. The term “alternative” is used to refer to the entire “test procedure and reaction system”. This term includes all ingredients, whether material or otherwise, required for implementing the method.

[SOURCE: ISO 16140-6:2019, 3.2, modified — Note 2 to entry has been deleted.]

### 3.2 bias

measurement bias  
estimate of a systematic measurement error, or the systematic difference between the quantitative assigned value and the average of measurement replicate results

[SOURCE: ISO 16140-1:2016, 2.9]

### 3.3 (food) category

group of (*food*) types (3.18) of the same origin

EXAMPLE Food category: heat-processed milk and dairy products. Food type: pasteurized dairy products. Food item: crème brûlée.

Note 1 to entry: The (food) categories are listed in [Annex A](#).

[SOURCE: ISO 16140-1:2016, 2.11, modified — In the term, “(food)” has been added before “category”. In the definition, “(food)” has replaced “sample”. The example has been modified to align with the terms used in [Annex A](#). Note 1 to entry has been added.]

### 3.4 estimated bias

**eBias**  
determination of the *bias* (3.2) based on the experimental design described in this document (i.e. ISO 16140-3)

Note 1 to entry: An accurate determination of the bias is not possible as the number of samples tested is small. Therefore, the term “estimated bias” (“eBias”) is used in this document.

### 3.5 estimated LOD<sub>50</sub>

**eLOD<sub>50</sub>**  
determination of the LOD<sub>50</sub> (level of detection at 50 % probability of detection) based on the experimental design described in this document

Note 1 to entry: An accurate determination of the LOD<sub>50</sub> is not possible as the number of samples tested is small in comparison to the number of samples required in ISO 16140-2:2016. Therefore, the term “estimated LOD<sub>50</sub>” (“eLOD<sub>50</sub>”) is used in this document.

Note 2 to entry: LOD<sub>50</sub> is defined in ISO 16140-1:2016, 2.35.

### 3.6 exclusivity study

study involving pure *non-target strains* (3.11), which can be potentially cross-reactive, but are not expected to be detected or enumerated by the alternative method

[SOURCE: ISO 16140-1:2016, 2.22]

### 3.7

#### **inclusivity study**

study involving pure *target strains* (3.15) to be detected or enumerated by the alternative method

[SOURCE: ISO 16140-1:2016, 2.31]

### 3.8

#### **(food) item**

single specified food, feed, environmental or primary production *matrix* (3.10)

EXAMPLE Food category: heat-processed milk and dairy products. Food type: pasteurized dairy products. Food item: crème brûlée.

[SOURCE: ISO 16140-1:2016, 2.34, modified — In the term, “(food)” has been added before “item”. The example has been modified to align with the terms used in [Annex A](#).]

### 3.9

#### **laboratory sample**

sample prepared for sending to the laboratory and intended for inspection or testing

[SOURCE: ISO 6887-1:2017, 3.1]

### 3.10

#### **matrix**

all the components of the sample

[SOURCE: ISO 16140-1:2016, 2.38, modified — In the term, “(product)” has been deleted.]

### 3.11

#### **non-target strain**

strain, defined according to the scope of the reference method that would not reasonably be expected to be confirmed, detected or enumerated by the alternative method

[SOURCE: ISO 16140-1:2016, 2.44, modified — In the definition, “confirmed” has been added to “detected or enumerated”.]

### 3.12

#### **reference material**

material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process

Note 1 to entry: Properties can be quantitative or qualitative, e.g. identity of substances or species.

Note 2 to entry: Uses may include the calibration of a measurement system, assessment of a measurement procedure, assigning values to other materials, and quality control.

[SOURCE: ISO Guide 30:2015, 2.1.1, modified — The original Notes 1 and 4 to entry have been omitted and the notes have been renumbered.]

### 3.13

#### **scope of laboratory application**

categories, matrices, analytes and concentrations for an analytical method that a *user laboratory* (3.19) claims to be capable of satisfactorily testing in its laboratory

Note 1 to entry: A method may have been validated to a broader range (scope) of analytes, matrices and concentrations than the scope that will be claimed by a user laboratory. The scope of laboratory application is  $\leq$  the *scope of validation* (3.14).

### 3.14

#### **scope of validation**

categories, matrices, analytes and concentrations for which a validated method of analysis can be used satisfactorily

[SOURCE: ISO 16140-1:2016, 2.70, modified — “categories” has been added and “matrices” has been moved before “analytes”.]

### 3.15

#### **target strain**

strain, defined according to the scope of the reference method, that is expected to be confirmed, detected or enumerated by the alternative method

[SOURCE: ISO 16140-1:2016, 2.74, modified — In the definition, “confirmed” has been added to “detected or enumerated”.]

### 3.16

#### **test portion**

measured (volume or mass) representative sample taken from the *laboratory sample* (3.9) for use in the preparation of the initial suspension

Note 1 to entry: Sometimes preparation of a *test sample* (3.17) from the laboratory sample is required before the test portion is taken, but this is infrequently used in microbiological examinations.

[SOURCE: ISO 6887-1:2017, 3.5, modified — In the Note 1 to entry, “a test sample from” has been added before “the laboratory sample”.]

### 3.17

#### **test sample**

sample prepared from the *laboratory sample* (3.9) according to the procedure specified in the test method and from which *test portions* (3.16) are taken

Note 1 to entry: Preparation of the laboratory sample before the test portion is taken is infrequently used in microbiological examinations.

Note 2 to entry: For confirmation and typing methods, the sample is an isolated colony on defined selective or non-selective agar plates.

[SOURCE: ISO 6887-1:2017, 3.4, modified — In the definition, “test method” has replaced “method of test” and Note 2 to entry has been added.]

### 3.18

#### **(food) type**

for a given *(food) category* (3.3), a group of *(food) items* (3.8) processed in a similar way, with similar intrinsic characteristics and a similar microbial ecology

EXAMPLE Food category: heat-processed milk and dairy products. Food type: pasteurized dairy product.

[SOURCE: ISO 16140-1:2016, 2.78, modified — In the term and the definition, “(food)” has been added before “type”, “category” and “items”.]

### 3.19

#### **user laboratory**

laboratory that implements a validated alternative method and/or a validated reference method

Note 1 to entry: Some reference methods (including ISO or CEN standards) are not yet (fully) validated. For verification of these methods, the protocols are described in [Annex F](#).

### 3.20 validation

establishment of the performance characteristics of a method and provision of objective evidence that the performance requirements for a specified intended use are fulfilled

[SOURCE: ISO 16140-1:2016, 2.81]

### 3.21 verification

demonstration that a validated method performs, in the user's hands, according to the method's specifications determined in the *validation* (3.20) study and is fit for its intended purpose

Note 1 to entry: Some reference methods (including ISO or CEN standards) are not yet (fully) validated. For verification of these methods, the protocols are described in [Annex F](#).

[SOURCE: ISO 16140-1: 2016, 2.83, modified — In the definition, “performs” has replaced “functions” and “intended” has been added before “purpose”. Note 1 to entry has been replaced.]

## 4 General principles of verification of qualitative (detection) methods and quantification methods

### 4.1 General

The verification of qualitative (detection) methods and quantitative methods is undertaken in two parts:

- implementation verification;
- (food) item verification.

The verification focuses on (food) items that are within the scope of validation and within the scope of laboratory application.

Before performing method verification, the user laboratory shall refer to the validation report(s) published by recognized standards bodies and/or method certification bodies as the source(s) for the scope of validation and to select appropriate (food) items for verification.

Implementation verification occurs before (food) item verification. The technical rules for performing implementation verification and (food) item verification are given in [Clause 5](#) for qualitative methods and [Clause 6](#) for quantitative methods.

For the verification of non-validated reference methods, the user laboratory shall use the technical protocols as described in [Annex F](#).

### 4.2 Implementation verification

Implementation verification aims to demonstrate the competence of the user laboratory to perform the validated method. This is achieved by its ability to obtain the expected results on a (food) item.

The user laboratory shall:

- review the validation data for the method;
- for qualitative methods:
  - select one (food) item tested during the validation study that belongs within the scope of laboratory application of the user laboratory;
  - when the (food) items included in the validation study do not belong within the scope of laboratory application of the user laboratory, the user laboratory shall obtain one of the (food) items; this is necessary because the limit of detection of the method is affected by the (food) item;



- use this selected (food) item and the sample size that was used in the validation study to perform the implementation verification;
- for quantitative methods: select any (food) item that belongs within the scope of validation of the method (but not necessarily tested during the validation).

### 4.3 (Food) item verification

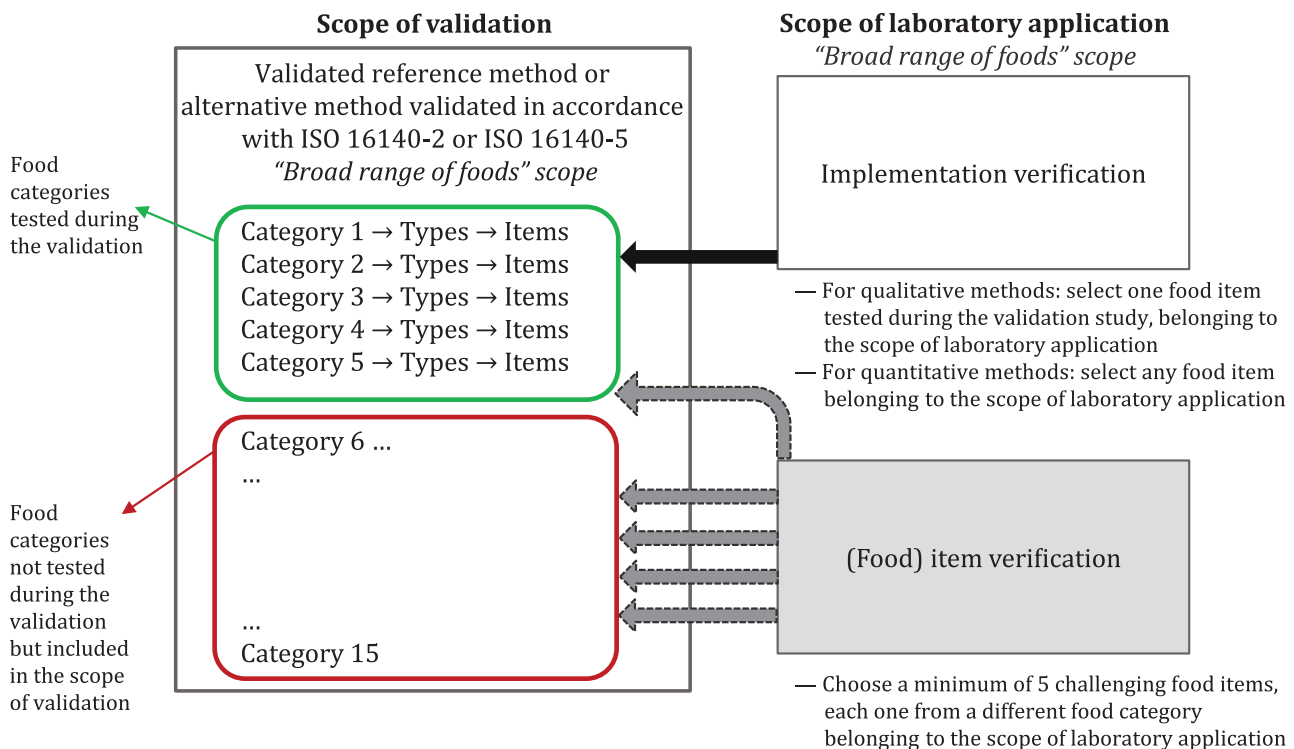
The (food) item verification aims to demonstrate the competence of the user laboratory to perform the validated method with (food) items that are tested in the user laboratory.

The user laboratory shall:

- select one challenging (food) item from each (food) category listed within the scope of validation (see 4.4 for details) that is also a (food) category tested within the scope of laboratory application of the user laboratory;
- use this (food) item and the sample size (or a smaller sample size if routinely used in the user laboratory) used in the validation study to perform the (food) item verification.

### 4.4 Requirements for implementation verification and (food) item verification

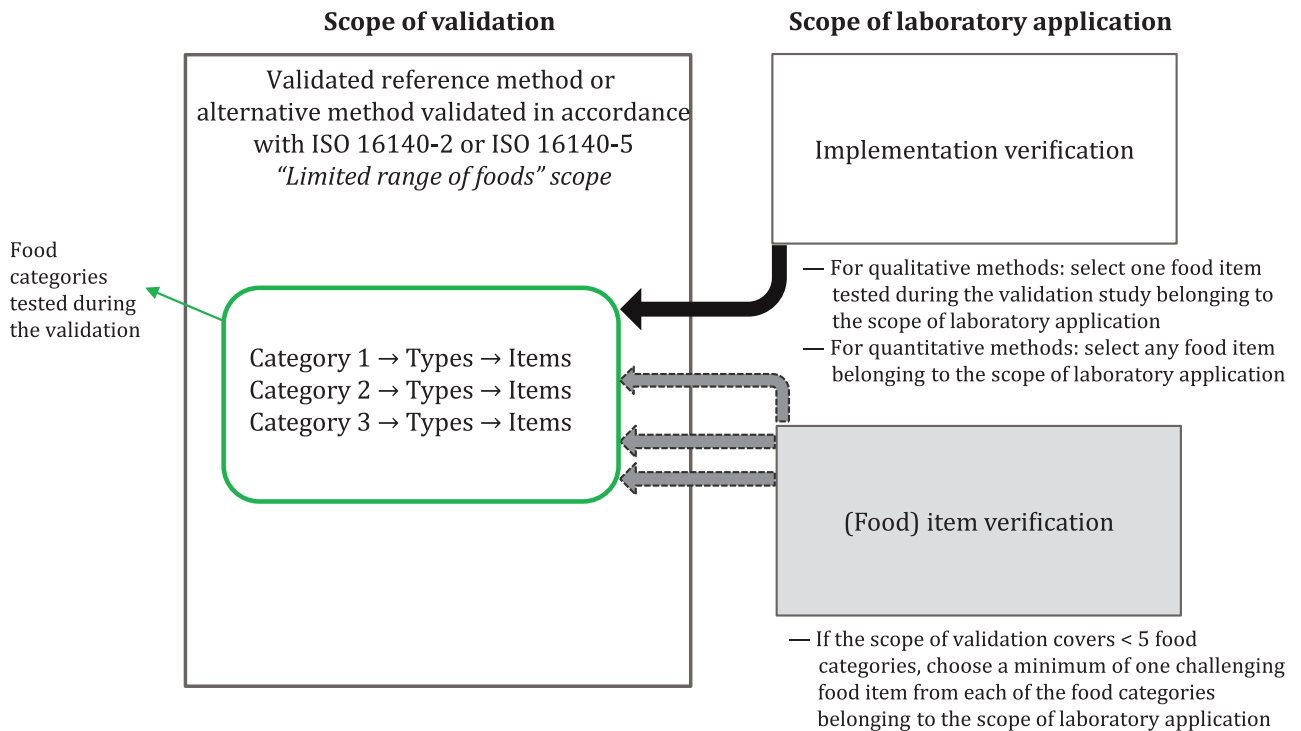
Figures 4, 5 and 6 show the number of (food) items required for implementation verification and (food) item verification under different circumstances. Figures 4 and 5 only refer to food categories. Figure 6 includes other categories.



**Figure 4 — Food items required when verifying a method for a "broad range of foods" scope**

In Figure 4, the selection of the categories for (food) item verification is given only as an example (arrows with dotted outlines). In contrast to implementation verification, there is no obligation to select one food item from a category tested during the validation (in the case of qualitative methods) and four food items from four food categories not tested during the validation. The user laboratory can make its own selection from the 15 food categories.

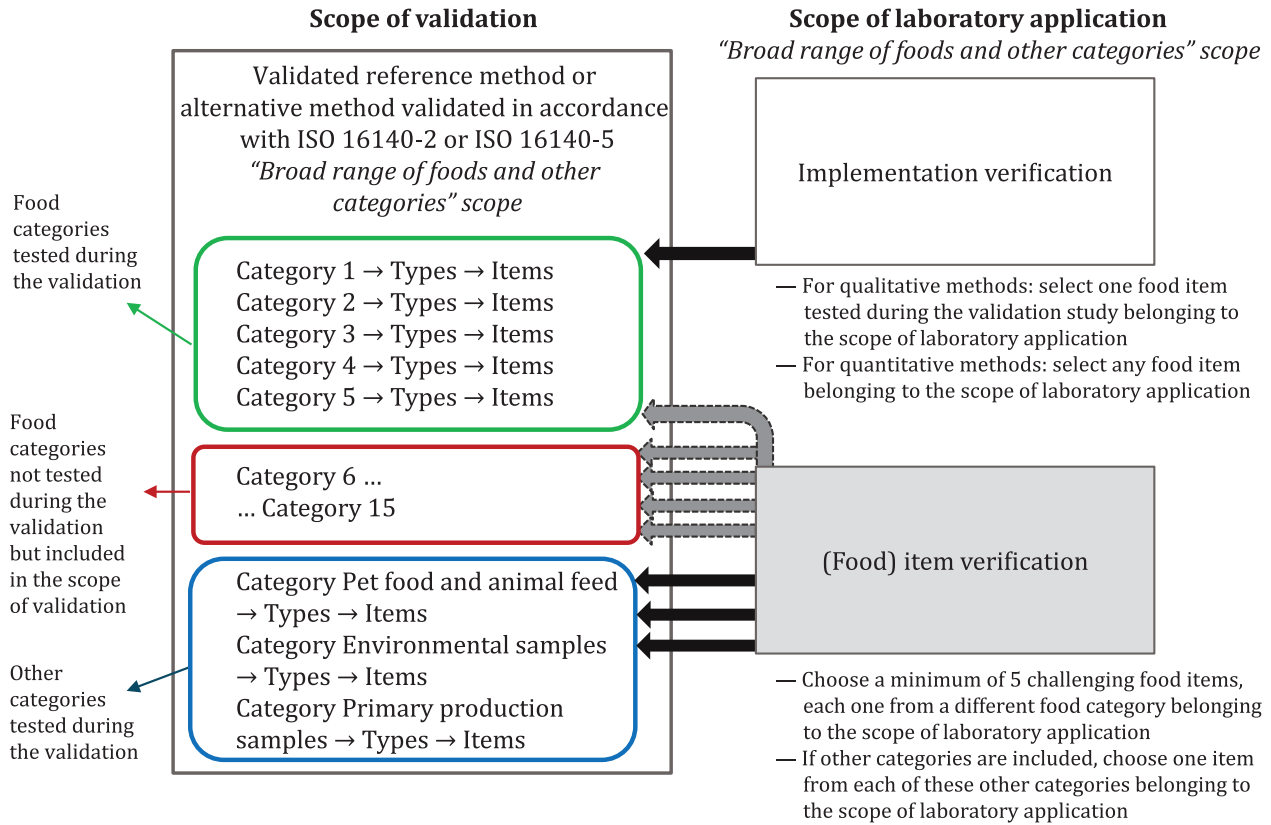
The scope of laboratory application shown in [Figure 4](#) is for a “broad range of foods”, meaning that the user laboratory has included five or more food categories in its verification study and can therefore claim application for a “broad range of foods”. If the scope of laboratory application is smaller than the scope of validation, the user laboratory shall only test food items from its restricted food categories. For example, if the scope of laboratory application is limited to three food categories, then the user laboratory shall verify a minimum of one food item from each of the three food categories.



**Figure 5 — Food items required when verifying a method for a “limited range of foods” scope**

In [Figure 5](#), the selection of the categories for (food) item verification is given only as an example. For the “limited range of foods” scope, a limited number of food categories is tested during the validation. It means the scope of validation is restricted to the tested categories. Consequently, the user laboratory shall not verify the method with categories outside of the limited scope. If the scope of laboratory application is smaller than the scope of validation, the user laboratory shall only test food items from its restricted food categories (arrows with dotted outlines). When the scope of the validation is limited to one category, both implementation verification and (food) item verification shall still be performed, using a minimum of two items from the category: one item for implementation verification and another food item for the (food) item verification.

[Figure 6](#) shows the number of items required when food and other categories are validated and included in the scope of laboratory application. These categories include pet food and animal feed, environmental samples (food or feed production) and primary production samples (PPS). If any of these other categories was included in the validation study and if it is claimed to be within the scope of laboratory application of the user laboratory, then one item from each claimed category shall also be included in the (food) item verification.



**Figure 6 — Items required when verifying a method for a “broad range of foods and other categories” scope**

Table 1 summarizes the minimum number of (food) items required for the different scenarios.

**Table 1 — Summary of the minimum number of (food) items required for verification**

| Scope of validation   | Number of samples           |  |   |
|---|-----------------------------|--|---|
|   | Implementation verification | (Food) item verification   | Total   |
| “Broad range of foods” scope $\geq 5$ food categories   | 1                           | $\geq 5$   | $\geq 6$  |
| “Limited range of foods” scope $N_{\text{food}}$ categories   | 1                           | $N_{\text{food}} \leq 4$   | $(N_{\text{food}} + 1) \leq 5$                    |
| “Broad range of foods” + other categories ( $N_{\text{other}}$ ) scope                                | 1                           | $\geq 5$ food items<br>+<br>1 item from each of the $N_{\text{other}}$ other categories      | $\geq 6 + N_{\text{other}}$                       |
| “Limited range of foods” $N_{\text{food}}$ categories + other categories ( $N_{\text{other}}$ ) scope | 1                           | $N_{\text{food}} \leq 4$<br>+<br>1 item from each of the $N_{\text{other}}$ other categories | $(N_{\text{food}} + N_{\text{other}} + 1) \leq 8$ |
| Other categories ( $N_{\text{other}}$ ) scope only  | 1                           | $N_{\text{other}} \leq 3$  | $(N_{\text{other}} + 1) \leq 4$                   |

Table A.1 provides the list of (food) categories and corresponding (food) items. Annex B provides further guidance on the selection of a challenging (food) item from each (food) category for (food) item verification. The (food) items chosen from each (food) category shall be items that reflect the range

of the laboratory samples received by the user laboratory, and should, as much as possible, be items with components such as natural antimicrobial properties, vitamins, flavours and probiotics that may interfere with the detection of the target microorganism.

#### 4.5 Performance characteristics

[Table 2](#) lists the required performance characteristics for method verification.

**Table 2 — Required performance characteristics to be determined for verification**

| Method       | Performance characteristic                                      | Implementation verification | (Food) item verification |
|--------------|---|-----------------------------|--------------------------|
| Qualitative  | Estimated LOD <sub>50</sub> (eLOD <sub>50</sub> )               | ✓                           | ✓                        |
| Quantitative | Intralaboratory reproducibility standard deviation ( $S_{IR}$ ) | ✓                           | Not applicable           |
|              | Estimated bias (eBias)  | Not applicable              | ✓                        |

NOTE 1 The relationship between intralaboratory reproducibility standard deviation ( $S_{IR}$ ) and ISO 19036 is explained in [6.1](#).

NOTE 2 For the verification of qualitative method, three protocols are proposed to the user laboratory. The protocol 3 does not require a determination of an eLOD<sub>50</sub> but to target a concentration of 3 cfu to 5 cfu/test portion.

## 5 Qualitative methods — Technical protocol for verification

### 5.1 Estimated LOD<sub>50</sub> (eLOD<sub>50</sub>) determination

The eLOD<sub>50</sub> determination is required for both the implementation verification and the (food) item verification.

- The user laboratory first follows one of the selected technical protocols outlined below in its entirety to complete the implementation verification, demonstrating its ability to perform the validated method correctly.
- The user laboratory then applies this same technical protocol for (food) item verification.

During the method verification, run the full procedure of the method as described, including the confirmation procedure (if there is one). A minimum of one individual test portion at each inoculation level needs to be confirmed, and the number of colonies for confirmation may be reduced to one.

### 5.2 Experimental design

The user laboratory shall select one of the three protocols described in [Table 3](#).

**Table 3 — Protocols to determine eLOD<sub>50</sub> and number of replicates needed per inoculation level**

| Protocol | Inoculation level of the test portion                 |  |  |                                 |       | Total number of replicates |
|----------|---|--|--|---------------------------------|-------|----------------------------|
|          | High level<br>9 × LOD <sub>50</sub> /<br>test portion | Intermediate<br>level<br>3 × LOD <sub>50</sub> /<br>test portion | Low level<br>1 × LOD <sub>50</sub> /<br>test portion | 3 cfu to 5 cfu<br>/test portion | Blank |                            |
| 1        | 1   | 4  | 4  | –                               | 1     | 10                         |
| 2        | –   | 3  | 5  | –                               | 1     | 9                          |
| 3        | –   | –  | –  | 7                               | 1     | 8                          |

NOTE The abbreviation of colony forming units is cfu.

The choice of protocol depends on the ability of the laboratory to achieve the desired level of contamination of the test portion. Laboratory grown cultures or reference materials can be used for inoculation (see [5.4.1](#)).

- Protocol 1 can be used when there is uncertainty of achieving the desired level of contamination of the test portions. This is relevant when a culture is used, without prior knowledge of the actual level of the inoculum, to inoculate the test portions.
- Protocol 3 can be used when the level of contamination of the inoculum is known, e.g. when using a reference material with known concentration.
- Protocol 2 can be used if the first chosen protocol did not work as anticipated, and the experiment needs to be repeated.

Additional dilutions to that prescribed for any of the protocols can be used to minimize the need to repeat the experiment when inoculation levels do not comply with the requirements or the verification test results cannot be interpreted (see [Tables 6](#) and [8](#)). This is, however, not mandatory, but the decision of the laboratory conducting the experiment.

The protocols shall be performed as follows.

- Prepare cultures of the target microorganisms for inoculating the (food) items.
- At a minimum, prepare the number of test portions of the same (food) item that are required for the selected protocol (see [Table 3](#)). Choose a (food) item that should not be naturally contaminated by the target microorganism.
- Inoculate the initial suspensions of the test portions according to the selected protocol in [Table 3](#).
- Determine the level of the target microorganism in the inoculum, at the same time as the test portions are inoculated, by plating on a non-selective medium (e.g. plate count agar) or by performing an MPN (e.g. 3 dilutions × 3 tubes). Enumerate in accordance with ISO 7218.

NOTE If the level of the culture used for inoculation is not known, additional test portions can be inoculated with extra dilutions to ensure that the target levels are included in the verification.

- Analyse the inoculated test portions using the full procedure of the method being verified.
- For protocol 1 and protocol 2: determine the eLOD<sub>50</sub> using the positive and negative results obtained (see [5.5](#) for details). For protocol 3, no eLOD<sub>50</sub> is determined. Instead, the results are evaluated based on the number of positives found out of the seven replicates tested.

See also [Annex C](#) for additional guidance and examples.

### 5.3 Selection of (food) items

One (food) item is required for the implementation verification. Any (food) item that is included in the validation and within the scope of laboratory application can be selected.

For the (food) item verification, the user laboratory shall test a minimum of one (food) item, preferably a challenging one, from each of the required (food) categories. Details on the required number of (food) categories to test according to the scope of validation or to the scope of laboratory application are described in [4.4](#). [Annex B](#) provides guidance on how to choose challenging (food) items.

### 5.4 Artificial contamination

#### 5.4.1 Selection of strains

Strains can be from:

- culture collections;

- user laboratory collections;
- reference materials (including commercial reference materials, e.g. a freeze-dried strain with known concentration).

When choosing the test strains, the majority should originate from the (food) categories selected for the verification study and cover the recognized range of the target analyte with respect to the diversity in identification characteristics (e.g. biochemical, serotype, phage type), geographical distribution and incidence (see ISO 16140-2:2016, Annex E).

NOTE Preferably, the strains used in the verification are from sources relevant to the (food) item being verified and a different strain is used for each of the (food) items to be tested.

#### 5.4.2 Inoculation of the test portions

Use the LOD<sub>50</sub> data of the corresponding (food) categories from the validation study of the method to determine the level of contamination (this should be between one to nine times the LOD<sub>50</sub>, see [Table 3](#)) that will be used to inoculate the test portion. For protocol 3, use 3 cfu to 5 cfu/test portion.

If no corresponding (food) categories are available in the validation study [e.g. for a challenging (food) item tested in (food) item verification], the LOD<sub>50</sub> value is assumed to be equal to or lower than 1 cfu/test portion.

The following guidance is given as an example of procedures suitable for producing inocula.

- The selected strain is grown in a culture medium under conditions that enable the optimal growth of the strain (e.g. overnight culture). Follow the procedures specified in ISO 11133:2014, 5.4.

NOTE In this document, overnight culture is specified as 16 h to 24 h of incubation.

- Enumerate the culture on a non-selective medium to determine the concentration of the strain in cfu/ml. It is assumed that this level will be consistently achieved when the same culture conditions are used.
- Repeat the culture under the same conditions and take into account the previously determined concentration to prepare dilutions to cover the range for inoculation. This step is not required if the stability of the strain is known by the user laboratory (e.g. viability after storage at 4 °C overnight).

If the user laboratory works with ready-to-use target strains with known levels (e.g. reference material), the steps described above are not required.

The prepared inoculum is introduced directly into the initial suspension of the individual test portions. After inoculation, the suspension is mixed thoroughly. The use of stressed cultures is recommended but is not required (see ISO 16140-2:2016, Annex C).

[Table 4](#) provides a guide on how to achieve the inoculation levels for each protocol.

**Table 4 — Inoculation levels for each protocol**

| Protocol | High level<br>9 × LOD <sub>50</sub> /test<br>portion                       | Intermediate level<br>3 × LOD <sub>50</sub> /test<br>portion                               | Low level<br>1 × LOD <sub>50</sub> /test<br>portion                                     | 3 cfu to 5 cfu/test<br>portion   |
|----------|--|--|---|--|
| 1        | This should be at a maximum of nine times the expected LOD <sub>50</sub> . | From the high inoculation level, perform a 1:3 dilution to achieve the intermediate level. | From the intermediate inoculation level, perform 1:3 dilution to achieve the low level. | –  |
| 2        | –  | This should be at a maximum of three times the expected LOD <sub>50</sub> .                | From the intermediate inoculation level, perform 1:3 dilution to achieve the low level. | –  |
| 3        | –  | –  | –   | The level of contamination of the inoculum is known, (e.g. reference material with known concentration). |

More dilutions can be tested to ensure that the target levels are included. Use as many dilutions as needed but always take into account a 1:3 dilution factor between the levels.

To determine the inoculum level, enumerate, at the time the test portions are inoculated, the high-level inoculum when using protocol 1, the intermediate-level inoculum when using protocol 2 or the 3 cfu to 5 cfu/test portion inoculum when using protocol 3, in accordance with ISO 7218 (using a non-selective medium, e.g. plate count agar). Take into account the fact that the level of contamination of the inoculum is very low and thus more replicates and/or a larger volume of the inoculum shall be analysed to obtain a valid result in accordance with ISO 7218. The concentration of the low and intermediate levels using protocol 1 or 2 will be calculated using the counts obtained and the dilution factors used.

Alternatively, an MPN determination of the inocula can be performed using a 3 dilutions × 3 tubes MPN approach; the use of a non-selective medium for enrichment (e.g. Brain Heart Infusion broth or Tryptone Soy Broth) is suitable (see also [Annex C](#) for more information). In this case, the results are determined according to [Table C.1](#).

**EXAMPLE** A user laboratory wants to verify the *Salmonella* method (see ISO 6579-1) using protocol 1.

- Based on the LOD<sub>50</sub> (2,5 cfu/test portion) determined in the validation study, the range of contamination for (food) item A will, theoretically, be 22,5 cfu/test portion, 7,5 cfu/test portion and 2,5 cfu/test portion.
- An overnight culture is prepared. Based on the preliminary enumeration, this is assumed to contain 6 × 10<sup>8</sup> cfu/ml.
- As the actual count of the new overnight culture is unknown, the user laboratory can use several dilutions to cover the three target levels, using each dilution and test portions required in [Table 5](#). In this case:
  - Dilution A (60 cfu/ml): use 1 ml of 10<sup>-7</sup> dilution of the overnight culture;
  - Dilution B (20 cfu/ml): use 1 ml of 1:3 dilution of A;
  - Dilution C (6,7 cfu/ml): use 1 ml of 1:3 dilution of B;
  - Dilution D (2,2 cfu/ml): use 1 ml of 1:3 dilution of C;
  - Dilution E (0,7 cfu/ml): use 1 ml of 1:3 dilution of D;
  - Dilution F (0,2 cfu/ml): use 1 ml of 1:3 dilution of E.



In this particular example, six dilutions are used to make sure the right dilutions are included. In total, 21 inoculated test portions will be examined together with one blank test portion. Only the three relevant levels and the blank level (not inoculated) will be retained for the eLOD<sub>50</sub> determination.

**Table 5 — Example of dilutions and corresponding number of replicates for protocol 1, 2, and 3 using more than the minimum number of required dilutions**

| Protocol | Dilution A<br>(10 <sup>-7</sup> ) | Dilution B<br>(1:3 of A) | Dilution C<br>(1:3 of B) | Dilution D<br>(1:3 of C) | Dilution E<br>(1:3 of D) | Dilution F<br>(1:3 of E) |
|----------|-----------------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| 1        | 1                                 | 4                        | 4                        | 4                        | 4                        | 4                        |
| 2        | –                                 | 3                        | 5                        | 5                        | 5                        | 5                        |
| 3        | –                                 | –                        | 7                        | 7                        | 7                        | 7                        |

- In this example, the count making the final average contamination level for this dilution is 54 cfu/ml.
- Using this selected dilution (A) for the preparation of the dilutions according to [Table 5](#) will result in dilution B containing 18 cfu/ml; dilution C (1:3 dilution of dilution B) containing 6 cfu/ml and dilution D (1:3 dilution C) containing 2 cfu/ml. Dilutions B, C and D are considered to be the three relevant levels as a 1 ml inoculum of dilution D is closest to the LOD<sub>50</sub> (2,5 cfu/test portion) of the method. See also [Annex C](#) for additional guidance and examples.

## 5.5 Evaluation of results

### 5.5.1 Determination of eLOD<sub>50</sub> using protocol 1

Record the number of positive results obtained at each inoculum level and use [Table 6](#) to determine the eLOD<sub>50</sub>. The blank level shall not produce a positive result. If a positive result is obtained for the blank level, the experiment shall be repeated for all levels.

For the evaluation of the results using protocol 1, the high-level inoculum (9 × LOD<sub>50</sub>) shall produce only positive results. If negative results are obtained, the experiment shall be repeated for all levels. Some of the MPN combinations indicated as “unreliable MPN result” (see [Tables 6](#) and [7](#)) are very unlikely to occur and the experiment shall therefore be repeated.

When more dilutions are used, the three dilutions, with the low inoculation level closest to the LOD<sub>50</sub> level, shall be used to evaluate the data according to [Table 6](#).

**Table 6 — Determination of eLOD<sub>50</sub> based on the number of positive results per level of contamination using protocol 1**

| <b>High inoculation level</b><br>targeted 9 × LOD <sub>50</sub> /<br>test portion | <b>Intermediate inoculation level</b><br>targeted 3 × LOD <sub>50</sub> /<br>test portion | <b>Low inoculation level</b><br>targeted 1 × LOD <sub>50</sub> /<br>test portion | <b>Blank level</b> | <b>eLOD<sub>50</sub></b><br><br>cfu/test portion |
|---|---|--|--------------------|--|
| 1/1   | 4/4   | 4/4  | 0/1                | < 1,0 × LIL <sup>a</sup>                         |
| 1/1   | 4/4   | 3/4  | 0/1                | = 0,5 × LIL                                      |
| 1/1   | 4/4   | 2/4  | 0/1                | = 0,7 × LIL                                      |
| 1/1   | 4/4   | 1/4  | 0/1                | = 1,0 × LIL                                      |
| 1/1   | 4/4   | 0/4  | 0/1                | = 1,5 × LIL                                      |
| 1/1   | 3/4   | 4/4  | 0/1                | = 0,7 × LIL                                      |
| 1/1   | 3/4   | 3/4  | 0/1                | = 1,0 × LIL                                      |
| 1/1   | 3/4   | 2/4  | 0/1                | = 1,3 × LIL                                      |
| 1/1   | 3/4   | 1/4  | 0/1                | = 1,7 × LIL                                      |
| 1/1   | 3/4   | 0/4  | 0/1                | = 2,3 × LIL                                      |
| 1/1   | 2/4   | 4/4  | 0/1                | = 1,1 × LIL                                      |
| 1/1   | 2/4   | 3/4  | 0/1                | = 1,5 × LIL                                      |
| 1/1   | 2/4   | 2/4  | 0/1                | = 1,9 × LIL                                      |
| 1/1   | 2/4   | 1/4  | 0/1                | = 2,6 × LIL                                      |
| 1/1   | 2/4   | 0/4  | 0/1                | = 3,7 × LIL                                      |
| 1/1   | 1/4   | 4/4  | 0/1                | Unreliable MPN result <sup>b</sup>               |
| 1/1   | 1/4   | 3/4  | 0/1                | = 2,1 × LIL                                      |
| 1/1   | 1/4   | 2/4  | 0/1                | = 2,8 × LIL                                      |
| 1/1   | 1/4   | 1/4  | 0/1                | = 4,0 × LIL                                      |
| 1/1   | 1/4   | 0/4  | 0/1                | = 6,3 × LIL                                      |
| 1/1   | 0/4   | 4/4  | 0/1                | Unreliable MPN result <sup>b</sup>               |
| 1/1   | 0/4   | 3/4  | 0/1                | = 3,0 × LIL                                      |
| 1/1   | 0/4   | 2/4  | 0/1                | = 4,3 × LIL                                      |
| 1/1   | 0/4   | 1/4  | 0/1                | = 6,7 × LIL                                      |
| 1/1   | 0/4   | 0/4  | 0/1                | = 14,0 × LIL                                     |

<sup>a</sup> LIL = low inoculation level.

<sup>b</sup> Unreliable MPN result: MPN combination is very unlikely to occur. The experiment shall be repeated.

Using the actual inoculum level given in the example described in 5.4.2, Table 6 can be used to determine the eLOD<sub>50</sub>. This is presented in Table 7. In this example, a high-level contamination of 18 cfu/test portion is used with the corresponding intermediate level contamination of 6 cfu/test portion and low-level contamination of 2 cfu/test portion.

**Table 7 — Example for the determination of the eLOD<sub>50</sub> based on the number of positive results per level of contamination using protocol 1**

| High inoculation level<br>= 18 cfu/test portion | Intermediate inoculation level<br>= 6 cfu/test portion | Low inoculation level<br>= 2 cfu/test portion | Blank level | eLOD <sub>50</sub><br>cfu/test portion |
|---|--|---|-------------|--|
| 1/1   | 4/4  | 4/4   | 0/1         | < 2,0                                  |
| 1/1   | 4/4  | 3/4   | 0/1         | = 1,0                                  |
| 1/1   | 4/4  | 2/4   | 0/1         | = 1,4                                  |
| 1/1   | 4/4  | 1/4   | 0/1         | = 2,0                                  |
| 1/1   | 4/4  | 0/4   | 0/1         | = 3,0                                  |
| 1/1   | 3/4  | 4/4   | 0/1         | = 1,4                                  |
| 1/1   | 3/4  | 3/4   | 0/1         | = 2,0                                  |
| 1/1   | 3/4  | 2/4   | 0/1         | = 2,6                                  |
| 1/1   | 3/4  | 1/4   | 0/1         | = 3,4                                  |
| 1/1   | 3/4  | 0/4   | 0/1         | = 4,6                                  |
| 1/1   | 2/4  | 4/4   | 0/1         | = 2,2                                  |
| 1/1   | 2/4  | 3/4   | 0/1         | = 3,0                                  |
| 1/1   | 2/4  | 2/4   | 0/1         | = 3,8                                  |
| 1/1   | 2/4  | 1/4   | 0/1         | = 5,2                                  |
| 1/1   | 2/4  | 0/4   | 0/1         | = 7,4                                  |
| 1/1   | 1/4  | 4/4   | 0/1         | Unreliable MPN result <sup>a</sup>     |
| 1/1   | 1/4  | 3/4   | 0/1         | = 4,2                                  |
| 1/1   | 1/4  | 2/4   | 0/1         | = 5,6                                  |
| 1/1   | 1/4  | 1/4   | 0/1         | = 8,0                                  |
| 1/1   | 1/4  | 0/4   | 0/1         | = 12,6                                 |
| 1/1   | 0/4  | 4/4   | 0/1         | Unreliable MPN result <sup>a</sup>     |
| 1/1   | 0/4  | 3/4   | 0/1         | = 6,0                                  |
| 1/1   | 0/4  | 2/4   | 0/1         | = 8,6                                  |
| 1/1   | 0/4  | 1/4   | 0/1         | = 13,4                                 |
| 1/1   | 0/4  | 0/4   | 0/1         | = 28,0                                 |

<sup>a</sup> Unreliable MPN result: MPN combination is very unlikely to occur. The experiment shall be repeated.

### 5.5.2 Determination of eLOD<sub>50</sub> using protocol 2

If protocol 2 was used in the verification for the example in 5.4.2, then 23 test portions would have been examined (see Table 5) together with one blank test portion. Record the number of positive results obtained at each inoculum level and use Table 8 to determine the eLOD<sub>50</sub>.

The blank level shall not produce a positive result. If a positive result is obtained for the blank level, the experiment shall be repeated for all levels.

For the evaluation of the results using protocol 2, both the intermediate and low inoculation levels can have positive and negative results. When only negative results are obtained, the experiment shall be repeated. Some of the MPN combinations indicated as “unreliable MPN result” (see Tables 8 and 9) are very unlikely to occur and the experiment shall therefore be repeated.

When more dilutions are used, the two dilutions, with the low inoculation level closest to the LOD<sub>50</sub>, shall be used to evaluate the data according to Table 8.

**Table 8 — Determination of eLOD<sub>50</sub> based on the number of positive results per level of contamination using protocol 2**

| Intermediate inoculation level<br>targeted $3 \times \text{LOD}_{50}$ /test portion | Low inoculation level<br>targeted $1 \times \text{LOD}_{50}$ /test portion | Blank level | eLOD <sub>50</sub><br>cfu/test portion |
|---|--|-------------|--|
| 3/3   | 5/5  | 0/1         | $< 1,0 \times \text{LIL}^a$            |
| 3/3   | 4/5  | 0/1         | $= 0,4 \times \text{LIL}$              |
| 3/3   | 3/5  | 0/1         | $= 0,7 \times \text{LIL}$              |
| 3/3   | 2/5  | 0/1         | $= 1,0 \times \text{LIL}$              |
| 3/3   | 1/5  | 0/1         | $= 1,4 \times \text{LIL}$              |
| 3/3   | 0/5  | 0/1         | $= 2,0 \times \text{LIL}$              |
| 2/3   | 5/5  | 0/1         | $= 0,7 \times \text{LIL}$              |
| 2/3   | 4/5  | 0/1         | $= 0,9 \times \text{LIL}$              |
| 2/3   | 3/5  | 0/1         | $= 1,2 \times \text{LIL}$              |
| 2/3   | 2/5  | 0/1         | $= 1,6 \times \text{LIL}$              |
| 2/3   | 1/5  | 0/1         | $= 2,3 \times \text{LIL}$              |
| 2/3   | 0/5  | 0/1         | $= 3,7 \times \text{LIL}$              |
| 1/3   | 5/5  | 0/1         | Unreliable MPN result <sup>b</sup>     |
| 1/3   | 4/5  | 0/1         | $= 1,4 \times \text{LIL}$              |
| 1/3   | 3/5  | 0/1         | $= 1,8 \times \text{LIL}$              |
| 1/3   | 2/5  | 0/1         | $= 2,6 \times \text{LIL}$              |
| 1/3   | 1/5  | 0/1         | $= 4,1 \times \text{LIL}$              |
| 1/3   | 0/5  | 0/1         | $= 8,6 \times \text{LIL}$              |
| 0/3   | 5/5  | 0/1         | Unreliable MPN result <sup>b</sup>     |
| 0/3   | 4/5  | 0/1         | Unreliable MPN result <sup>b</sup>     |
| 0/3   | 3/5  | 0/1         | $= 2,9 \times \text{LIL}$              |
| 0/3   | 2/5  | 0/1         | $= 4,5 \times \text{LIL}$              |
| 0/3   | 1/5  | 0/1         | $= 9,4 \times \text{LIL}$              |

<sup>a</sup> LIL = low inoculation level.  
<sup>b</sup> Unreliable MPN result: MPN combination is very unlikely to occur. The experiment shall be repeated.

Using the actual inoculum level given in the example described in 5.4.2, Table 8 can be used to determine the eLOD<sub>50</sub>. This is presented in Table 9. In this example, an intermediate-level contamination of 6 cfu/test portion and low-level contamination of 2 cfu/test portion are used.

**Table 9 — Example for the determination of the eLOD<sub>50</sub> based on the number of positive results per level of contamination using protocol 2**

| Intermediate inoculation level<br>= 6 cfu/test portion | Low inoculation level<br>= 2 cfu/test portion | Blank level | eLOD <sub>50</sub><br>cfu/test portion |
|--|---|-------------|--|
| 3/3  | 5/5   | 0/1         | < 2,0                                  |
| 3/3  | 4/5   | 0/1         | = 0,8                                  |
| 3/3  | 3/5   | 0/1         | = 1,4                                  |
| 3/3  | 2/5   | 0/1         | = 2,0                                  |
| 3/3  | 1/5   | 0/1         | = 2,8                                  |
| 3/3  | 0/5   | 0/1         | = 4,0                                  |
| 2/3  | 5/5   | 0/1         | = 1,4                                  |
| 2/3  | 4/5   | 0/1         | = 1,8                                  |
| 2/3  | 3/5   | 0/1         | = 2,4                                  |
| 2/3  | 2/5   | 0/1         | = 3,2                                  |
| 2/3  | 1/5   | 0/1         | = 4,6                                  |
| 2/3  | 0/5   | 0/1         | = 7,4                                  |
| 1/3  | 5/5   | 0/1         | Unreliable MPN result <sup>a</sup>     |
| 1/3  | 4/5   | 0/1         | = 2,8                                  |
| 1/3  | 3/5   | 0/1         | = 3,6                                  |
| 1/3  | 2/5   | 0/1         | = 5,2                                  |
| 1/3  | 1/5   | 0/1         | = 8,2                                  |
| 1/3  | 0/5   | 0/1         | = 17,2                                 |
| 0/3  | 5/5   | 0/1         | Unreliable MPN result <sup>a</sup>     |
| 0/3  | 4/5   | 0/1         | Unreliable MPN result <sup>a</sup>     |
| 0/3  | 3/5   | 0/1         | = 5,8                                  |
| 0/3  | 2/5   | 0/1         | = 9,0                                  |
| 0/3  | 1/5   | 0/1         | = 18,6                                 |

<sup>a</sup> Unreliable MPN result: MPN combination is very unlikely to occur. The experiment shall be repeated.

### 5.5.3 Use of protocol 3

The blank level shall not produce a positive result. If a positive result is obtained for the blank level, the experiment shall be repeated for all levels.

Results from protocol 3 should only be used for evaluation when the level of contamination of the test portions is within the stated limits of between 3 cfu and 5 cfu/test portion. This level of contamination shall be determined either by enumeration or by MPN as outlined in 5.4.2. If the level of contamination is > 5 cfu/test portion, the results cannot be used, and the experiment shall be repeated. If the level of contamination is < 3 cfu/test portion and the acceptability limit is met, the results can be used. Otherwise, the experiment shall be repeated.

No eLOD is determined using protocol 3. The results shall be evaluated based on the number of positives found out of the seven replicates tested. The acceptability limit for this is presented in 5.6.

## 5.6 Acceptability limits

The eLOD<sub>50</sub>, determined according to protocol 1 (see 5.5.1) or protocol 2 (see 5.5.2) shall be compared to the LOD<sub>50</sub> from the validation study. For implementation verification, use the LOD<sub>50</sub> value corresponding to the tested (food) item.

For (food) item verification, the eLOD<sub>50</sub> shall not be  $> 4 \times \text{LOD}_{50}$  observed in the validation study. If no LOD<sub>50</sub> value corresponds to the tested (food) item, the eLOD<sub>50</sub> shall not be  $> 4$  cfu/test portion. This acceptability limit is based on the theoretical value of a LOD<sub>50</sub> of 1 cfu/test portion.

For protocol 3, there shall be a minimum of six positive results out of the seven replicates tested.

[Clause 8](#) provides a summary of the acceptability limits.

### NOTE

- The eLOD<sub>50</sub> of the verification study is valid and acceptable only if it is obtained from the same or a smaller test portion (e.g. 25 g, 100 ml, 375 g) used in the validation study.
- The LOD<sub>50</sub> observed in the validation study can be expressed as cfu/g, cfu/ml or cfu/test portion. The validation LOD<sub>50</sub> will need to be expressed in cfu/test portion to be able to compare the result with the result of the verification study.
- In cases where the LOD<sub>50</sub> observed in the validation study is given as cfu/g or cfu/ml, then the LOD<sub>50</sub> needs to be multiplied by the size of the test portion used. Therefore, an LOD<sub>50</sub> of 0,1 cfu/g or cfu/ml will give an LOD<sub>50</sub> of 2,5 when a 25 g or 25 ml test portion is used.
- If, for example, the LOD<sub>50</sub> from the validation study is 2,5 cfu/test portion, the maximum acceptable value for the eLOD<sub>50</sub> will be 10 cfu/test portion (maximum of  $4 \times \text{LOD}_{50}$ ).

## 5.7 Root cause analysis

When the verification result exceeds the acceptability limits (e.g. the eLOD<sub>50</sub> is  $> 4 \times \text{LOD}_{50}$  observed in the validation study), perform a root cause analysis to provide an explanation for the observed results.

It can be useful to re-run verification of a validated alternative method in parallel with the validated reference method on this food (item). This is to investigate if this (food) item is performing similarly for both methods in the hands of the user laboratory.

The root cause analysis shall be conducted to determine concerns such as (but not limited to):

- analytical error due to poor laboratory practice;
- analytical error in protocol application (e.g. incorrect inoculation level);
- (food) item specificity [e.g. very challenging (food) items that required a higher dilution factor in the initial suspension].

When the problems have been identified, implement corrective actions and repeat the experiment.

Information (based on investigations, e.g. root cause analysis) can be given in the verification study report to provide an explanation of the findings.

When the verification of a particular (food) item does not meet the acceptability limits, it is recommended that the user laboratory informs a relevant organization (e.g. standardization body, supplier, certification body) depending on the method.

## 6 Quantitative methods — Technical protocol for verification

### 6.1 Intralaboratory reproducibility standard deviation determination

#### 6.1.1 General

The intralaboratory reproducibility standard deviation determination only applies to implementation verification.

Implementation verification is performed in a single laboratory, and the reproducibility is expressed as the intralaboratory reproducibility standard deviation ( $S_{IR}$ ).

The determination of the intralaboratory reproducibility standard deviation ( $S_{IR}$ ) in the implementation verification corresponds to the determination of the technical uncertainty, which is one of the three main uncertainty components (technical, matrix and distributional) described in ISO 19036. The intralaboratory reproducibility standard deviation ( $S_{IR}$ ) determination is based on ISO 19036:2019, 5.2.2.

During the implementation verification, run the full procedure of the method as described, including the confirmation procedure for each individual test portion.

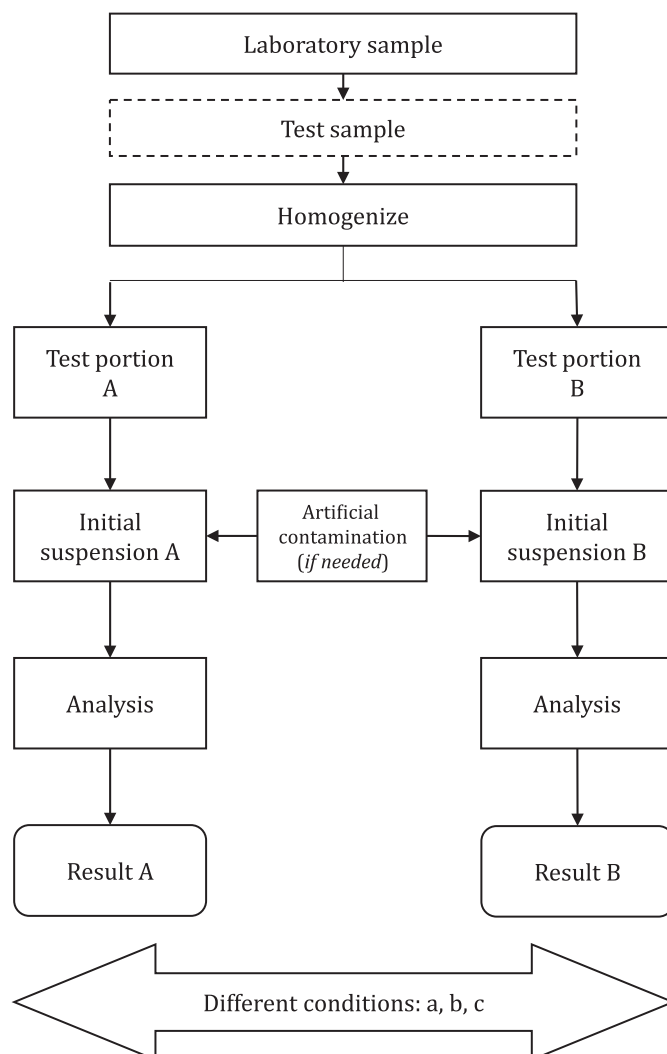
#### 6.1.2 Experimental design

The protocol shall be performed as follows.

- A minimum of 10 laboratory samples, belonging to the same (food) item, are required. These may be from a maximum number of different *batches* in cases where the user laboratory is linked to a production facility or from different *manufacturers* for private laboratories servicing different manufacturers. More samples may be tested to cover the possible loss of data from some samples due to practical errors/mishaps during testing.
- The contamination levels used shall be representative of the range of the natural contamination found in the samples tested in the user laboratory.
- Each laboratory (or test) sample shall be mixed or homogenized before two test portions are taken (see [Figure 7](#)). This is essential for the uniform distribution of the microorganisms. For liquid products, mixing shall be performed by shaking the laboratory sample (or test sample) by hand (e.g. 25 times through an arc of 25 cm). For solid products, the homogenization may be performed by mechanical means, which could include stomachers and blenders. For details, follow the procedure in the ISO 6887 series.
- If artificial contamination is used, inoculate the initial suspension of each test portion with a known level of the strain.
- Naturally contaminated test portions can be analysed directly after homogenization of the laboratory sample (or test sample).

See also [D.1](#) for additional guidance and examples.





**Figure 7 — Experimental protocol to estimate the intralaboratory reproducibility standard deviation ( $S_{IR}$ )**

The test sample, as defined in 3.17, is infrequently used in microbiological examinations. Most of the time, the laboratory sample is directly used for homogenization.

Test conditions used in the analyses of test portion A and test portion B shall be varied in as many ways as possible within the scope of validation. These shall include, unless the user laboratory can justify otherwise, but not be limited to:

- a) technicians;
- b) batches of culture media and reagents (optional: when relevant, different strains may also be used to inoculate different laboratory samples);
- c) apparatus (e.g. incubators, vortex mixer, pipettes).

Test conditions a) and b) are considered to cause the most variability in the results of a method and shall be varied unless the user laboratory can justify otherwise. Test condition c) shall be varied based on the availability of the apparatus in the user laboratory. If the inoculated (food) item can be shown to be sufficiently stable, the analysis may be conducted on different days. Results shall be assessed according to 6.1.6.

NOTE Culture media batches can be generated from different preparations/productions from the same batch of powder.

### 6.1.3 Selection of the (food) item

One (food) item is required for the implementation verification. The intralaboratory reproducibility standard deviation ( $S_{IR}$ ), as determined according to [Figure 7](#), is independent of the matrix as the experiments are designed to exclude contributions from the heterogeneity of the matrix, so any (food) item within the scope of validation can be selected. It is recommended to select a (food) item that can be effectively homogenized in order to minimize the matrix uncertainty.

### 6.1.4 Natural contamination

Whenever possible, use naturally contaminated items. For the (food) item chosen, the individual test portions evaluated shall have contamination levels representative of the range of the natural contamination found in the samples analysed in the user laboratory.

If the expected level of natural contamination is less than 10 cfu/g in the test portion, artificial contamination is used (see [6.1.5](#) for details) to cover the range of use of the method.

### 6.1.5 Artificial contamination

#### 6.1.5.1 Selection of the strain

The strain can be from:

- a culture collection;
- a user laboratory collection;
- a reference material (including commercial reference material, e.g. a freeze-dried strain with known concentration).

NOTE Preferably, the strain used in the verification is from a source relevant to the (food) item being verified.

#### 6.1.5.2 Inoculation of the test portion

When inoculating the test portion, the contamination levels used shall be representative of the range of the natural contamination found in the laboratory samples analysed in the user laboratory.

The following guidance is given as an example of procedures suitable for producing inocula.

- The selected strain is grown in a culture medium under conditions that enable optimal growth of the strain (e.g. overnight culture). Follow the procedures specified in ISO 11133:2014, 5.4.

NOTE In this document, overnight culture is specified as 16 h to 24 h of incubation.

- Enumerate the culture on non-selective media to determine the concentration of the strain in cfu/ml. It is assumed that this level will be consistently achieved when the same culture conditions are used.
- Repeat the culture under the same conditions and take into account the previously determined concentration to prepare dilutions to cover the representative range of natural contamination. This step is not required if the stability of the strain is known by the user laboratory (e.g. viability after storage at 4 °C overnight).

If the user laboratory works with ready-to-use target strains with known levels (e.g. reference material), the steps described above are not required.

The prepared inoculum is introduced directly into the initial suspension of the individual test portions. After inoculation, the suspension is mixed thoroughly. The use of stressed cultures is recommended but is not required (see ISO 16140-2:2016, Annex C).

**EXAMPLE** A user laboratory wants to verify the *Enterobacteriaceae* enumeration method (see ISO 21528-2). The validation of this method was performed using one (food) item within each of four food categories and one other category:

- a) food category: heat-processed milk and dairy products; food type: pasteurized milk-based products; food item: pasteurized milk;
- b) food category: raw meat and ready-to-cook meat products (except poultry); food type: fresh meats (unprocessed); food item: raw meat (minced pork);
- c) food category: eggs and egg products (derivatives); food type: egg product (heat-processed) without additives; food item: egg product (whole liquid egg);
- d) food category: chocolate, bakery products and confectionary; food type: pastries; food item: tiramisu;
- e) other category: pet food and animal feed; (food) type: animal origin ingredients; (food) item: animal feed (meat and bone meal).

For implementation verification, the food item “tiramisu” was chosen and *E. coli* was chosen as the strain for the artificial inoculation. Note that tiramisu is given as an example since any (food) item can be chosen for implementation verification.

- Based on the range of contamination levels representative of the natural contamination found in the samples analysed in the user laboratory, the tiramisu will be inoculated between 30 (1,5 log<sub>10</sub>) cfu/g to 30 000 (4,5 log<sub>10</sub>) cfu/g.
- A minimum of 10 different (brands, lots) laboratory samples of tiramisu will be prepared and each divided into two test portions: A and B (see [Figure 7](#)).
- Both test portions, A and B, originating from the same laboratory sample (see [Figure 7](#)) will be inoculated with the same inoculum. Each set of the 10 or more laboratory samples will be inoculated at different levels (and possibly with different strains) between 30 cfu/g and 30 000 cfu/g. The culture will be inoculated into the initial suspensions, which have been prepared using 10 g test portions.
- In order to do this, an overnight culture is prepared and assumed to contain 10<sup>9</sup> cfu/ml (based on the results of previous enumerations).
- To contaminate at the level of 30 cfu/g, six serial decimal dilutions of the overnight culture are prepared, to reduce the initial level from 10<sup>9</sup> cfu/ml to 10<sup>3</sup> cfu/ml.

Different contamination levels covering the range of 30 cfu/g and 30 000 cfu/g can be obtained with different dilutions and/or inoculation volume. The user laboratory shall ensure that the inoculum does not affect the integrity of the matrix. The results from this example are summarized in [Table 10](#). [D.1](#) provides details of the experimental process for this example.

### 6.1.6 Evaluation of results

The intralaboratory reproducibility standard deviation ( $S_{IR}$ ) is calculated, based on a minimum of 10 laboratory samples, according to [Formula \(1\)](#):

$$S_{IR} = \sqrt{\frac{1}{2n} \sum_{i=1}^n (y_{iA} - y_{iB})^2} \quad (1)$$

where

- $S_{IR}$  is the intralaboratory reproducibility standard deviation;
- $i$  is the index of the laboratory sample,  $i = 1$  to  $n$  ( $n \geq 10$ );
- $n$  is the number of samples;
- $y_{iA}, y_{iB}$  are the log-transformed data, in  $\log_{10}$  (cfu/g) or  $\log_{10}$  (cfu/ml), from conditions a, b and c, respectively.

An example of a manual calculation is given in [Table 11](#).

### 6.1.7 Acceptability limit

The intralaboratory reproducibility standard deviation ( $S_{IR}$ ) of the verified method shall be  $\leq 2 \times$  the lowest mean value of the interlaboratory reproducibility standard deviation ( $S_R$ ) of the (food) items used in the validation study. When only one ( $S_R$ ) value is determined in the validation study, the ( $S_{IR}$ ) of the verified method shall be  $\leq 2 \times$  interlaboratory reproducibility standard deviation ( $S_R$ ).

[Clause 8](#) provides a summary of the acceptability limits.

#### EXAMPLE

- The user laboratory has examined 12 laboratory samples of tiramisu for the level of *Enterobacteriaceae* (using ISO 21528-2) following the experimental design given in [Figure 7](#). The results (calculated as cfu/g in test portions A and B of the laboratory sample) given in [Table 10](#) were obtained.

**Table 10 — Test results**

| Laboratory sample number | Expected contamination level<br>cfu/g | Result A ( $x_{iA}$ )<br>cfu/g | Result B ( $x_{iB}$ )<br>cfu/g | Log <sub>10</sub> result A<br>$y_{iA} = \log_{10}(x_{iA})$ | Log <sub>10</sub> result B<br>$y_{iB} = \log_{10}(x_{iB})$ |
|--------------------------|---------------------------------------|--------------------------------|--------------------------------|--|--|
| 1                        | 30                                    | < 40 (10)                      | < 40 (30)                      | $\leq 1,60$  | $\leq 1,60$  |
| 2                        | 300                                   | 110                            | 182                            | 2,04   | 2,26   |
| 3                        | 300                                   | 410                            | 620                            | 2,61   | 2,79   |
| 4                        | 600                                   | 640                            | 330                            | 2,81   | 2,52   |
| 5                        | 600                                   | 690                            | 570                            | 2,84   | 2,76   |
| 6                        | 600                                   | 780                            | 640                            | 2,89   | 2,81   |
| 7                        | 600                                   | 620                            | 1 300                          | 2,79   | 3,11   |
| 8                        | 600                                   | 870                            | 1 500                          | 2,94   | 3,18   |
| 9                        | 6 000                                 | 8 600                          | 6 400                          | 3,93   | 3,81   |
| 10                       | 6 000                                 | 16 000                         | 5 000                          | 4,20   | 3,70   |
| 11                       | 6 000                                 | > 15 000                       | 13 400                         | > 4,18   | 4,13   |
| 12                       | 30 000                                | 20 000                         | 32 000                         | 4,30   | 4,51   |

- The results of laboratory samples 1 and 11 cannot be used because one of the counts was either too high (“>” result) or too low (below the permitted counting range in accordance with ISO 7218). The results of 10 laboratory samples remain for the calculation.
- Based on the 10 remaining laboratory samples, the  $S_{IR}$  can be calculated as shown in [Table 11](#).

**Table 11 — Calculation of  $S_{IR}$**

| Laboratory sample number | Log <sub>10</sub> result A   | Log <sub>10</sub> result B   | Absolute difference         | Squared difference    |
|--------------------------|------------------------------|------------------------------|-----------------------------|-----------------------|
|                          | $y_{iA} = \log_{10}(x_{iA})$ | $y_{iB} = \log_{10}(x_{iB})$ | $ y_{iA} - y_{iB} $         | $ y_{iA} - y_{iB} ^2$ |
| 1                        | ≤ 1,602 1                    | ≤ 1,602 1                    | Not used                    | Not used              |
| 2                        | 2,041 4                      | 2,260 1                      | 0,218 7                     | 0,047 8               |
| 3                        | 2,612 8                      | 2,792 4                      | 0,179 6                     | 0,032 3               |
| 4                        | 2,806 2                      | 2,518 5                      | 0,287 7                     | 0,082 8               |
| 5                        | 2,838 8                      | 2,755 9                      | 0,083 0                     | 0,006 9               |
| 6                        | 2,892 1                      | 2,806 2                      | 0,085 9                     | 0,007 4               |
| 7                        | 2,792 4                      | 3,113 9                      | 0,321 6                     | 0,103 4               |
| 8                        | 2,939 5                      | 3,176 1                      | 0,236 6                     | 0,056 0               |
| 9                        | 3,934 5                      | 3,806 2                      | 0,128 3                     | 0,016 5               |
| 10                       | 4,204 1                      | 3,699 0                      | 0,505 1                     | 0,255 2               |
| 11                       | > 4,176 1                    | 4,127 1                      | Not used                    | Not used              |
| 12                       | 4,301 0                      | 4,505 1                      | 0,204 1                     | 0,041 7               |
|                          |                              |                              | Sum                         | 0,650 0               |
|                          |                              |                              | Sum/(2 × 10)                | 0,032 5               |
|                          |                              |                              | $S_{IR} = \sqrt{(0,032 5)}$ | 0,18                  |

- The calculated  $S_{IR}$  value of 0,18 is compared to the results of the validation study (data taken over from ISO 21528-2). [Table 12](#) lists the  $S_R$  values obtained from that validation study.

**Table 12 — Summary of  $S_R$  values from the validation study for ISO 21528-2**

| (Food) item      | $S_R$ values from the validation study |                                |                        |  |
|------------------|--|--------------------------------|------------------------|--|
|                  | Low inoculation level                  | Intermediate inoculation level | High inoculation level | Mean value of three inoculation levels |
| Egg product      | 0,32                                   | 0,50                           | 0,48                   | 0,43                                   |
| Raw meat         | 0,28                                   | 0,36                           | 0,57                   | 0,40                                   |
| Animal feed      | 0,18                                   | 0,17                           | 0,20                   | 0,18                                   |
| Pasteurized milk | 0,24                                   | 0,18                           | 0,19                   | 0,20                                   |
| Tiramisu         | 0,22                                   | 0,28                           | 0,13                   | 0,21                                   |

- The experiment is designed to not consider the effect of the (food) item. The  $S_{IR}$  obtained is compared to the lowest mean value of  $S_R$  for any of the items tested in the validation study. In this example, the lowest mean value of  $S_R$  was 0,18 (for animal feed).
- The  $S_{IR}$  found in the verification study (0,18) is assessed against  $2 \times S_R$  ( $2 \times 0,18$ ) from the validation study.
- As the  $S_{IR}$  of the verification study (0,18) is ≤ 0,36 ( $2 \times 0,18$ ), the conclusion is that the acceptability limit for the implementation verification is met.

NOTE When only one  $S_R$  value is determined in the validation study, the  $S_{IR}$  value is compared to that  $S_R$  value.

### 6.1.8 Root cause analysis

When the verification result does not meet the acceptability limits, perform a root cause analysis to provide an explanation for the observed results.

It can be useful to re-run verification of a validated alternative method in parallel with the validated reference method on this food (item). This is to investigate if this (food) item is performing similarly for both methods in the hands of the user laboratory.

The root cause analysis shall be conducted to determine concerns such as (but not limited to):

- analytical error due to poor laboratory practice;
- analytical error in protocol application (e.g. incorrect inoculation level).

When the problems have been identified, implement corrective actions and repeat the experiment.

Information (based on investigations, e.g. root cause analysis) can be given in the verification study report to provide an explanation of the findings.

When the verification of a particular (food) item does not meet the acceptability limits, it is recommended that the user laboratory informs a relevant organization (e.g. standardization body, supplier, certification body) depending on the method.

## **6.2 Estimated bias (eBias) determination**

### **6.2.1 General**

The eBias determination is only required for (food) item verification (see [Table 2](#)).

During the (food) item verification, run the full procedure of the method as described, including the confirmation procedure (if there is one) for each individual test portion.

### **6.2.2 Experimental design**

The protocol shall be performed as follows.

- Select the (food) item(s) for testing (see [6.2.3](#)).
- Artificially contaminate the (food) item(s) at three inoculation levels that cover the range of use of the method by the user laboratory. The artificial contamination is done in the initial suspension. Each level is performed in duplicate. Preferably, use a different laboratory sample or a different batch produced of the same (food) item for each of the three inoculation levels.
- Enumerate, using the method to be verified, the artificially contaminated (food) item and the (pure culture) suspension used to inoculate the (food) item.
- Test the uninoculated test portion for each laboratory sample or batch to determine the background contamination level. The results of these negative controls are recorded and can provide useful information when a root cause analysis is required (see [6.2.7](#)).

See also [D.2](#) for additional guidance and examples.

### **6.2.3 Selection of (food) items**

For the (food) item verification, the user laboratory shall test a minimum of one (food) item, preferably a challenging one, from each of the required (food) categories. Details on the required number of (food) categories to test according to the scope of validation or to the scope of laboratory application are described in [4.4. Annex B](#) provides guidance on how to choose challenging (food) items.

### **6.2.4 Artificial contamination**

#### **6.2.4.1 Selection of strains**

Strains can be from:

- culture collections;
- user laboratory collection;

- reference materials (including commercial reference materials, e.g. a freeze-dried strain with known concentration).

When choosing the test strains, the majority should originate from the (food) categories selected for the verification study and cover the recognized range of the target analyte with respect to the diversity in identification characteristics (e.g. biochemical, serotype, phage type), geographical distribution and incidence (see ISO 16140-2:2016, Annex E).

NOTE Preferably, the strains used in the verification are from sources relevant to the (food) item being verified and a different strain is used for each of the (food) items to be tested.

#### 6.2.4.2 Inoculation of the test portions

When inoculating the test portions, the three contamination levels used shall be representative of the range of the natural contamination found in the laboratory samples analysed in the user laboratory.

NOTE 1 If the user laboratory sets up more than three inoculum levels (e.g. five or six), it is more likely to obtain the three levels required for comparison studies.

The following guidance is given as an example of procedures suitable for producing inocula.

- The selected strain is grown in a culture medium under conditions that enable optimal growth of the strain (e.g. overnight culture). Follow the procedures specified in ISO 11133:2014, 5.4.

NOTE 2 In this document, overnight culture is specified as 16 h to 24 h of incubation.

- Repeat the culture under the same conditions and take into account the previously determined concentration to prepare dilutions to cover the targeted range of contamination. This step is not required if the stability of the strain is known by the user laboratory (e.g. viability after storage at 4 °C overnight).

If the user laboratory works with ready-to-use strains with known levels (e.g. reference material), the steps described above are not required.

The prepared inoculum is introduced directly into the initial suspension of the individual test portions. After inoculation, the suspension is mixed thoroughly. The use of stressed cultures is recommended but is not required (see ISO 16140-2:2016, Annex C).

EXAMPLE A user laboratory usually expects to find between  $10^2$  cfu/g and  $10^6$  cfu/g in submitted samples. The verification study required inoculation of the initial suspension to the levels of  $10^1$  cfu/ml,  $10^3$  cfu/ml and  $10^5$  cfu/ml (as the initial suspension is a 10-fold dilution of the test portion, this is equivalent to  $10^2$  cfu/g,  $10^4$  cfu/g and  $10^6$  cfu/g of the test portion). It is assumed that the test portion is 10 g and the final volume of the initial suspension is 100 ml.

- A fresh overnight culture of the required microorganism (see [6.2.4.1](#)) was prepared and assumed through previous measurement/experience to have  $10^7$  cfu/ml. Appropriate 10-fold dilutions were made covering their target range of  $10^3$  cfu/ml to  $10^7$  cfu/ml (= the undiluted overnight culture) for inoculation.
- One ml of inoculum was transferred into duplicate initial suspensions, giving assumed concentrations in the initial suspensions of:  $10^1$  cfu/ml,  $10^3$  cfu/ml and  $10^5$  cfu/ml (this was equivalent to  $10^2$  cfu/g,  $10^4$  cfu/g and  $10^6$  cfu/g in the test portions). An uninoculated test portion was included. Additional dilutions (to cover a wider range from  $10^0$  cfu/ml to  $10^6$  cfu/ml of the initial suspensions) were used as the actual concentration of microorganisms of the inoculum is unknown at this stage.
- The uninoculated test portion and each of the inoculated initial suspensions were then enumerated using the method to be verified.
- The overnight culture and its dilutions used to inoculate the initial suspensions were also enumerated, using the method to be verified, to determine the actual concentration of microorganisms.
- However, after incubation and counting of the inoculum, it was determined that the overnight culture actually contained  $5 \times 10^8$  cfu/ml (i.e.  $1 \log_{10} >$  the assumed level). Therefore, the actual levels in the initial suspensions were  $10^2$  cfu/ml to  $10^6$  cfu/ml, and the calculated levels in the test portions were  $10^3$  cfu/g to  $10^7$  cfu/g.



- Because of the additional dilutions used, the user laboratory was then still able to select the results relating to  $10^2$  cfu/g,  $10^4$  cfu/g and  $10^6$  cfu/g of the test portion ( $10^1$  cfu/ml,  $10^3$  cfu/ml and  $10^5$  cfu/ml of the initial suspensions) and compare actual results for the method being verified, with the counts of the inoculum.

### 6.2.5 Evaluation of results

Compare the results of the artificially contaminated (food) item to the results of the inoculum suspension [being the specific (diluted) suspension used to contaminate the initial suspension of the (food) item]. Both the (food) item and the specific (diluted) inoculum suspension are tested using the method to be verified.

For the comparison, the results for the (food) item shall be expressed in  $\log_{10}$  cfu/per test portion and the results for the inoculum suspension shall be expressed in  $\log_{10}$  cfu/ml.

The results of the uninoculated test portion (negative control) provides information on the level of natural contamination, if present, of the (food) item with the target microorganism(s).

### 6.2.6 Acceptability limit

It is expected that, at each level, the absolute difference between the results of the artificially contaminated (food) item in  $\log_{10}$  cfu/test portion and that of the inoculum suspension is equal to or less than  $0,5 \log_{10}$ . However, this may not be the case if the (food) item used was naturally contaminated prior to inoculation. The results of the uninoculated test portions (negative controls) can assist when a root cause analysis is required (see 6.2.7).

Clause 8 provides a summary of the acceptability limits.

**EXAMPLE** A user laboratory wants to verify the eBias of a validated alternative enumeration method for *Enterobacteriaceae*, using the food item “boiled pasta”. The expected range of contamination is between  $10^2$  cfu/g and  $10^4$  cfu/g. The results of the tests are given in Table 13.

**Table 13 — Test results obtained using the method to be verified**

|   | Mean result<br>Artificially<br>contaminated<br>(food) item<br>( $\log_{10}$ cfu/g<br>or ml) <sup>a</sup> | For comparison   |   | eBias:<br>absolute difference<br>in results between<br>artificially contami-<br>nated (food) item per<br>test portion and the<br>inoculum suspension |
|---|--|--|---|--|
|   |  | Result<br>Artificially contami-<br>nated (food) item<br>( $\log_{10}$ cfu/<br>test portion) <sup>a</sup> | Result<br>Inoculum suspension<br>[without (food) item]<br>( $\log_{10}$ cfu/ml) |  |
| Laboratory sample 1<br>(from batch 1), test portion 1 | 2,06<br>(average of 1,87<br>and 2,25)  | 3,06   | 3,17  | 0,11   |
| Laboratory sample 1<br>(from batch 1), test portion 2 |  |  |   |  |
| Laboratory sample 2<br>(from batch 2), test portion 1 | 3,11<br>(average of 3,16<br>and 3,06)  | 4,11   | 4,05  | 0,06   |
| Laboratory sample 2<br>(from batch 2), test portion 2 |  |  |   |  |
| Laboratory sample 3<br>(from batch 3), test portion 1 | 3,99<br>(average of 3,93<br>and 4,04)  | 4,99   | 5,29  | 0,30   |
| Laboratory sample 3<br>(from batch 3), test portion 2 |  |  |   |  |

<sup>a</sup> This example is based on the use of a 10-gram test portion inoculated with 1 ml of inoculum.

The results indicate that at each level of contamination the absolute difference between the two results is less than  $0,5 \log_{10}$ , so the method to be verified works correctly in the user laboratory.



### 6.2.7 Root cause analysis

When the verification result does not meet the acceptability limits, perform a root cause analysis in order to provide an explanation for the observed results.

It can be useful to re-run verification of a validated alternative method in parallel with the validated reference method on this food (item). This is to investigate if this (food) item is performing similarly for both methods in the hands of the user laboratory.

The root cause analysis shall be conducted to determine concerns such as (but not limited to):

- analytical error due to poor laboratory practice;
- analytical error in protocol application (e.g. incorrect inoculation level).

When the problems have been identified, implement corrective actions and repeat the experiment.

Information (based on investigations, e.g. root cause analysis) can be given in the verification study report to provide an explanation of the findings when the eBias is  $> 0,5 \log_{10}$  cfu/g.

When the verification of a particular (food) item does not meet the acceptability limits, it is recommended that the user laboratory informs a relevant organization (e.g. standardization body, supplier, certification body) depending on the method.

## 7 Validated alternative confirmation and typing methods — Technical protocol for verification

### 7.1 General

The verification of validated alternative confirmation and typing methods only requires implementation verification. The sample is an isolated colony on defined selective or non-selective agar plates.

### 7.2 Implementation verification

Implementation verification aims to demonstrate the competence of the user laboratory to perform the validated alternative confirmation or typing method. This is achieved by its ability to obtain the expected results on an isolated colony from specified selective or non-selective agar(s).

The user laboratory shall:

- review the validation data for the method (the validation data can be obtained from the alternative methods validation report);
- select one selective agar plate tested during the validation study that, if possible, belongs within the scope of the laboratory;
- use this selective agar plate to perform implementation verification. If no selective agar plate was tested, select and use one non-selective agar plate tested during the validation study to perform the implementation.

NOTE Detailed examples on verification of an alternative confirmation method and on verification of an alternative typing method are given in [Annex E](#).

### 7.3 Experimental design

#### 7.3.1 General

For the implementation verification, the number of strains to be tested is given in [Table 14](#).

**Table 14 — Number of strains for implementation verification of validated alternative confirmation or typing methods**

| Level of the confirmation                                   | Inclusivity study | Exclusivity study |
|---|-------------------|-------------------|
| Family  | 5                 | 5                 |
| Genus   |                   |                   |
| Species   |                   |                   |
| Microbial (sub)type (e.g. serotyping of <i>Salmonella</i> ) |                   |                   |

### 7.3.2 Strain selection

Strains can be from:

- culture collections;
- user laboratory collection;
- reference materials (including commercial reference materials, e.g. freeze-dried strains).

When choosing test strains, the majority should originate from the (food) categories within the scope of laboratory application and cover the recognized range of the target analyte with respect to the diversity in identification characteristics, e.g. biochemical, serotype, phage type, geographical distribution and incidence (see ISO 16140-2:2016, Annex E).

For the implementation verification, select five target strains and five non-target strains for the inclusivity and exclusivity study, respectively. The selection of the strains can be based on the strains tested in the validation study. The exclusivity strains shall be relevant (e.g. *L. innocua* shall be selected for a validated *L. monocytogenes* alternative confirmation method).

### 7.4 Evaluation of results

Test the selected inclusivity and exclusivity strains according to the validated alternative confirmation or typing method being verified.

Tabulate the results for the inclusivity and the exclusivity studies as shown in [Table 15](#). Report the agreements and deviations between the expected confirmation or typing result and the result of the confirmation or typing method being verified.

**Table 15 — Overview of verification results for a validated alternative confirmation or typing method**

| Tested strains | Inclusivity/exclusivity | Characteristics of the strain | Expected confirmation/typing result | Result of the confirmation/typing method being verified | Interpretation <sup>a</sup> |
|----------------|-------------------------|-------------------------------|-------------------------------------|---|-----------------------------|
| 1              |                         |                               |                                     |   |                             |
| 2              |                         |                               |                                     |   |                             |
| ...            |                         |                               |                                     |   |                             |
| 9              |                         |                               |                                     |   |                             |
| 10             |                         |                               |                                     |   |                             |

<sup>a</sup> Agreement or deviation between the expected result and the result of the tested confirmation or typing method.

NOTE Characteristics of the individual strains are as a minimum: the name of the strain, (culture) collection number and origin of the strain. Other available characteristics can be added as well.

## 7.5 Acceptability limit

The result of the alternative confirmation or typing method being verified shall be the same as the expected confirmation or typing result for all strains tested. Therefore, there should be 100 % agreement.

## 7.6 Root cause analysis

When the result does not meet the acceptance limit, perform a root cause analysis in order to provide an explanation for the observed results.

The root cause analysis shall be conducted to determine concerns such as (but not limited to):

- analytical error due to poor laboratory practice;
- analytical error in protocol application (e.g. incorrect incubation time or temperature);
- the formulation of culture medium/media;
- the correct identity of test strains.

## 8 Summary of acceptability limits for the verification of validated methods

[Table 16](#) summarizes the acceptability limits that are used for method verification of validated methods.

As stated in [4.3](#) and [5.6](#), the LOD<sub>50</sub> of the validation study and the eLOD<sub>50</sub> of the verification are valid and acceptable only if both are derived from test portions of the same size (or a smaller sample size if routinely used in the user laboratory).

**Table 16 — Acceptability limits for the verification of validated methods**

| Method                    | Performance characteristics | Acceptability limits   |
|---------------------------|-----------------------------|--|
| Qualitative               | eLOD <sub>50</sub>          | For protocols 1 and 2: eLOD <sub>50</sub> ≤ 4 × LOD <sub>50</sub><br>For protocol 3: ≥ 6 out of 7 positive results   |
| Quantitative              | S <sub>IR</sub>             | S <sub>IR</sub> ≤ 2 × lowest S <sub>R</sub> mean value <sup>a</sup><br>determined in the validation study  |
|                           | eBias                       | log <sub>10</sub> cfu/ml (inoculum) – mean log <sub>10</sub> cfu/test portion<br>(artificially contaminated [food] item)  <br>≤ 0,5 log <sub>10</sub> for each of the inoculation levels |
| Confirmation<br>or typing | inclusivity and exclusivity | 100 % agreement between methods  |

<sup>a</sup> S<sub>IR</sub> ≤ 2 × S<sub>R</sub> for validation studies with only one S<sub>R</sub> value.

## Annex A (informative)

### Classification of (food) categories and suggested target combinations for verification studies

[Table A.1](#) outlines the classification of foods, feeds, primary production and environmental samples to guide user laboratories in the selection of (food) items in their corresponding (food) categories when performing method verification.

The intrinsic properties of foods such as levels of indigenous microbiota, fat content, pH, salt content, water activity and the presence of antimicrobial compounds can have a substantial influence on the outcome of a method. The main physico-chemical properties of foods have been considered to the extent possible in the classification of foods.

Regulatory authorities in different jurisdictions can have slightly different requirements regarding the classification of foods.

Points to note when using [Table A.1](#):

- ISO 16140-2:2016, Annex A, is the source for [Table A.1](#) and the notes shown at the end of the table;
- the symbol “Y” in [Table A.1](#) indicates that, for that sample, it is relevant to test for the indicated microorganism;
- IMF is the abbreviation for "intermediate moisture food".

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 enterocolitica | Vibrio spp. | Bacillus cereus (vegetative cells or spores) | Clostridium fringens (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                                |                 |               |                  |                                      |                  |               |                                      |             |  |   |  |
| Raw milk and dairy products | Raw milk-based products with high fat content and/or high back-ground microbiota | Raw butters  |                    |                      |                   |                    | Y                | Y                                | Y               | Y             | Y                | Y                                    |                  |               | Y                                    |             |  |   |  |
|                             |  | Raw creams   |                    |                      |                   |                    | Y                | Y                                | Y               | Y             | Y                | Y                                    |                  |               | Y                                    |             |  |   |  |
| Raw milk and dairy products | Raw milk-based products with high fat content and/or high back-ground microbiota | 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           |  |   |  |
| Raw milk and dairy products | Raw milk-based products with high fat content and/or high back-ground microbiota | 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. | (Pathogenic) Yersinia enterocolitica | 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  |  |  |
|  |   |  |                    |                      |                   |                    |                  |                                  |                 |               |                  |                                      |                  |                                      |             |  |  |  |  |
|  |   | Fermented/acidified pasteurized milk, yoghurts, dairy-based products |                    |                      | Y                 | Y                  | Y                | Y                                | Y               | Y             | Y                | Y                                    |                  |                                      |             | Y  |  |  |  |
|  |   |  |                    | Y                    |                   |                    |                  |                                  |                 |               |                  |                                      |                  |                                      |             |  |  |  |  |
|  |   | Pasteurized milks  |                    | Y                    |                   | Y                  | Y                | Y                                | Y               | Y             | Y                | Y                                    |                  |                                      |             | Y  |  |  |  |
|  |   |  |                    |                      |                   |                    |                  |                                  |                 |               |                  |                                      |                  |                                      |             |  |  |  |  |
|  |   | Butters  |                    |                      |                   | Y                  | Y                | Y                                | Y               | Y             | Y                | Y                                    |                  |                                      |             |  |  |  |  |
|  |   |  |                    |                      |                   |                    |                  |                                  |                 |               |                  |                                      |                  |                                      |             |  |  |  |  |
|  | Creams  |  |                    | Y                    | 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             | Y                |                                      |                  |                                      |             |  |  |  |  |
|  |   |  |                    |                      |                   |                    |                  |                                  |                 |               |                  |                                      |                  |                                      |             |  |  |  |  |
|  | Soft cheeses (e.g. Brie, Munster)   |  |                    |                      |                   | Y                  | Y                | Y                                | Y               | Y             | Y                |                                      |                  |                                      |             |  |  | Y  |  |
|  |   |  |                    |                      |                   |                    |                  |                                  |                 |               |                  |                                      |                  |                                      |             |  |  |  |  |
| Dry                                    | Milk powders  |  | Y                  |                      | Y                 | Y                  | Y                | Y                                | Y               | Y             | Y                |                                      |                  |                                      |             | Y  |  |  |  |
|  |   |  | Y                  |                      |                   |                    |                  |                                  |                 |               |                  |                                      |                  |                                      |             |  |  |  |  |
|  |   | Powder for milk-based desserts                                       | 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 enterocolitica | Vibrio spp. | Bacillus cereus (vegetative cells or spores) | Clostridium fringens (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           | Y                  |                      |                   | Y                  | Y                | Y                                | Y               | Y             | Y                | Y                                    |                  | Y             | Y                                    |             |  |   |  |
|   |                                     | Minced meat, meat preparations, carpaccio | Y                  |                      | Y                 | Y                  | Y                | Y                                | Y               | Y             | Y                | Y                                    |                  | Y             | Y                                    |             |  |   |  |
|   | Ready-to-cook (processed)           | Carcasses, swabs, rinsates                | Y                  |                      |                   | Y                  | Y                | Y                                | Y               | Y             | Y                | Y                                    |                  | Y             | Y                                    |             |  |   |  |
| 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  |
|   |                                     | 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  |
|   |                                     | Cobourgh ham, dry cured ham               | Y                  |                      |                   | Y                  | Y                | Y                                | Y               | Y             | Y                | Y                                    |                  | Y             | Y                                    |             | Y  | Y   | Y  |
| Canned meat (ambient stable)                              |                                     |   |                    |                      |                   |                    |                  |                                  |                 |               |                  |                                      |                  |               |                                      | 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 enterocolitica | 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      |  | Carcasses, meats, cuts                | Y                  |                      |                   | Y                  | Y                | Y                                | Y               | Y             | Y                |                                      |                  | Y             |                                      |             |  |  |  |
|   | Fresh meats (unprocessed)  | Carcasses, swabs, rinsates            | Y                  |                      | Y                 | Y                  | Y                | Y                                | Y               | Y             | Y                |                                      |                  | Y             |                                      |             |  |  |  |
|   |  | Minced meat, meat preparations        | Y                  |                      | Y                 | Y                  | Y                | Y                                | Y               | Y             | Y                |                                      |                  | Y             |                                      |             |  |  |  |
|   | Ready-to-cook products (processed)                                 | Seasoned chicken breasts              | Y                  |                      | Y                 | Y                  | Y                | Y                                | Y               | Y             | Y                |                                      |                  | Y             |                                      |             |  |  |  |
| Ready-to-eat, ready-to-reheat meat poultry 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  | Y  |
|   | Raw cured (smoked) ( $a_w > 0,92$ )                                | Smoked turkey filet                   | Y                  |                      | Y                 | Y                  | Y                | Y                                | Y               | Y             | Y                |                                      |                  | Y             |                                      |             | Y  | Y  | Y  |
|   | Canned (ambient stable)  | Canned poultry meat, canned duck pâté |                    |                      |                   |                    |                  |                                  |                 |               |                  |                                      |                  |               |                                      |             | Y  | Y  | Y  |
| Eggs and egg products (derivates)                   | Eggs (unprocessed)   | Shell eggs                            | Y                  |                      | 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  |  |



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 enterocolitica | Vibrio spp. | Bacillus cereus (vegetative cells or spores) | Clostridium fringens (vegetative cells or spores) | Clostridium botulinum (vegetative cells or spores) |
|---|--|---|--------------------|----------------------|-------------------|--------------------|------------------|----------------------------------|-----------------|---------------|------------------|--------------------------------------|------------------|---------------|--------------------------------------|-------------|--|---|--|
| 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           |  |   |  |
| Ready-to-eat, ready-to-re-heat 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  |
|   | Acidified and marinated fishery products                       | Roll herring, anchovy   | Y                  | Y                    | Y                 | Y                  | Y                | Y                                | Y               | Y             | Y                |                                      |                  |               |                                      |             | Y  |   |  |
| Ready-to-eat, ready-to-re-heat fishery products       | 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 enterocolitica | Vibrio spp. | Bacillus cereus (vegetative cells or spores) | Clostridium perfringens (vegetative cells or spores) | Clostridium botulinum (vegetative cells or spores) |  |
|--------------------------|---|--|--------------------|----------------------|-------------------|--------------------|------------------|----------------------------------|-----------------|---------------|------------------|--------------------------------------|------------------|---------------|--------------------------------------|-------------|--|--|--|--|
| Fresh produce and fruits | Cutready-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                                    | 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) | Campylobacter | (Pathogenic) Yersinia enterocolitica | Vibrio spp. | Bacillus cereus (vegetative cells or spores) | Clostridium fringens (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 and fruits | Y                  |                      |                   | Y                  | Y                | Y                                | Y               | Y             | Y                |                                      |               |                                      |             | Y  | Y   | Y  |
| Dried cereals, fruits, nuts, seeds and vegetables | Fermented/acidified vegetables                | Fermented cabbage, pickle                      | Y                  |                      |                   | Y                  | Y                | Y                                | Y               | Y             | Y                |                                      |               |                                      |             | Y  | Y   | Y  |
|   | 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  |   |  |
| Dried cereals, fruits, nuts, seeds and vegetables | 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 enterocolitica | 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  |  |  |
|                                   | Non-probiotic ingredients    | Dehydrated milk, dehydrated yoghurt, dehydrated berries              | 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  |  |  |
|                                   | Probiotic infant formula     | Whey-based (dairy), soy-based (vegetables) fortification formulation | Y                  |                      |                   | Y                  | Y                | Y                                | Y               |               |                  |                                      | Y                |               |                                      |             | Y  |  |  |
|                                   | Non-probiotic infant cereals | Infant cereals   | Y                  |                      | Y                 | Y                  | Y                | Y                                | Y               |               |                  |                                      |                  |               |                                      |             | Y  |  |  |
|                                   | Probiotic infant cereals     | Probiotic infant cereals   | 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 enterocolitica | Vibrio spp. | Bacillus cereus (vegetative cells or spores) | Clostridium fringens (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                |                                      |                  |               |                                      |             | Y  |   |  |
|  | Low moisture  | Crackers, breads, cookies   | Y                  |                      | Y                 |                    | Y                | Y                                | Y               |               |                  |                                      |                  |               |                                      |             | Y  |   |  |
|  | Dry and sugared low moisture ( $a_w < 0,85$ )                           | Cake, pralines, marzipan  | Y                  |                      | 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  |   |  |
| Multi-component foods or meal components     | Composite foods with substantial raw ingredients (excluding patisserie) | Refrigerated pasta salads, sandwiches, chocolate mousse, bavaoais | 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   | Y  |
|  | Ready-to-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  |

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 enterocolitica | Vibrio spp. | Bacillus cereus (vegetative cells or spores) | Clostridium fringens (vegetative cells or spores) | Clostridium botulinum (vegetative cells or spores) |
|--|---|--|--------------------|----------------------|-------------------|--------------------|------------------|----------------------------------|-----------------|---------------|------------------|--------------------------------------|------------------|---------------|--------------------------------------|-------------|--|---|--|
| Multi-component foods or meal components | Ready-to-(re)heat food: frozen                                      | Frozen fries, pizza, stuffed croissants          | Y                  |                      | Y                 | Y                  | Y                | Y                                | Y               | Y             | Y                |                                      |                  |               |                                      |             | Y  | Y   | Y  |
|  | 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  |
|  | Mayon-<br>naise-based deli salads (acid) with raw ingredients       | Raw vegetable salads with dressing               | Y                  | Y                    | Y                 | Y                  | Y                | Y                                | Y               | Y             | Y                | Y                                    |                  |               |                                      |             | Y  |   | Y  |
|  | Mayon-<br>naise-based deli salads (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  |   | 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 enterocolitica | Vibrio spp. | Bacillus cereus (vegetative cells or spores) | Clostridium fringens (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                        | Corn meal, soybean meal, vegetables                                    | Y                  |                      | Y                 | Y                  | Y                | Y                                | Y               |               |                  |                                      |                  |               |                                      |             | Y  |   |  |
|  | Other ingredients                               | Microbial products such as yeast extracts and 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  |
|  | 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  |   |  |
|  | 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  |
| 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  |

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 enterocolitica | Vibrio spp. | Bacillus cereus (vegetative cells or spores) | Clostridium perfringens (vegetative cells or spores) | Clostridium botulinum (vegetative cells or spores) |
|----------------------------------|--------------------------------------|---|--------------------|----------------------|-------------------|--------------------|------------------|----------------------------------|-----------------|---------------|------------------|--------------------------------------|------------------|---------------|--------------------------------------|-------------|--|--|--|
| 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, e.g. see References [17], [18] and [19].

NOTE 3 Unprocessed products, according to REGULATION (EC) No 852/2004<sup>[15]</sup>, 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. It is important to check with the appropriate authority in the jurisdiction.

EXAMPLE Fresh meat [see REGULATION (EC) No 853/2004<sup>[16]</sup>] 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 REGULATION (EC) No 852/2004<sup>[15]</sup> 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 can contain ingredients that are necessary for their manufacture or to give them specific characteristics. Different jurisdictions have different definitions for processed and unprocessed products. It is important to check with the appropriate authority in the jurisdiction.

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

NOTE 6 Poultry meat preparations include marinated and spiced meat cuts, chicken fillets and chicken wings, i.e. an 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 is 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 microorganisms of concern.

NOTE 9 Ready-to-cook (RTC) food is 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 is food designed by the producer or the manufacturer as suitable for direct human consumption without the need for cooking, but which can 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<sup>[14]</sup>.

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

NOTE 13 If specific sample sizes of a considered item are to be tested in a food category, e.g. 375 g ground beef, a complete technical protocol is tested 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 probiotics, the items containing probiotics are selected and validated as a full category.

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

## Annex B (informative)

### Guidance on how to choose challenging (food) item(s) for (food) item verification

#### B.1 General

It is important to select (food) items that are representative of those encountered in the user laboratory. This annex is specifically applicable to (food) item verification.

The (food) item can affect the outcome of an analysis. The composition of the food, its background microbiota and other contaminants can interfere with the test method and invalidate the result. It is therefore expected that the user laboratory will ensure that the method is fit-for-purpose for the (food) items of interest to them. Even if a method is validated for a broad range of foods, not all (food) items have been tested during validation. Therefore, it is important that the user laboratory demonstrates that the method is applicable to the (food) items tested in the laboratory. As only one (food) item is required from each (food) category, it is then important to perform the (food) item verification with the most challenging one.

#### B.2 Matrix effects to consider

##### B.2.1 Microbial characteristics

Unless the food has been sterilized (e.g. canned food), (food) items can contain (naturally or intentionally introduced in the manufacturing process) microorganisms, which can be categorized as:

- technological microbiota such as microbial cultures and probiotics, e.g. fermented and cured foods, probiotic food products inoculated with a level of microorganisms from  $10^6$  cfu/g to  $10^9$  cfu/g;
- high background microbiota samples, e.g. poultry minced meat, faecal samples, raw milk;
- spoilage microorganisms: the presence of this native microbiota can influence the recovery and growth of the target microorganism.

##### B.2.2 Physical and chemical characteristics

The following physical and chemical parameters are known to affect the recovery of microorganisms and/or the method performance:

- composition, e.g. high fat content, lecithin, thickener, nutrient content;
- pH, e.g. pH < 4 to 5 (e.g. beverages, sauces);
- oxidation reduction potential;
- water activity, e.g.  $a_w < 0,85$  (flour, low moisture foods);
- antimicrobial constituents and growth inhibitors, e.g. polyphenols, enzymes, molecular inhibitors;
- physical structure of the food, e.g. viscosity, solubility;
- colour, e.g. food dyes.

### B.2.3 Food process induced characteristics

The manufacturing process of the considered matrix can often have a treatment step (e.g. heating, high pressure process) that could result in injuring microbial cells. This affects the viability and the culturability of the cells and therefore affects the recovery of the microorganism of concern.

### B.3 Selection of (food) items for verification

The microbial, physical, chemical and process induced characteristics mentioned above can be found in (food) items amongst all (food) categories described in [Annex A](#).

When selecting a challenging (food) item from each category, the user laboratory shall choose, among the (food) items tested in its laboratory, those (food) items which show one or more of the challenging characteristics. For instance, a (food) item having a combination of two challenging characteristics (e.g. pH +  $a_w$ ) is preferable as this represents the worst-case scenario.

For a broad range of foods scope, a minimum of five food items selected from five food categories is required (see 4.4 for details). When possible, each of the five food items shall have a different challenging characteristic or a combination of these characteristics in order to cover different cases. [Table B.1](#) provides an example.

**Table B.1 — Example of (food) items and its characteristics**

| Category | Item | Challenging characteristic        |
|----------|------|-----------------------------------|
| 1        | 1    | pH                                |
| 2        | 2    | Viscosity                         |
| 3        | 3    | Fat content                       |
| 4        | 4    | High background microbiota and pH |
| 5        | 5    | Polyphenol                        |

The selection of (food) items also depends on the principle of the method that can guide the user laboratory in the selection of the (food) items. [Table B.2](#) gives examples of food characteristics that, depending on the method principle, can affect the performance of the method.

**Table B.2 — Examples of characteristics of (food) items that can affect performance, categorized by method principles**

| Method principle | High number of competitive (micro)organisms |                                      | Physical characteristics |       |                      |        | Chemical compound   |        |             |                       |
|------------------|---|--------------------------------------|--------------------------|-------|----------------------|--------|---------------------|--------|-------------|-----------------------|
|                  | Technological microbiota                    | High background microbiota, spoilage | pH                       | $a_w$ | Solubility/viscosity | Colour | Vanillin, salt, ... | Enzyme | Polyphe-nol | Molec-ular inhibi-tor |
| Cultural method  | x   | x                                    | x                        | x     | x                    | x      | x                   | x      | x           |                       |
| Immuno-enzymatic | x   | x                                    | x                        | x     | x                    | x      | x                   | x      | x           |                       |
| Molecular test   | x   | x                                    | x                        | x     | x                    | x      | x                   | x      | x           | x                     |
| Flow cytometry   | x   | x                                    | x                        | x     | x                    | x      | x                   |        |             |                       |
| ATP              | x   | x                                    | x                        | x     | x                    | x      | x                   |        |             |                       |

## Annex C (informative)

### Qualitative method verification — Example

#### C.1 Method to be verified

The user laboratory wishes to verify ISO 6579-1. The  $LOD_{50}$  for the method, obtained from review of the validation data for the method, was found to be 2,5 cfu/test portion.

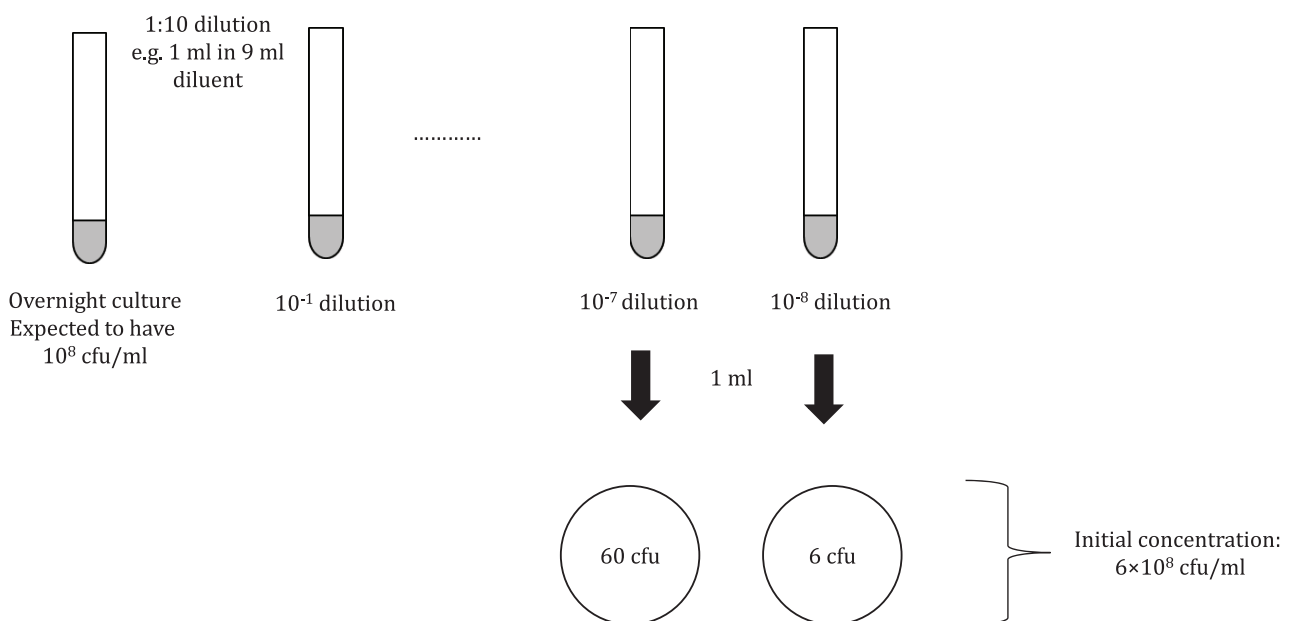
#### C.2 Preparation for verification

A preliminary enumeration of the microbial suspension that will be used for the method verification is performed to provide an estimate of the concentration of the inoculum that will be used. The procedure is as follows.

- Prepare a culture of the microorganism under appropriate conditions (medium, temperature, time of incubation) and check the purity. If this fails, re-isolate, select and identify the pure colonies and restart subculture. Follow the procedures specified in ISO 11133:2014, 5.4.
- Perform the enumeration of the culture on a non-selective medium to determine the concentration in cfu/ml. For decimal dilution and enumeration details, follow ISO 7218 and ISO 6887-1.

The result of the enumeration will be used as the starting point for the dilutions to inoculate the test portions. In this example, the initial concentration of the overnight culture was previously determined to be  $6 \times 10^8$  cfu/ml (see [Figure C.1](#)).

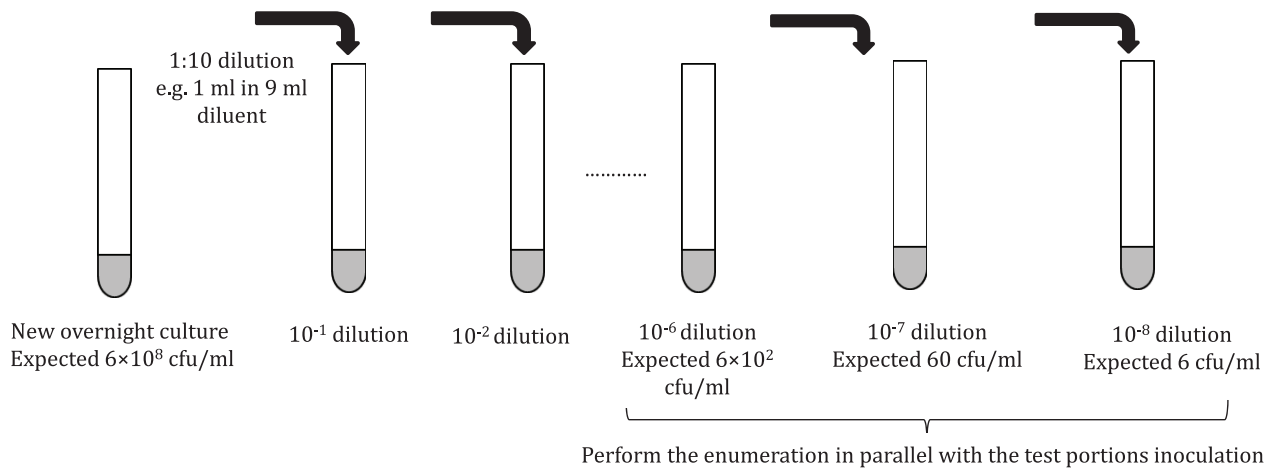
**NOTE** Overnight culture is intended to obtain microorganisms in a stationary phase of growth. The culture conditions are modified if the target microorganism requires longer incubation times.



**Figure C.1 — Example of preliminary determination of the inoculum level**

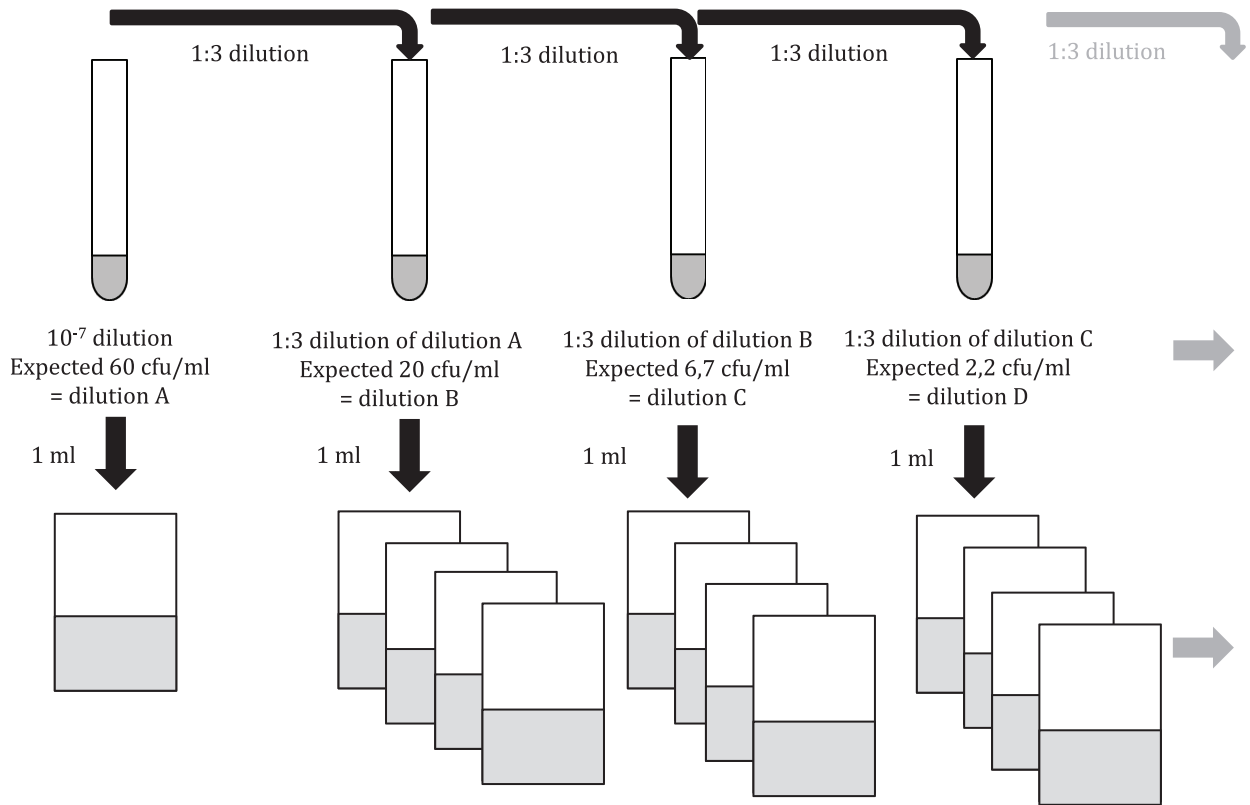
### C.3 Verification

Using the previously determined concentration ( $6 \times 10^8$  cfu/ml for this example), prepare dilutions to cover target contamination levels (see [Figure C.2](#)). The dilutions selected for inoculation are based on the validation  $LOD_{50}$  (2,5 cfu/test portion) from the validation report for ISO 6579-1.

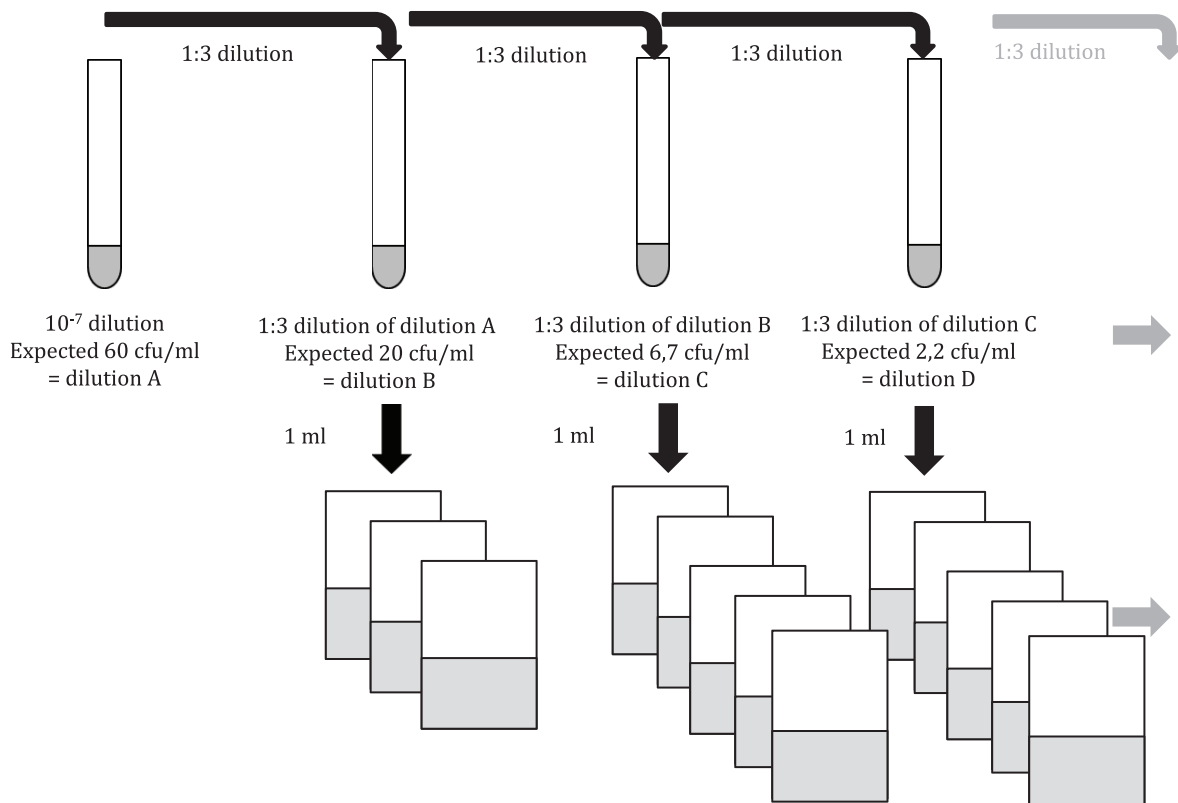


**Figure C.2 — Example of the preparation of the inoculum**

- In theory, three contamination levels (high, intermediate and low) and one blank level are required using protocol 1. However, as the actual count is not known at the time of inoculation, it is recommended that a “range” of serial dilutions, that would include the three target contamination levels, be performed (see [Figure C.3](#)). Inoculate 1 ml of the selected dilution into the initial suspension of the individual test portions.
- In the example shown in [Figure C.3](#), the expected high-level inoculum is prepared using the  $10^{-7}$  dilution, in case the new overnight culture has a different concentration than expected.
- [Figure C.4](#) shows the process for the inoculation of test portions if protocol 2 was used for the verification.
- [Figure C.5](#) shows the process for inoculation of test portions if protocol 3 was used. The reference material is prepared to ensure an inoculum of 3 cfu to 5 cfu/test portion.
- Perform the method to be verified.
- Record the positive and negative results.
- Calculate the actual contamination based on the enumerated result (see [5.4.2](#)).
- In the example shown in [Figure C.6](#), the concentration of the new overnight culture is  $5,4 \times 10^8$  cfu/ml and not  $6 \times 10^8$  cfu/ml.



**Figure C.3 — Example of the inoculation of the test portions when using protocol 1**



**Figure C.4 — Example of the inoculation of the test portions when using protocol 2**

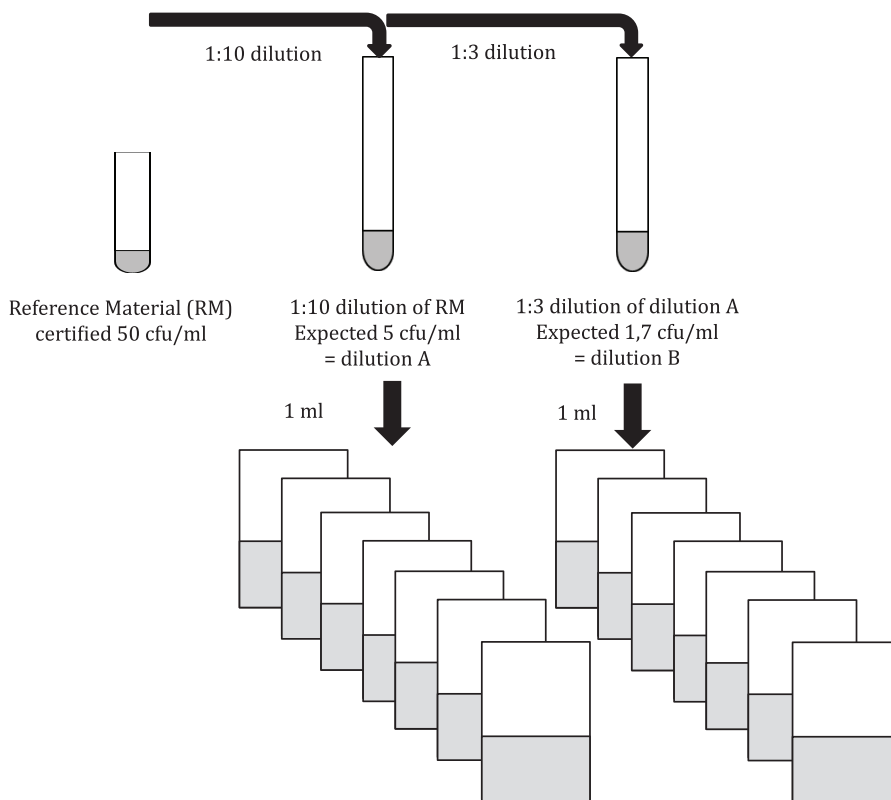


Figure C.5 — Example of the inoculation of the test portions when using protocol 3

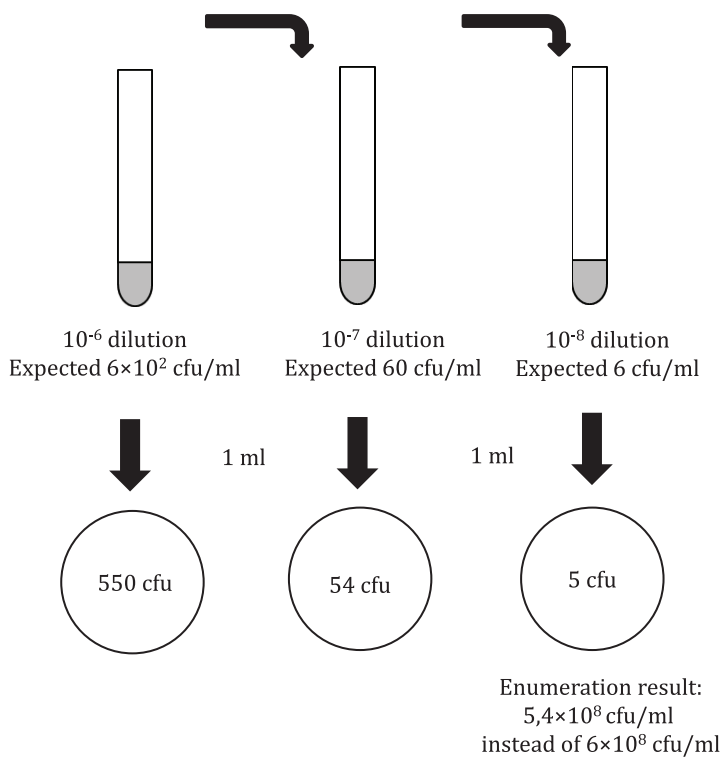
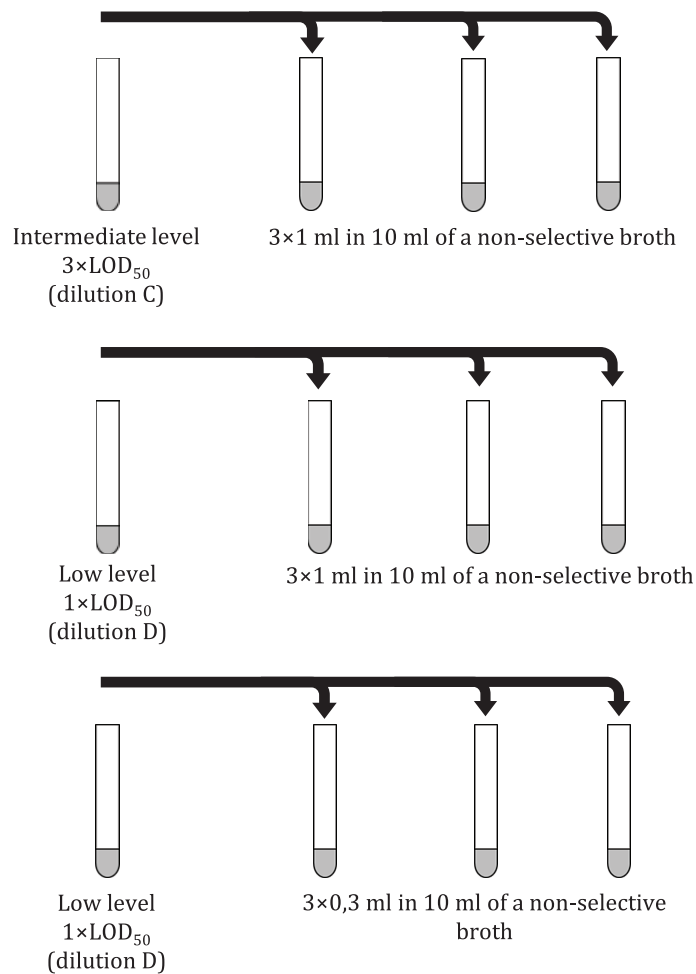


Figure C.6 — Example of the enumeration of the actual inoculum level

- Alternatively, an MPN approach can be used to determine the level of contamination of the inoculum.
- For protocol 1 and 2, the MPN is performed using  $3 \times 1$  ml of dilutions C and D and  $3 \times 0,3$  ml of dilution D. See also [Figure C.7](#).
- For protocol 3, the MPN is performed using  $3 \times 3$  ml,  $3 \times 1$  ml and  $3 \times 0,3$  ml of the inoculum. See also [Figure C.8](#).

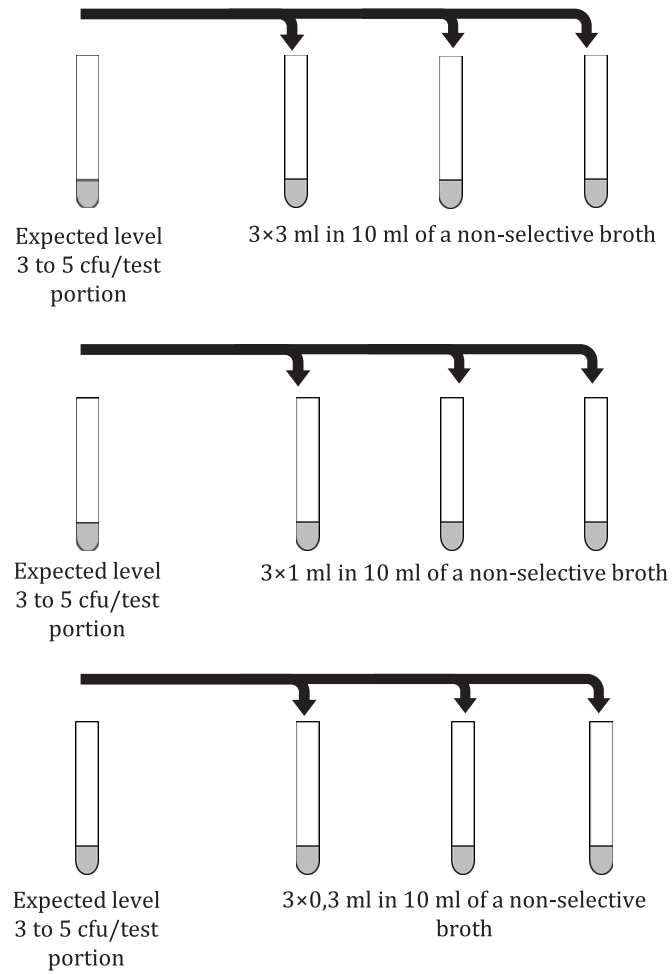
The results of the MPN (expressed as MPN/ml of the lowest inoculum level) are determined using [Table C.1](#).

Use the MPN results obtained and refer to [Table 7](#) (for protocol 1) or [Table 9](#) (for protocol 2) to determine the  $eLOD_{50}$ .



**Figure C.7 — MPN determination of the inoculum level for protocols 1 and 2**





**Figure C.8 — MPN determination of the inoculum level for protocol 3**

Table C.1 — MPN table for the calculation of the inoculum level using protocols 1, 2 or 3

| Number positive results for inoculum volume (ml) |                 |                 |                   | MPN per ml<br>of dilution D<br>(protocols 1 and 2)<br>or of inoculum<br>(protocol 3) | Rarity<br>category <sup>a</sup> |
|--|-----------------|-----------------|-------------------|--|---------------------------------|
| Protocol 1                                       | 1 ml dilution C | 1 ml dilution D | 0,3 ml dilution D |  |                                 |
| Protocol 2                                       | 1 ml dilution C | 1 ml dilution D | 0,3 ml dilution D |  |                                 |
| Protocol 3                                       | 3 ml inoculum   | 1 ml inoculum   | 0,3 ml inoculum   |  |                                 |
|  | 3               | 3               | 3                 | ∞  | 1                               |
|  | 3               | 3               | 2                 | 4,1  | 1                               |
|  | 3               | 3               | 1                 | 2,4  | 1                               |
|  | 3               | 3               | 0                 | 1,5  | 1                               |
|  | 3               | 2               | 3                 | 2,5  | 1                               |
|  | 3               | 2               | 2                 | 1,8  | 1                               |
|  | 3               | 2               | 1                 | 1,3  | 1                               |
|  | 3               | 2               | 0                 | 0,9  | 1                               |
|  | 3               | 1               | 3                 | 1,5  | 2                               |
|  | 3               | 1               | 2                 | 1,1  | 1                               |
|  | 3               | 1               | 1                 | 0,8  | 1                               |
|  | 3               | 1               | 0                 | 0,6  | 1                               |
|  | 3               | 0               | 3                 | 1,0  | 3                               |
|  | 3               | 0               | 2                 | 0,8  | 1                               |
|  | 3               | 0               | 1                 | 0,6  | 1                               |
|  | 3               | 0               | 0                 | 0,4  | 1                               |
|  | 2               | 3               | 3                 | 1,3  | 3                               |
|  | 2               | 3               | 2                 | 1,1  | 2                               |
|  | 2               | 3               | 1                 | 0,9  | 1                               |
|  | 2               | 3               | 0                 | 0,7  | 1                               |
|  | 2               | 2               | 3                 | 1,0  | 3                               |
|  | 2               | 2               | 2                 | 0,8  | 1                               |
|  | 2               | 2               | 1                 | 0,7  | 1                               |
|  | 2               | 2               | 0                 | 0,5  | 1                               |
|  | 2               | 1               | 3                 | 0,8  | 3                               |
|  | 2               | 1               | 2                 | 0,6  | 1                               |
|  | 2               | 1               | 1                 | 0,5  | 1                               |
|  | 2               | 1               | 0                 | 0,3  | 1                               |
|  | 2               | 0               | 3                 | 0,6  | 3                               |
|  | 2               | 0               | 2                 | 0,5  | 2                               |
|  | 2               | 0               | 1                 | 0,3  | 1                               |
|  | 2               | 0               | 0                 | 0,2  | 1                               |
|  | 1               | 3               | 3                 | 0,8  | 3                               |
|  | 1               | 3               | 2                 | 0,7  | 3                               |
|  | 1               | 3               | 1                 | 0,5  | 2                               |
|  | 1               | 3               | 0                 | 0,4  | 2                               |
|  | 1               | 2               | 3                 | 0,6  | 3                               |
|  | 1               | 2               | 2                 | 0,5  | 2                               |
|  | 1               | 2               | 1                 | 0,4  | 1                               |

<sup>a</sup> If the result of the rarity category is 3, the MPN combination is very unlikely to occur. In this case, the experiment shall be repeated.

Table C.1 (continued)

| Number positive results for inoculum volume (ml) |                 |                 |                   | MPN per ml of dilution D (protocols 1 and 2) or of inoculum (protocol 3) | Rarity category <sup>a</sup> |
|--|-----------------|-----------------|-------------------|--|------------------------------|
| Protocol 1                                       | 1 ml dilution C | 1 ml dilution D | 0,3 ml dilution D |  |                              |
| Protocol 2                                       | 1 ml dilution C | 1 ml dilution D | 0,3 ml dilution D |  |                              |
| Protocol 3                                       | 3 ml inoculum   | 1 ml inoculum   | 0,3 ml inoculum   |  |                              |
|  | 1               | 2               | 0                 | 0,3  | 1                            |
|  | 1               | 1               | 3                 | 0,5  | 3                            |
|  | 1               | 1               | 2                 | 0,4  | 2                            |
|  | 1               | 1               | 1                 | 0,3  | 1                            |
|  | 1               | 1               | 0                 | 0,2  | 1                            |
|  | 1               | 0               | 3                 | 0,4  | 3                            |
|  | 1               | 0               | 2                 | 0,3  | 2                            |
|  | 1               | 0               | 1                 | 0,2  | 1                            |
|  | 1               | 0               | 0                 | 0,1  | 1                            |
|  | 0               | 3               | 3                 | 0,6  | 3                            |
|  | 0               | 3               | 2                 | 0,5  | 3                            |
|  | 0               | 3               | 1                 | 0,4  | 3                            |
|  | 0               | 3               | 0                 | 0,3  | 3                            |
|  | 0               | 2               | 3                 | 0,4  | 3                            |
|  | 0               | 2               | 2                 | 0,4  | 3                            |
|  | 0               | 2               | 1                 | 0,3  | 2                            |
|  | 0               | 2               | 0                 | 0,2  | 1                            |
|  | 0               | 1               | 3                 | 0,3  | 3                            |
|  | 0               | 1               | 2                 | 0,3  | 3                            |
|  | 0               | 1               | 1                 | 0,2  | 2                            |
|  | 0               | 1               | 0                 | 0,1  | 1                            |
|  | 0               | 0               | 3                 | 0,2  | 3                            |
|  | 0               | 0               | 2                 | 0,2  | 3                            |
|  | 0               | 0               | 1                 | 0,1  | 1                            |
|  | 0               | 0               | 0                 | 0,0  | 1                            |

<sup>a</sup> If the result of the rarity category is 3, the MPN combination is very unlikely to occur. In this case, the experiment shall be repeated.

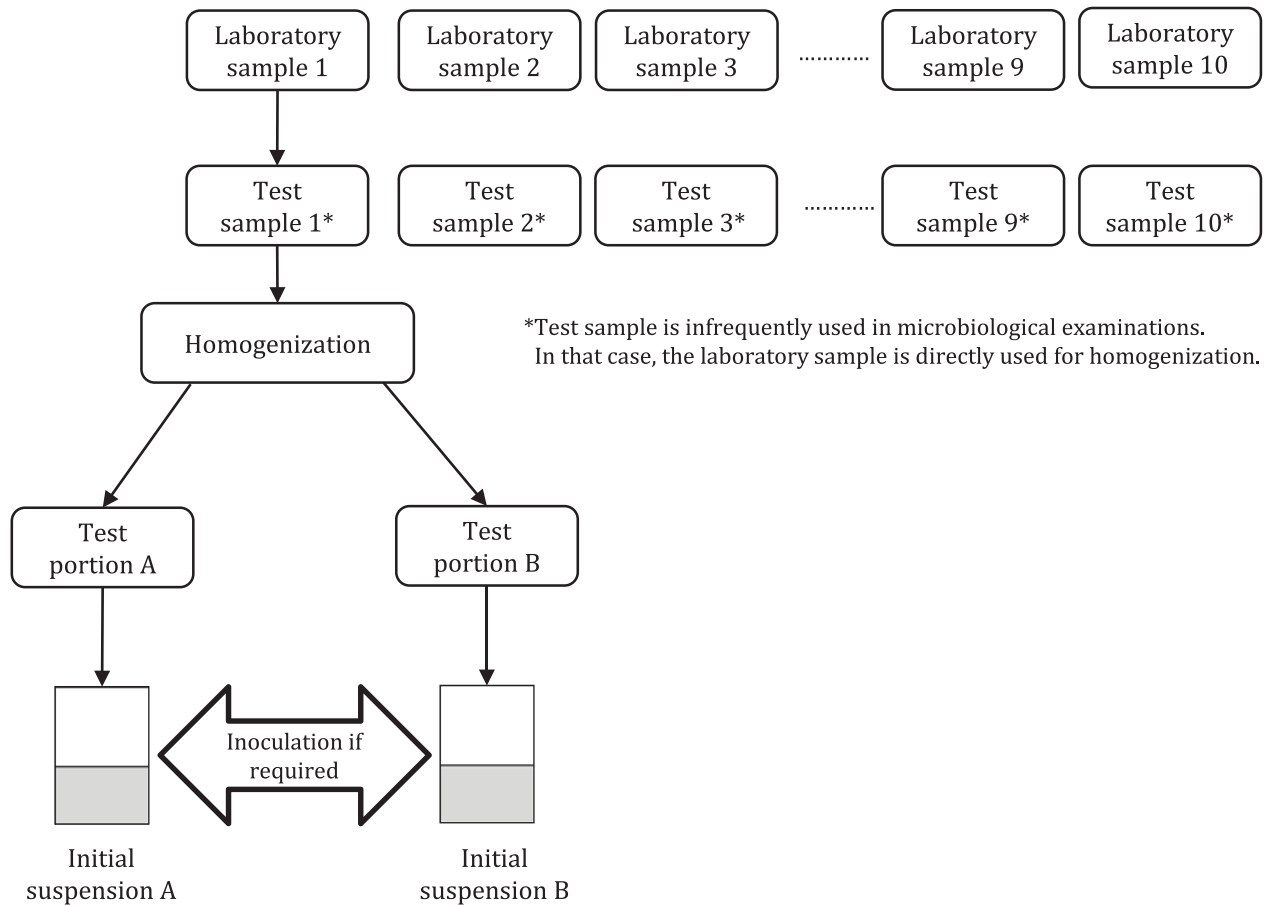
## Annex D (informative)

### Quantitative method verification — Example

#### D.1 Determination of intralaboratory reproducibility standard deviation — Example

The contamination levels used shall be representative of the range of the natural contamination found in the samples tested in the user laboratory.

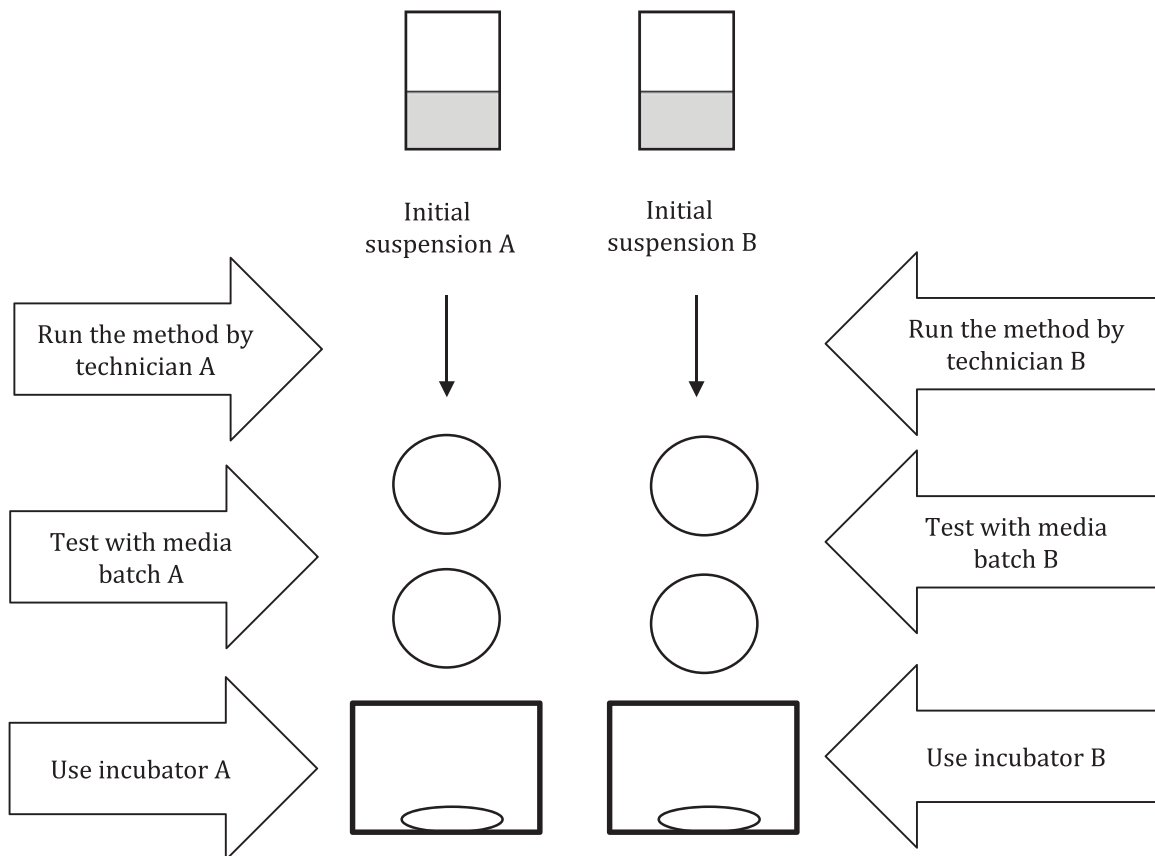
This annex describes the preparation of the laboratory samples and test portions (see also [Figure D.1](#)).



**Figure D.1 — Preparation of samples for intralaboratory reproducibility standard deviation determination**

- For one (food) item, e.g. tiramisu, a minimum of 10 laboratory samples are collected. Each laboratory sample is thoroughly homogenized (in order to exclude contributions from heterogeneity within the laboratory sample/test sample) and divided into two test portions.
- If no (food) item with natural contamination is available (or contamination < 10 cfu/g), inoculate the initial suspension with a selected strain. If artificial contamination is used, enumerate, in parallel, the inoculum suspension (used to inoculate the initial suspension) using a non-selective medium.

- Each initial suspension is then analysed using the method protocol. Test conditions used in the analysis of the test portions A and B shall be different in as many ways as possible within the scope of validation (e.g. technicians, batches of culture media and reagents, and, when relevant, apparatus and days, if the inoculated laboratory sample can be shown to be sufficiently stable). Different strains can also be used for different laboratory samples but not for the test portions A and B. See [Figure D.2](#).
- From the results obtained, calculate the intralaboratory reproducibility standard deviation  $S_{IR}$  (see [6.1.6](#) and [6.1.7](#)).



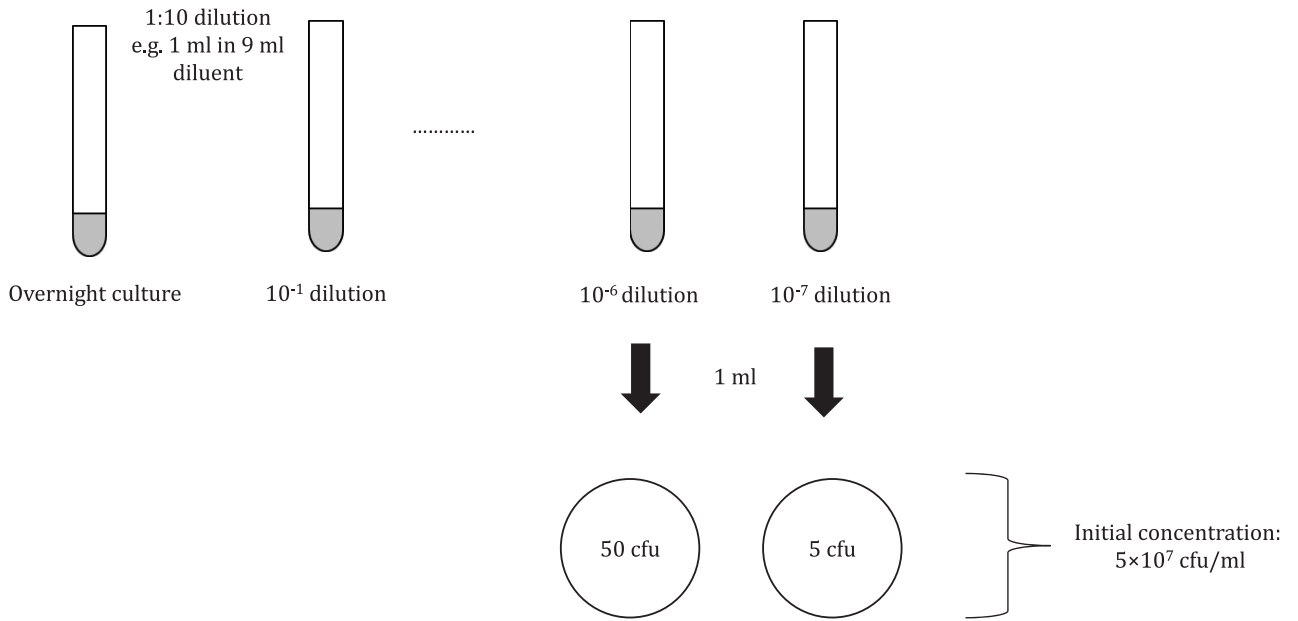
**Figure D.2 — Suggestions for variations for intralaboratory reproducibility standard deviation determination**

## D.2 Determination of eBias — Example

### D.2.1 Preparation for verification

A preliminary enumeration of the microbial suspension that will be used for the method verification is performed to provide an estimate of the concentration of the inoculum that will be used (see [Figure D.3](#)).

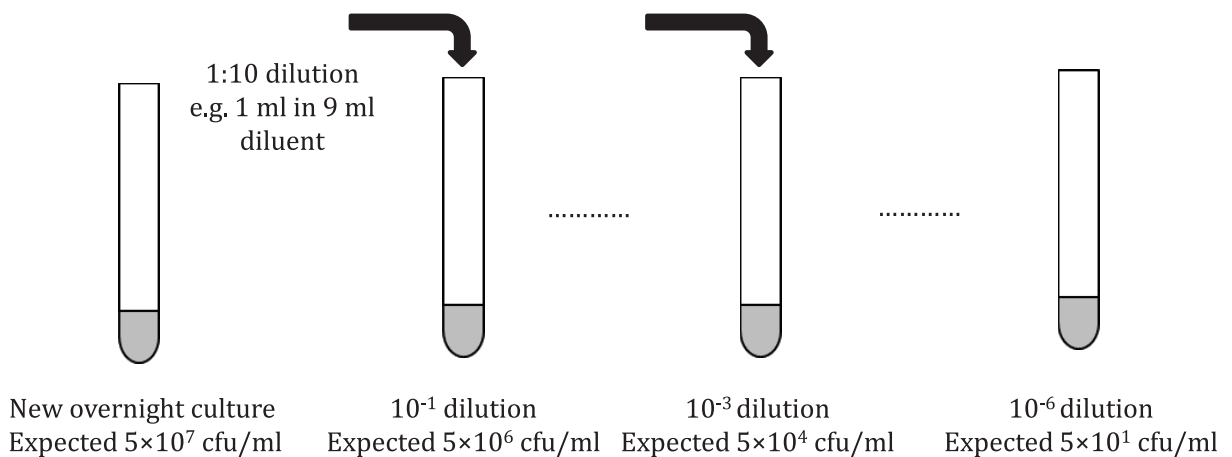
Prepare a culture of the microorganism under appropriate conditions (medium, temperature, time of incubation) and check the purity. If this fails, re-isolate, select and identify the pure colonies and restart subculture. Follow the procedures specified in ISO 11133:2014, 5.4.



**Figure D.3 — Example of the preliminary determination of the inoculum level**

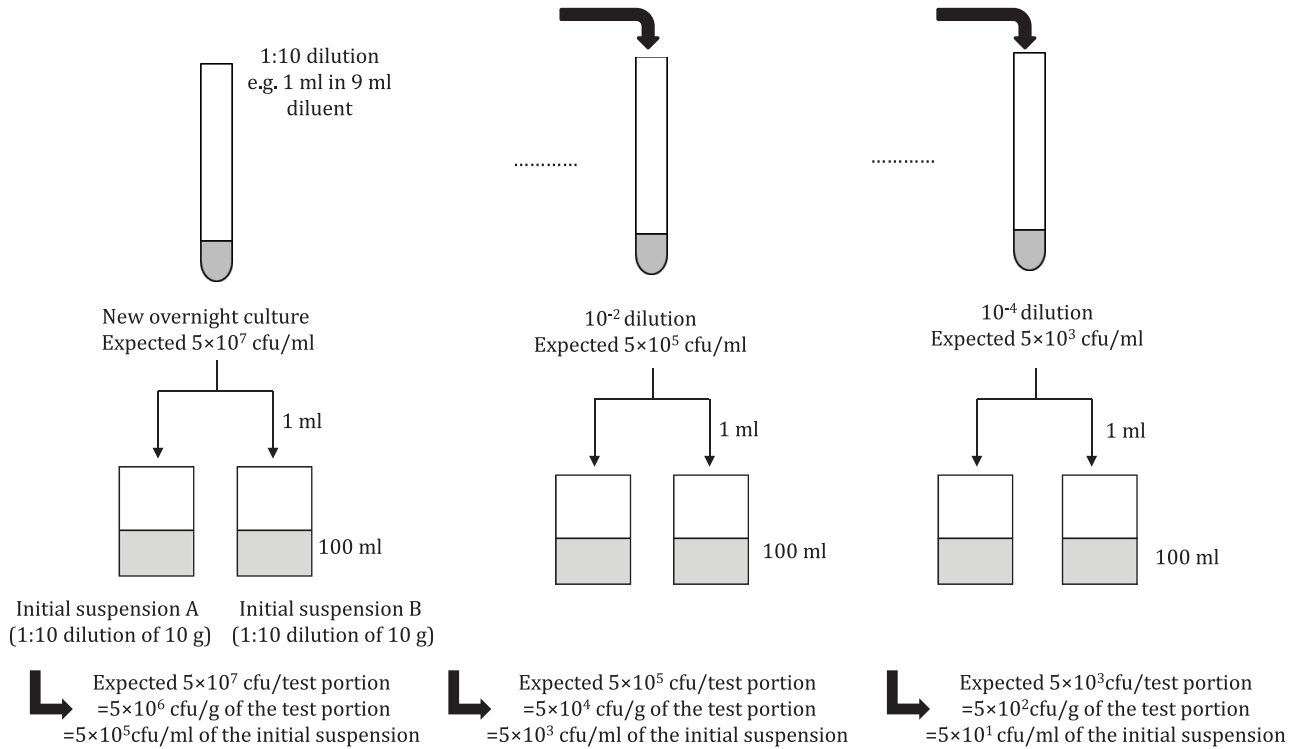
### D.2.2 Verification

- Repeat the culture, taking into account the concentration previously determined.
- A user laboratory usually expects to find between  $10^2$  cfu/g and  $10^6$  cfu/g in submitted samples. For this eBias determination, the inoculation of the initial suspension to the levels of  $10^1$  cfu/ml,  $10^3$  cfu/ml and  $10^5$  cfu/ml is required. Based on the results of the previous enumeration test, appropriate dilutions are made covering the presumed wide range of the inoculum of  $10^1$  cfu/ml to  $10^7$  cfu/ml (see [Figure D.4](#)).



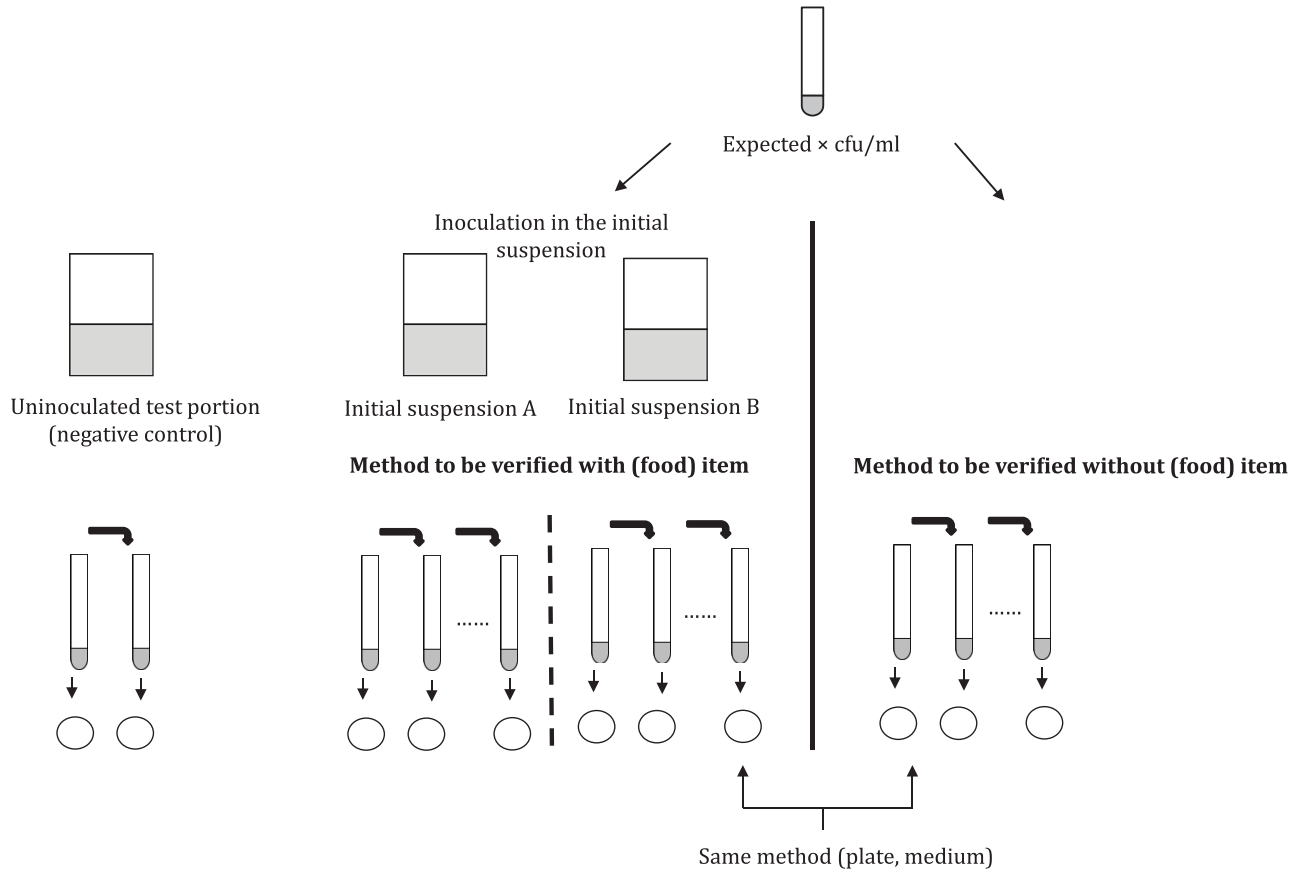
**Figure D.4 — Example of the preparation of the inoculum**

- Inoculate 1 ml of each dilution into duplicate initial suspensions to give final concentrations of:  $10^1$  cfu/ml,  $10^3$  cfu/ml and  $10^5$  cfu/ml (see [Figure D.5](#)). Additional dilutions (to cover the wider range from  $10^0$  cfu/ml to  $10^6$  cfu/ml of the initial suspensions) can be prepared so that the three levels required for comparison studies are more likely to be included.



**Figure D.5 — Example of the inoculation of the test portions**

- Enumerate using the method to be verified (see [Figure D.6](#)):
  - the uninoculated test portion;
  - the inoculated test portions (A and B);
  - the inoculum suspension (used to prepare the initial suspension).



**Figure D.6 — Example of quantitative method verification (eBias) using artificial contamination**

- Compare the results of the artificially contaminated (food) item to that of the inoculum suspension tested with the same method (see [Table 13](#)). The results of the negative control (uninoculated test portion) can provide useful information when a root cause analysis is required (see [6.2.7](#)).



## Annex E (informative)

### Validated alternative confirmation or typing method verification — Examples

#### E.1 Alternative confirmation method verification — Example

This subclause shows an example of the verification of a validated alternative confirmation method to the species level for *Listeria monocytogenes*, which was validated in accordance with ISO 16140-6 (also see the example on the validation in ISO 16140-6:2019, Annex B). The reference confirmation method is the ISO 11290 series for detection and enumeration of *Listeria monocytogenes*. Isolation is on Agar *Listeria* according to Ottaviani and Agosti, followed by the confirmation tests for haemolysis and fermentation of L-rhamnose and D-xylose as a minimum. The validated alternative confirmation method is a commercially available PCR-test, directly applied on colonies isolated on Agar *Listeria* according to Ottaviani and Agosti.

Select a variety of five target strains of *Listeria monocytogenes*.

Select a variety of five non-target strains, at least including a *Listeria innocua* strain.

Test the selected inclusivity and exclusivity strains according to the alternative confirmation method being verified.

The verification results are tabulated in [Table E.1](#).

**Table E.1 — Overview of verification results for the validated alternative confirmation method**

| Tested strains | Inclusivity/exclusivity | Characteristics of the strain  | Expected confirmation result <sup>a</sup> | Result of the confirmation method being verified <sup>a</sup> | Interpretation <sup>b</sup> |
|----------------|-------------------------|--|---|---|-----------------------------|
| 1              | Inclusivity             | <i>L. monocytogenes</i><br>(serotype 4b)<br>WDCM 00021<br>Human isolate        | +   | +   | Agreement                   |
| 2              | Inclusivity             | <i>L. monocytogenes</i><br>(serotype 1/2a)<br>WDCM 00109<br>Guinea-pig isolate | +   | +   | Agreement                   |
| 3              | Inclusivity             | <i>L. monocytogenes</i><br>(genotype IV)<br>12MOB112LM<br>Meat isolate         | +   | +   | Agreement                   |

<sup>a</sup> +: positive result, indicating the strain is confirmed to be the target;

–: negative result, indicating the strain is not confirmed to be the target.

<sup>b</sup> Agreement or deviation between the expected result and the result of the confirmation method being verified.

<sup>c</sup> Not able to grow on Agar *Listeria* according to Ottaviani and Agosti, and therefore tested from a non-selective agar plate.

Table E.1 (continued)

| Tested strains | Inclusivity/exclusivity | Characteristics of the strain   | Expected confirmation result <sup>a</sup> | Result of the confirmation method being verified <sup>a</sup> | Interpretation <sup>b</sup> |
|----------------|-------------------------|---|---|---|-----------------------------|
| 4              | Inclusivity             | <i>L. monocytogenes</i><br>(genotype II)<br>12MOB118LM<br>Dairy isolate | +   | +   | Agreement                   |
| 5              | Inclusivity             | <i>L. monocytogenes</i><br>Field strain LM01<br>Smoked salmon isolate   | +   | +   | Agreement                   |
| 6              | Exclusivity             | <i>L. innocua</i><br>WDCM 00017   | -   | -   | Agreement                   |
| 7              | Exclusivity             | <i>L. ivanovii</i><br>WDCM 00018  | -   | -   | Agreement                   |
| 8              | Exclusivity             | <i>Bacillus cereus</i><br>WDCM 00001 <sup>c</sup>                       | -   | -   | Agreement                   |
| 9              | Exclusivity             | <i>Enterococcus faecalis</i><br>WDCM 00009 <sup>c</sup>                 | -   | -   | Agreement                   |
| 10             | Exclusivity             | <i>Staphylococcus aureus</i><br>WDCM 00034 <sup>c</sup>                 | -   | -   | Agreement                   |

<sup>a</sup> +: positive result, indicating the strain is confirmed to be the target;  
-: negative result, indicating the strain is not confirmed to be the target.

<sup>b</sup> Agreement or deviation between the expected result and the result of the confirmation method being verified.

<sup>c</sup> Not able to grow on Agar *Listeria* according to Ottaviani and Agosti, and therefore tested from a non-selective agar plate.

## E.2 Alternative typing method verification — Example

This subclause shows an example of the verification of an alternative typing method to the *Salmonella* serovar level, which was validated in accordance with ISO 16140-6 (also see the example on the validation in ISO 16140-6:2019, Annex C). The reference *Salmonella* serotyping method is ISO/TR 6579-3. The alternative serotyping method is a commercially available PCR-based test. The alternative serotyping method claims to be able to serotype the following 15 *Salmonella enterica* subsp. *enterica* serovars: *S. Agona*, *S. Anatum*, *S. Brandenburg*, *S. Enteritidis*, *S. Hadar*, *S. Heidelberg*, *S. Indiana*, *S. Infantis*, *S. Mbandaka*, *S. Montevideo*, *S. Lexington*, *S. Livingstone*, *S. Senftenberg*, *S. Typhimurium* and *S. Virchow*.

Select a variety of five target strains.

Select a variety of five non-target strains.

Test the selected inclusivity and exclusivity strains according to the alternative typing method being verified.

The verification results are tabulated in [Table E.2](#).

Table E.2 — Overview of verification results for the alternative typing method

| Tested strains | Inclusivity/exclusivity | Characteristics of the strain   | Expected typing result <sup>a</sup> | Result of the typing method being verified <sup>a</sup> | Interpretation <sup>b</sup> |
|----------------|-------------------------|---|-------------------------------------|---|-----------------------------|
| 1              | Inclusivity             | <i>S. Anatum</i><br>(3,{10}{15}{15,34}:e,h:1,6)<br>Field strain Salm01<br>Dairy product isolate | <i>S. Anatum</i>                    | <i>S. Anatum</i>  | Agreement                   |
| 2              | Inclusivity             | <i>S. Enteritidis</i><br>(1,9,12:g,m:-)<br>WDCM 00030   | <i>S. Enteritidis</i>               | <i>S. Enteritidis</i>                                   | Agreement                   |
| 3              | Inclusivity             | <i>S. Hadar</i><br>(6,8:z <sub>10</sub> :e,n,x)<br>Field strain Salm02<br>Poultry meat isolate  | <i>S. Hadar</i>                     | <i>S. Hadar</i>   | Agreement                   |
| 4              | Inclusivity             | <i>S. Infantis</i><br>(6,7,14:r:1,5)<br>Field strain Salm03<br>Egg product isolate              | <i>S. Infantis</i>                  | <i>S. Infantis</i>                                      | Agreement                   |
| 5              | Inclusivity             | <i>S. Typhimurium</i><br>(1,4,[5],12:i:1,2)<br>WDCM 00031<br>Chicken tissue isolate             | <i>S. Typhimurium</i>               | <i>S. Typhimurium</i>                                   | Agreement                   |
| 6              | Exclusivity             | <i>S. Panama</i><br>(1,9,12:l,v:1,5)<br>Field strain Salm04<br>Human isolate                    | –                                   | –   | Agreement                   |
| 7              | Exclusivity             | <i>S. Saintpaul</i><br>(1,4,[5],12:e,h:1,2)<br>Field strain Salm05<br>Turkey isolate            | –                                   | –   | Agreement                   |
| 8              | Exclusivity             | <i>Citrobacter freundii</i><br>WDCM 00006   | –                                   | –   | Agreement                   |
| 9              | Exclusivity             | <i>Escherichia coli</i><br>WDCM 00012   | –                                   | –   | Agreement                   |
| 10             | Exclusivity             | <i>Hafnia alvei</i><br>WDCM 00095   | –                                   | –   | Agreement                   |

<sup>a</sup> –: negative result, indicating the strain is not confirmed to be one of the 15 target *Salmonella* serovars.  
<sup>b</sup> Agreement or deviation between the expected result and the result of the typing method being verified.

## Annex F (normative)

### Protocol for the verification of non-validated reference methods in a single laboratory

#### F.1 General

For non-validated reference methods, only (food) item verification is undertaken for verification of qualitative (detection) methods and quantitative methods. Implementation verification does not apply to non-validated reference methods, because there is no validation data available for comparison.

The verification focuses on (food) items that are within the scope of the reference method and within the scope of laboratory application.

The technical rules for performing (food) item verification are given in [F.5](#) for qualitative methods and [F.6](#) for quantitative methods.

#### F.2 (Food) item verification

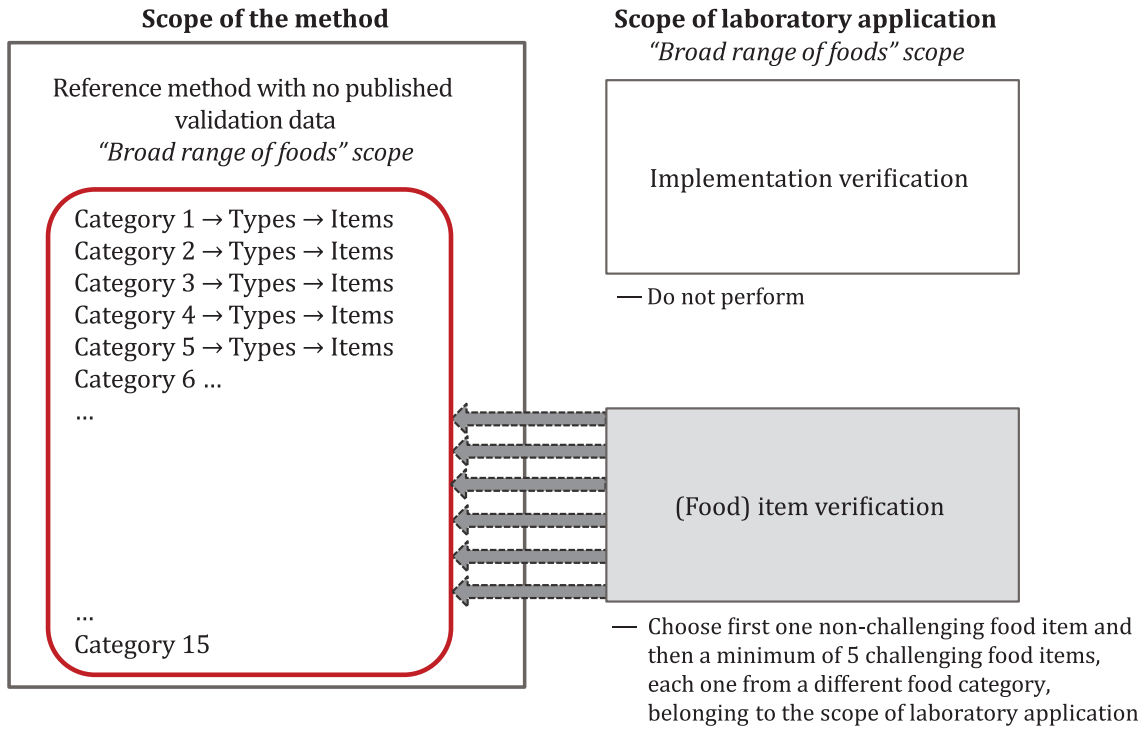
The (food) item verification aims to demonstrate the competence of the user laboratory to perform the non-validated reference method with (food) items that are tested in the user laboratory.

The user laboratory shall:

- select one non-challenging (food) item from a (food) category claimed in the scope of the reference method, that is also a (food) category tested within the scope of laboratory application of the user laboratory;
- select a minimum of one challenging (food) item from each (food) category claimed in the scope of the reference method, that is also a (food) category which is tested within the scope of laboratory application of the user laboratory;
- use these (food) items and the sample size as used in the reference method (or a smaller sample size if routinely used in the user laboratory) to perform the (food) item verification.

#### F.3 Requirements for (food) item verification

[Figure F.1](#) shows the case of a “broad range of foods” scope with no validation data.

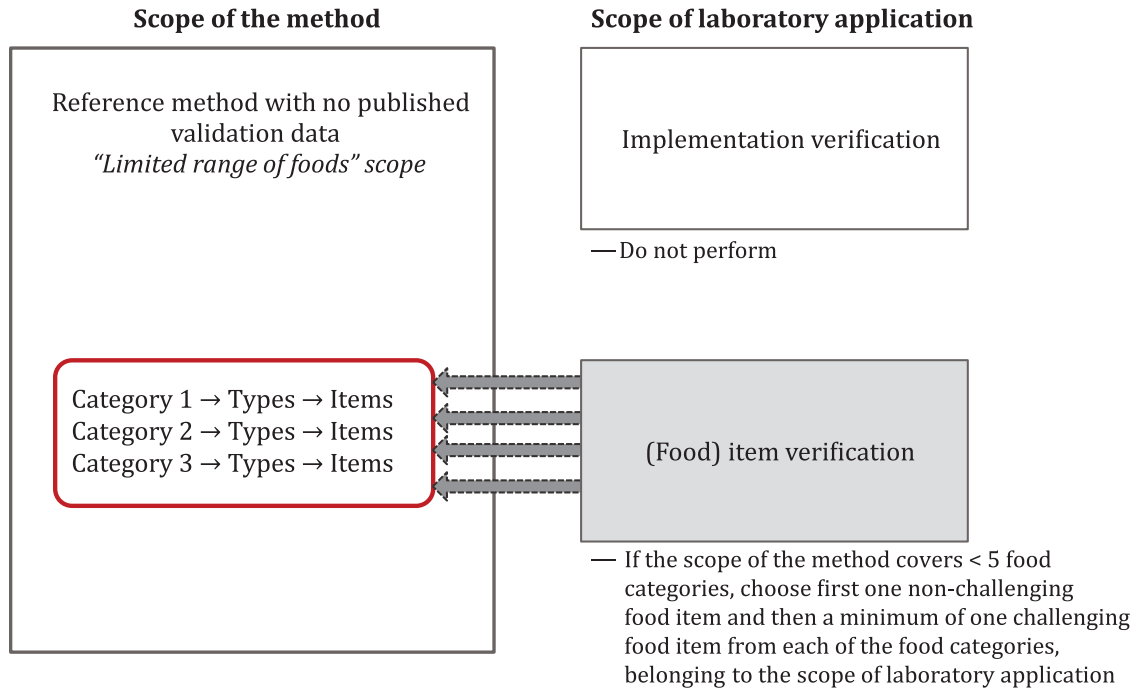


**Figure F.1 — Food items required when verifying a non-validated reference method for a “broad range of foods” scope**

The user laboratory first demonstrates its competence to conduct the method correctly. To do so, it selects and tests one non-challenging food item within the scope of laboratory application. After the user laboratory has demonstrated it can perform the method correctly, it selects and tests a minimum of five challenging food items, each one from a different food category and belonging to the scope of laboratory application.

The scope of laboratory application shown in [Figure F.1](#) is for a “broad range of foods”, meaning that the user laboratory has included five or more food categories in its verification study and can therefore claim application for a “broad range of foods”. If the scope of laboratory application is smaller than the scope of the method, the user laboratory shall only test food items from its restricted food categories. For example, if the scope of laboratory application is limited to three food categories, then the user laboratory shall verify a minimum of one challenging food item from each of the three food categories.

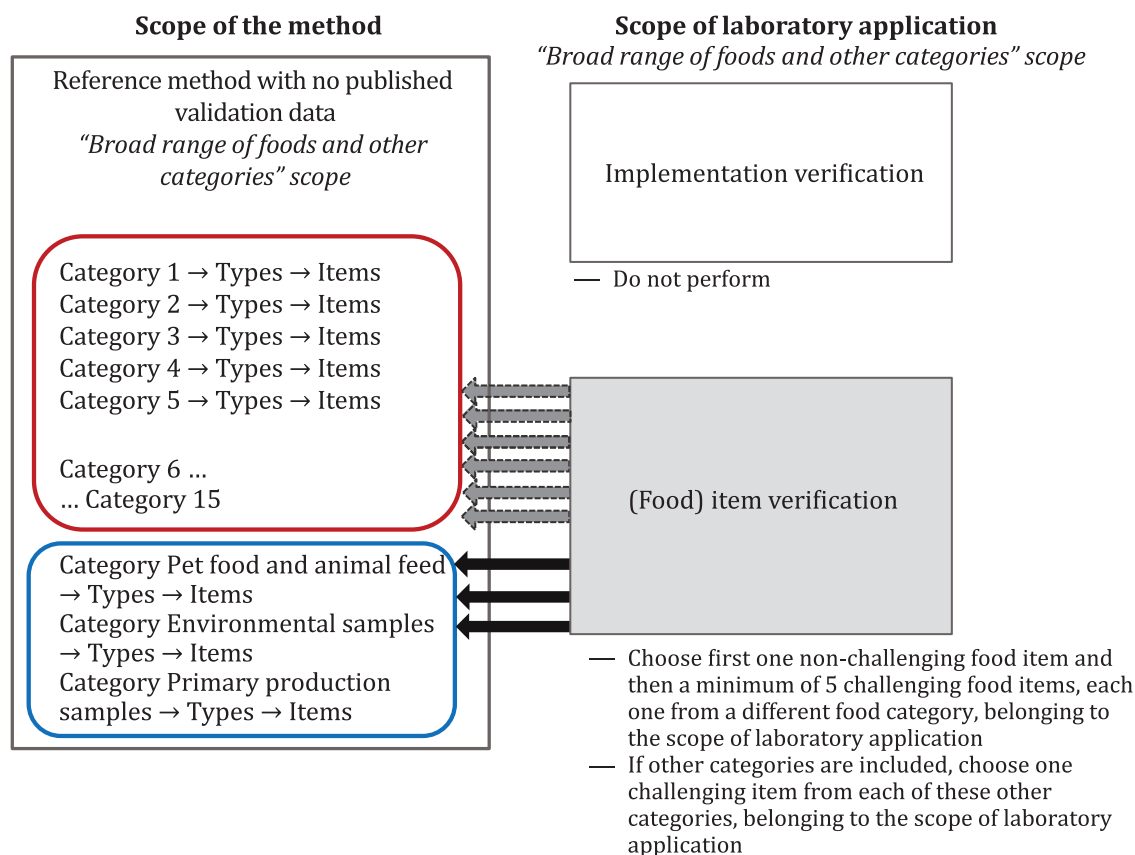
[Figure F.2](#) shows the case of a “limited range of foods” scope with no validation data.



**Figure F.2 — Food item required when verifying a non-validated reference method for a "limited range of foods" scope**

In this case, the user laboratory still demonstrates its competence to conduct the method correctly. To do so, it selects and tests one non-challenging food item within the scope of laboratory application. After the user laboratory has demonstrated it can perform the method correctly, it selects a minimum of one challenging food item from each of the food categories and belonging to the scope of laboratory application. If the scope of laboratory application is smaller than the method, the user laboratory shall only test food items from its restricted food categories.

[Figure F.3](#) shows the number of items required when food and other categories are included in the scope of laboratory application. These categories include pet food and animal feed, environmental samples (food or feed production) and primary production samples (PPS). If any of these other categories is claimed to be within the scope of laboratory application of the user laboratory, then one challenging item from each claimed category shall also be included in the (food) item verification.



**Figure F.3 — Items required when verifying a method for a “broad range of foods and other categories” scope**

Table F.1 summarizes the minimum number of (food) items required for the different scenarios of a non-validated reference method.

**Table F.1 — Summary of the minimum number of (food) items required for verification of a non-validated reference method**

| Scope of the reference method   | Number of samples           |   |   |
|---|-----------------------------|---|---|
|   | Implementation verification | (Food) item verification  | Total   |
| “Broad range of foods” scope<br>≥ 5 food categories   | Not applicable              | 1 non-challenging +<br>$N_{\text{food}} \geq 5$ challenging food items  | ≥ 6   |
| “Limited range of foods” scope<br>$N_{\text{food}}$ categories  | Not applicable              | 1 non-challenging +<br>$N_{\text{food}} \leq 4$ challenging food items  | $(N_{\text{food}} + 1) \leq 5$                    |
| “Broad range of foods” +<br>other categories ( $N_{\text{other}}$ ) scope                                   | Not applicable              | 1 non-challenging +<br>$N_{\text{food}} \geq 5$ challenging food items<br>+<br>1 challenging item from each of<br>the $N_{\text{other}}$ other categories | ≥ 6 + $N_{\text{other}}$                          |
| “Limited range of foods”<br>$N_{\text{food}}$ categories<br>+ other categories ( $N_{\text{other}}$ ) scope | Not applicable              | 1 non-challenging +<br>$N_{\text{food}} \leq 4$ challenging food items<br>+<br>1 challenging item from each<br>of the $N_{\text{other}}$ other categories | $(N_{\text{food}} + N_{\text{other}} + 1) \leq 8$ |
| Other categories ( $N_{\text{other}}$ )<br>scope only   | Not applicable              | 1 non-challenging (food or<br>other) + $N_{\text{other}} \leq 3$ challenging<br>items   | $(N_{\text{other}} + 1) \leq 4$                   |

[Table A.1](#) provides the list of (food) categories and corresponding (food) items. [Annex B](#) provides further guidance on the selection of a challenging (food) item from each (food) category for (food) item verification.

## F.4 Performance characteristics

[Table F.2](#) lists the required performance characteristics for method verification.

**Table F.2 — Required performance characteristics to be determined for verification of a non-validated reference method**

| Method       | Performance characteristic   | Implementation verification | (Food) item verification |
|--------------|--|-----------------------------|--------------------------|
| Qualitative  | Estimated LOD <sub>50</sub> (eLOD <sub>50</sub> )                            | Not applicable              | ✓                        |
| Quantitative | Intralaboratory reproducibility standard deviation ( <i>S<sub>IR</sub></i> ) | Not applicable              | Not applicable           |
|              | Estimated bias (eBias)   | Not applicable              | ✓                        |

NOTE For the verification of a qualitative method, three protocols are proposed to the user laboratory. The protocol 3 does not require a determination of an eLOD<sub>50</sub> but to target a concentration of 3 cfu to 5 cfu/test portion.

## F.5 Qualitative methods — Technical protocol for verification of a non-validated reference method

### F.5.1 Estimated LOD<sub>50</sub> (eLOD<sub>50</sub>) determination

For non-validated reference methods, the eLOD<sub>50</sub> determination is required for the (food) item verification.

During the verification, run the full procedure of the non-validated reference method as described, including the confirmation procedure (if there is one). A minimum of one individual test portion at each inoculation level needs to be confirmed, and the number of colonies for confirmation may be reduced to one.

### F.5.2 Experimental design

The user laboratory shall select one of the three protocols described in [Table F.3](#). For non-validated reference methods, the LOD<sub>50</sub> value is assumed to be equal to 1 cfu/test portion.

**Table F.3 — Protocols to determine eLOD<sub>50</sub> and number of replicates needed per inoculation level for a non-validated reference method**

| Protocol | Inoculation level of the test portion |  |                                    |                                 |       | Total number of replicates |
|----------|---------------------------------------|--|------------------------------------|---------------------------------|-------|----------------------------|
|          | High level<br>9 cfu/test<br>portion   | Intermediate<br>level<br>3 cfu/test<br>portion | Low level<br>1 cfu/test<br>portion | 3 cfu to 5 cfu/<br>test portion | Blank |                            |
| 1        | 1                                     | 4  | 4                                  | –                               | 1     | 10                         |
| 2        | –                                     | 3  | 5                                  | –                               | 1     | 9                          |
| 3        | –                                     | –  | –                                  | 7                               | 1     | 8                          |

NOTE The abbreviation of colony forming units is cfu.

For further details, see [5.2](#).



### F.5.3 Selection of (food) items

For (food) item verification, the user laboratory shall:

- first test one non-challenging (food) item from a (food) category claimed in the scope of the reference method and tested within the scope of laboratory application;
- then test a minimum of one challenging (food) item from each (food) category claimed in the scope of the reference method and tested within the scope of laboratory application.

### F.5.4 Artificial contamination

#### F.5.4.1 Selection of strains

See [5.4.1](#).

#### F.5.4.2 Inoculation of the test portions

See [5.4.2](#).

For non-validated reference methods, the LOD<sub>50</sub> value is assumed to be equal to 1 cfu/test portion.

[Table F.4](#) provides a guide on how to achieve the inoculation levels for each protocol.

**Table F.4 — Inoculation levels for each protocol for a non-validated reference method**

| Protocol | High level<br>9 cfu/test portion  | Intermediate level<br>3 cfu/test portion   | Low level<br>1 cfu/test portion   | 3 cfu to 5 cfu/test<br>portion   |
|----------|---|--|---|--|
| 1        | This should be at a maximum of nine times the assumed LOD <sub>50</sub> . | From the high inoculation level, perform a 1:3 dilution to achieve the intermediate level. | From the intermediate inoculation level, perform a 1:3 dilution to achieve the low level. | –  |
| 2        | –   | This should be at a maximum of three times the assumed LOD <sub>50</sub> .                 | From the intermediate inoculation level, perform a 1:3 dilution to achieve the low level. | –  |
| 3        | –   | –  | –   | The level of contamination of the inoculum is known, (e.g. reference material with known concentration). |

More dilutions can be tested in order to make sure the target levels are reached. Use as many dilutions as needed, but always take into account a 1:3 dilution factor between the levels.

### F.5.5 Evaluation of results

See [5.5](#).

For non-validated reference methods, the LOD<sub>50</sub> value as mentioned in [Table 6](#) and [Table 8](#) is assumed to be equal to 1 cfu/test portion.

### F.5.6 Acceptability limits

The eLOD<sub>50</sub>, determined according to protocol 1 (see [5.5.1](#)) or protocol 2 (see [5.5.2](#)) shall not be > 4 × LOD<sub>50</sub>. For non-validated reference methods, the LOD<sub>50</sub> value is assumed to be equal to 1 cfu/test portion. Therefore, the eLOD<sub>50</sub> shall not be > 4 cfu/test portion.

For protocol 3, there shall be a minimum of six positive results out of the seven replicates tested.

[F.7](#) provides a summary of the acceptability limits.

### F.5.7 Root cause analysis

When the result exceeds the acceptability limit (if the  $eLOD_{50}$  is  $> 4$  cfu/test portion), perform a root cause analysis to provide an explanation for the observed results.

For further details, see [5.7](#).

## F.6 Quantitative methods — Technical protocol for verification of a non-validated reference method

### F.6.1 Intralaboratory reproducibility standard deviation determination

For non-validated reference methods, only the determination of the estimated bias (eBias) is required. The determination of the intralaboratory reproducibility standard deviation is not required.

### F.6.2 Estimated bias (eBias) determination

For (food) item verification, the user laboratory shall:

- first test one non-challenging (food) item from a (food) category claimed in the scope of the reference method and tested within the scope of laboratory application;
- then test a minimum of one challenging (food) item from each (food) category claimed in the scope of the reference method and tested within the scope of laboratory application.

For further details, see [6.2](#).

## F.7 Summary of acceptability limits

[Table F.5](#) summarizes the acceptability limits that are used for method verification of non-validated reference methods.

**Table F.5 — Acceptability limits for the verification of non-validated reference methods**

| Method       | Performance characteristics | Acceptability limits  |
|--------------|-----------------------------|---|
| Qualitative  | $eLOD_{50}$                 | For protocols 1 and 2: $eLOD_{50} \leq 4$ cfu/test portion<br>For protocol 3: $\geq 6$ out of 7 positive results  |
| Quantitative | eBias                       | $ \log_{10} \text{ cfu/ml (inoculum)} - \text{mean } \log_{10} \text{ cfu/test portion (artificially contaminated [food] item)}  \leq 0,5 \log_{10}$ for each of the inoculation levels |

## Bibliography

- [1] ISO 6579-1, *Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of Salmonella — Part 1: Detection of Salmonella spp.*
- [2] ISO/TR 6579-3, *Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of Salmonella — Part 3: Guidelines for serotyping of Salmonella spp.*
- [3] ISO 11133:2014, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*
- [4] ISO 11290 (all parts), *Microbiology of the food chain — Horizontal method for the detection and enumeration of Listeria monocytogenes and of Listeria spp.*
- [5] ISO 16140-2:2016, *Microbiology of the food chain — Method validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method*
- [6] ISO 16140-4, *Microbiology of the food chain — Method validation — Part 4: Protocol for method validation in a single laboratory*
- [7] ISO 16140-5, *Microbiology of the food chain — Method validation — Part 5: Protocol for factorial interlaboratory validation for non-proprietary methods*
- [8] ISO 16140-6:2019, *Microbiology of the food chain — Method validation — Part 6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures*
- [9] ISO 17468:2016, *Microbiology of the food chain — Technical requirements and guidance on establishment or revision of a standardized reference method*
- [10] ISO 19036:2019, *Microbiology of the food chain — Estimation of measurement uncertainty for quantitative determinations*
- [11] ISO 21528-2, *Microbiology of the food chain — Horizontal method for the detection and enumeration of Enterobacteriaceae — Part 2: Colony-count technique*
- [12] ISO Guide 30:2015, *Reference materials — Selected terms and definitions*
- [13] *Transition period for the implementation of ISO 16140-3*. Available (viewed: 2020-09-15) from: <https://committee.iso.org/home/tc34sc9>
- [14] Council Directive 79/373/EEC of 2 April 1979 on the marketing of compound feedingstuffs. *Official Journal of the European Communities*. L 86, 6.4.1979, pp. 30-37
- [15] Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. *Official Journal of the European Communities*. L 139, 30.4.2004, pp. 1-54
- [16] Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. *Official Journal of the European Communities*. L 139, 30.4.2004, pp. 55-205
- [17] Health Canada (website). Available at: <https://www.hc-sc.gc.ca/>
- [18] United States Department of Agriculture (website). Available at: <http://www.fsis.usda.gov/>
- [19] US Food & Drug Administration (website). Available at: <http://www.fda.gov/>

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| <i>International Standard</i>   | <i>Corresponding Indian Standard</i>  | <i>Degree of Equivalence</i>      |
|---|---|-----------------------------------|
| ISO 6887-4 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 4: Specific rules for the preparation of miscellaneous products                              | IS 17447 : 2020 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of miscellaneous products                             | Identical with ISO 6887-4 : 2017  |
| ISO 6887-5 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 5: Specific rules for the preparation of milk and milk products                              | IS 17779 : 2021 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of milk and milk products                             | Identical with ISO 6887-5 : 2020  |
| ISO 6887-6 Microbiology of food and animal feed — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 6: Specific rules for the preparation of samples taken at the primary production stage | IS 16980 : 2018 Microbiology of food and animal feed — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination: Specific rules for the preparation of samples taken at the primary production stage | Identical with ISO 6887-6 : 2013  |
| ISO 7218 Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations  | IS 16122 : 2013 Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations   | Identical with ISO 7218 : 2007    |
| ISO 16140-1 Microbiology of the food chain — Method validation — Part 1: Vocabulary   | IS 17113 (Part 1) : 2019 Microbiology of the food chain — Method validation: Part 1 Vocabulary  | Identical with ISO 16140-1 : 2016 |

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