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Microbiology of the food chain — Technical requirements and guidance on the establishment or revision of a standardized reference method

Microbiologie de la chaîne alimentaire — Exigences et recommandations techniques pour le développement ou la révision d'une méthode de référence normalisée

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Foreword

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

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This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology,* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 463, *Microbiology of the food chain,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 17468:2016), which has been technically revised.

The main changes are as follows:

— a cross-reference is made not only to ISO 16140-2, but also to ISO 16140-4 and ISO 16140-6;

— a new optional step has been added, "method(s) optimization". In addition, a new annex providing guidance on method optimization studies is included, to compare two options during the development of a new standardized reference method or for its revision;

— the inclusion of the case of confirmation and typing methods;

— the assessment of the nature of a change (minor/major) during the revision of a standardized reference method.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Microbiology of the food chain — Technical requirements and guidance on the establishment or revision of a standardized reference method

1 Scope

This document gives technical requirements and guidance on the establishment or revision of standardized reference methods used for the analysis of microorganisms in:

— products intended for human consumption;

— products for feeding animals;

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

— samples from the primary production stage.

This document specifies the technical stages of the establishment of a new standardized reference method and of the revision of an existing standardized reference method. It includes, in particular, requirements and guidance on the validation of the selected method.

This document is intended to be implemented in particular by ISO/TC 34/SC 9 and its corresponding structure at CEN level, which is CEN/TC 463.

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

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

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

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

ISO 16140-2:2016/Amd.1:—[1)], *Microbiology of the food chain — Method validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method — Amendment 1: Revision of qualitative MCS data evaluation, RLOD calculations in the ILS, calculation and interpretation of the RT study, and inclusion of a commercial sterility testing protocol for specific products*

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*

3 Terms and definitions

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

1) Under preparation. Stage at time of publication ISO 16140-2:2016/DAmd.1:2023.

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

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
candidate reference method
method selected and likely to become the *standardized reference method* (3.7)

3.2
ILS organizer
organizing laboratory
laboratory with responsibility for managing all of the technical and statistical activities involved in the organization of the interlaboratory study

[SOURCE: ISO 16140-1:2016, 2.45, modified - "validation study, i.e. method comparison study and the interlaboratory study" has been replaced by "organization of the interlaboratory study" and the Note 1 to entry has been deleted.]

3.3
ILS participant
participating laboratory
individual laboratory technician, who works completely independently from other ILS participants, using different sets of blind samples or test portions

[SOURCE: ISO 16140-1:2016, 2.13, modified - "collaborator" has been replaced by "ILS participant".]

3.4
interlaboratory study
study performed by multiple laboratories testing identical samples at the same time, the results of which are used to estimate performance characteristics of the candidate reference method

Note 1 to entry: The aim of an interlaboratory study is to determine the variability of the results obtained in different laboratories using identical samples.

[SOURCE: ISO 16140-1:2016, 2.33, modified - "alternative-method performance parameters" has been replaced by "performance characteristics of the candidate reference method".]

3.5
pre-standardization stage
technical stage prior to the standardization stage and comprising the different steps described in this document

Note 1 to entry: The standardization stage starts with the proposal stage which is the approval of a New Work Item Proposal (ISO/NP) for inclusion of the Work Item on the work programme of ISO/TC 34/SC 9.

3.6
"real life" study
study of one or several methods, conducted in different laboratories, using their own routine samples and with preference given to naturally contaminated samples

3.7
standardized reference method
reference method described in a standard

Note 1 to entry: See ISO 16140-1 for the definition of "reference method".

4 Technical procedure for standardizing a new reference method

4.1 General

The validation of a method in view of its standardization as a reference method includes six technical steps (see 4.2):

— step 1: method(s) selection (mandatory);

— step 2: method(s) optimization (optional);

— step 3: method(s) evaluation study (recommended);

— step 4: "real life" study (recommended) (this step does not apply to confirmation and typing methods);

— step 5: selection of one candidate reference method for further validation (mandatory);

— step 6: interlaboratory study (mandatory).

The first five technical steps correspond to a pre-standardization stage and are usually performed before launching the standardization process. Step 6 (see 4.2.6) is usually performed during the standardization process [preferably after the committee stage (ISO/CD) and before the enquiry stage (ISO/DIS)].

The flow chart of the technical steps for the establishment of a new standardized reference method is given in Annex A.

The working group in charge of organizing the studies on a qualitative method shall consider the inclusion of a test portion size larger than, for example, 10 g or 25 g, when it is relevant and feasible. This will facilitate the use of the standardized reference method without the need for further validation when a larger test portion size is routinely used.

EXAMPLE Detection of *Salmonella* in 375 g infant formula test portions.

Information on the studies conducted in step 1 to step 6 should not be shared publicly until their completion. Once these studies are completed, all relevant data as obtained in step 1 to step 6 should become publicly available, by reporting it either in a scientific publication or in a report. The report associated to each standardized reference method can be made available on the ISO Standards Maintenance Portal (https://standards.iso.org/iso/) and/or by including a link in the Bibliography of the standardized reference method to the website where the report is publicly available.

NOTE Data, e.g. regarding inclusivity and exclusivity, can be derived from earlier studies as long as the information is traceable to the originally published data or made available on the ISO Standards Maintenance Portal associated to the standardized reference method.

The performance characteristics obtained from the interlaboratory study (step 6, also see 4.2.6) shall be incorporated into the corresponding standardized reference method.

4.2 Technical steps

4.2.1 Step 1: Method(s) selection (mandatory)

Information from different sources (e.g. national/regional standardized methods, scientific papers on methods with evaluation data, evaluation/validation reports on methods, practicability of the method) shall be collated for the choice of (a) candidate reference method(s). Based on the information available, the working group in charge of developing the standard (referred to as 'the working group' from this point forward) shall select one or several candidate reference methods.

4.2.2 Step 2: Method(s) optimization (optional)

If, after the step of method(s) selection (see 4.2.1), one (or several) candidate reference method(s) comprise(s) two options for one factor (e.g. culture medium, incubation time, incubation temperature), a method(s) optimization study can be conducted, comparing the two options.

Annex B provides guidance on how to compare these two options, and how to make a choice for the method to be standardized and to be further evaluated (see 4.2.3).

If the candidate reference method(s) does (do) not comprise(s) any factor with options, proceed directly to step 3 (see 4.2.3).

4.2.3 Step 3: Method(s) evaluation study (recommended)

4.2.3.1 General

An evaluation study of the candidate reference method(s) (see 4.2.1 and 4.2.2) should be conducted, normally by one laboratory, but more than one laboratory may also be involved.

If several candidate reference methods are evaluated at this step, the outcome of this evaluation study should enable the relevant working group to reduce the number of candidate reference methods, ideally to one (step 5, also see 4.2.5).

The working group shall check that the candidate reference method(s) work(s) using culture media prepared in the laboratory from individual ingredients described in the candidate reference method(s).

4.2.3.2 Detection and quantification methods

When conducted, the method(s) evaluation study enables the estimation of performance characteristics listed below and aims at assessing them for a large variety of (food) types and (food) items, within the (food) categories studied, representative of the scope of the method. In accordance with ISO 16140-2:2016, 5.1.3.1, if the method is to be applied to a broad range of food, at least five food categories shall be studied. If the method is to be validated for a restricted number of food categories, then only these categories need to be studied. In addition to the food categories, pet food and animal feed samples, environmental samples and primary production stage samples can be included as additional categories.

NOTE The working group can assess, possibly through an inventory among users, whether it is sufficient to validate the method for a restricted number of (food) categories.

This study should fulfil the requirements of the single-laboratory method validation study without comparison to a reference method, as stated in ISO 16140-4:

— for qualitative methods:
  — factorial approach (in accordance with ISO 16140-4:2020, 5.1.2): sensitivity, level of detection (LOD_{50}), inclusivity/exclusivity; or
  — conventional approach (in accordance with ISO 16140-4:2020, 6.1.2): specificity, LOD_{50}, sensitivity, inclusivity/exclusivity;
— for quantitative methods:
  — factorial approach (in accordance with ISO 16140-4:2020, 5.2.2): relative trueness, accuracy profile, in-house precision study (repeatability and in-house reproducibility), inclusivity/exclusivity; or
  — conventional approach (in accordance with ISO 16140-4:2020, 6.2.2): relative trueness, accuracy profile, in-house precision study (repeatability and in-house reproducibility), inclusivity/exclusivity, and if applicable, limit of quantification.

4.2.3.3 Confirmation and typing methods

When conducted, the method(s) evaluation study shall fulfil the requirements of the method comparison study for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures, as stated in ISO 16140-6:2019, Clause 6: inclusivity and exclusivity. As there is no comparison to a reference method, compare the inclusivity and exclusivity results directly to the identity of the strains (second interpretation in ISO 16140-6:2019, 6.5, Tables 2 and 4).

4.2.4 Step 4: "Real life" study (recommended)

The "real life" study should be conducted on the candidate detection/quantification reference method(s) (see 4.2.1), using a wide range of samples with preference given to naturally contaminated samples. In the case the "real life" study is conducted for revising a standardized reference method (see Clause 5), the original reference method shall be included. When conducted, this study shall be organized in different laboratories, located in different countries/different regions of the world to cover the largest diversity possible of:

a) matrices where the target microorganism can naturally be found;

b) strains of the target microorganism.

In particular, each laboratory shall use its own samples, reagents and culture media to reflect their diversity. If existing, different brands and types (from individual ingredients or from powders, or ready-to-use) of commercial media should be used. For culture media and reagents, follow the requirements in ISO 11133 on quality control.

If the outcome of this study is not assessed as acceptable by the relevant working group, this working group shall reconsider the choice of the candidate reference method(s) and go back to step 1.

This "real life" study may be conducted in parallel with step 1 (see 4.2.1) or step 3 (see 4.2.3).

NOTE The "real life" study is not applicable to confirmation and typing methods.

4.2.5 Step 5: Selection of one candidate reference method for further validation (mandatory)

Based on the information and data gained in the previous steps (see 4.2.1 to 4.2.4 as applicable), the relevant working group shall select one method for further validation (see step 6, 4.2.6).

4.2.6 Step 6: Interlaboratory study (mandatory)

An interlaboratory study (ILS) shall be conducted to adopt the new method, as selected at step 5 (see 4.2.5). In the exceptional case where it has been decided by ISO/TC 34/SC 9 and CEN/TC 463 to adopt a method which has not been validated with an ILS, this method should be published as an ISO Technical Specification.

NOTE In general, verification (see ISO 16140-3) is only applicable to reference methods that have been validated using an interlaboratory study. This underlines the importance of conducting an interlaboratory study before the adoption of a new method in the development of a standard.

The aim of the interlaboratory study is to determine the relevant performance characteristics of the selected method when implemented in different laboratories using identical samples. Whenever possible, the study conditions should reflect the normal variability between different laboratories using identical samples.

The interlaboratory study is usually performed during the standardization process [preferably after the committee stage (ISO/CD) and before the enquiry stage (ISO/DIS)].

For each (food) category tested, the interlaboratory study shall be conducted with ILS participants from more than one country. The ILS organizer (and the ILS participants) may differ per (food) category to be studied.

The interlaboratory study of detection/quantification methods shall be conducted in accordance with ISO 16140-2 and shall include one (food) item per (food) category studied in step 3 [see 4.2.3, in particular for the total number of (food) categories to be tested]. Each (food) item should be contaminated by a different target strain.

The artificial contamination of samples used in the interlaboratory study can be done:

— by the ILS organizer: using either a contamination by mixture, an artificial contamination of samples using a seeding protocol or a spiking protocol (see ISO 16140-2:2016, Annex C); or

— by the ILS participants: the ILS organizer sends:

  — pre-portioned samples from a matrix not contaminated with the target microorganism;

  — per sample blind-coded inoculum (e.g. inoculation solutions of the target microorganism), ensuring that the inoculum is homogeneous and stable.

Information on the preparation, check of homogeneity and stability of samples can be found in ISO 22117.

Shipment of samples shall take into account the applicable transport safety requirements.

The ILS organizer(s) should provide the ILS participants with adequate information on handling and storage of the samples upon arrival.

When a method has been validated for certain (food) categories, the scope of validation can be extended to other categories, e.g. to reach at least the five food categories for a validation applicable to a broad range of food, by organizing an additional interlaboratory study for the additional categories.

The experimental design for **qualitative** methods is described in ISO 16140-2:2016, 5.2.2, considering only the requirements concerning the reference method. The following performance characteristics shall be determined, with L_0, L_1 and L_2 defined in ISO 16140-2:2016, 5.2.2:

— Specificity (SP) = ((number of samples tested at L_0 – number of positive samples at L_0)/number of samples tested at L_0) × 100 %;

— Sensitivity at level L_1 (SE_{L1}) = (number of positive samples at L_1 /number of samples tested at L_1) × 100 %;

— Sensitivity at level L_2 (SE_{L2}) = (number of positive samples at L_2 /number of samples tested at L_2) × 100 %;

— LOD_{50} (and 95 % confidence interval) in accordance with ISO 16140-2:2016/Amd.1:—, Annex F. An Excel®-based program[2)] for the calculation of the LOD in accordance with this annex is freely available for download at https://standards.iso.org/iso/16140/-2/ed-1/en/amd/1/ (download the file "PODLOD-Interlab"). This Excel®-based program can be used for three inoculation levels, including the blank level.

The experimental design for **quantitative** methods is described in ISO 16140-2:2016, 6.2.2, considering only the requirements concerning the reference method. The following performance characteristics shall be determined: interlaboratory repeatability standard deviation (s_r) and interlaboratory reproducibility standard deviation (s_R). These performance characteristics shall be calculated in accordance with ISO 5725-2:

— per item and contamination level;

— for all contamination levels per item (X) as a median of the values per contamination level;

— if possible (if the values per item are close enough), for all contamination levels and items (Y), as a median of the values per contamination level and per item.

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

The interlaboratory study of **confirmation and typing methods** shall be conducted in accordance with ISO 16140-6:2019, Clause 7. As there is no comparison to a reference method, compare the inclusivity and exclusivity results directly to the identity of the strains (second interpretation in ISO 16140-6:2019, 6.5, Tables 2 and 4).

For qualitative methods, quantitative methods, as well as confirmation and typing methods, Reference [5] provides guidance and templates to incorporate the relevant performance characteristics obtained from the interlaboratory study into the corresponding standardized reference methods.

5 Technical procedure for revising a standardized reference method

The working group shall agree on the changes to be made to the existing standardized reference method and shall assess whether these changes are of a major or minor nature.

A major technical change is, for example, a modification in the method detection/quantification technology or a substantial modification of the procedure (e.g. nature of the enrichment broth or isolation agar, incubation time and temperature). It is expected to produce a different result.

A minor change is, for example, an editorial change to the text of the method or a minor technical change that is not expected to affect the result. An example of a minor technical change is shown in the ISO 10272 series for detection and enumeration of *Campylobacter* spp., where the confirmation tests on microaerobic growth at 25 °C and aerobic growth at 41,5 °C were replaced by aerobic growth at 25 °C.

If the technical change in the method is evaluated as being major, a re-validation of the method is needed. When the re-validation of the method is conducted, the impact on the performance characteristics shall be evaluated to determine if the changes are to be regarded as major (performance characteristics have substantially changed) or minor (no impact on performance characteristics observed). In certain cases, a major technical change in the method can be considered to be minor, if the re-validation of the method shows that it has no significant impact on the performance characteristics or test results.

For re-validation of a method, follow steps 1 (mandatory), 2 (optional), 4 (recommended), 5 (mandatory) and 6 (mandatory) as described in 4.2. Follow step 3 “method(s) evaluation study” (recommended, see 4.2.3) as described below.

Step 3 “method(s) evaluation study of detection and quantification methods” should enable estimation of performance characteristics and fulfil the requirements of the single-laboratory method validation study with comparison to a reference method (the existing standardized reference method), as stated in ISO 16140-4:

— for qualitative methods:

  — factorial approach (in accordance with ISO 16140-4:2020, 5.1.1): sensitivity, relative level of detection (RLOD), inclusivity/exclusivity; or

  — conventional approach (in accordance with ISO 16140-4:2020, 6.1.1): sensitivity, RLOD, inclusivity/exclusivity;

— for quantitative methods:

  — factorial approach (in accordance with ISO 16140-4:2020, 5.2.1): relative trueness, accuracy profile, in-house precision study (repeatability and in-house reproducibility), inclusivity/exclusivity; or

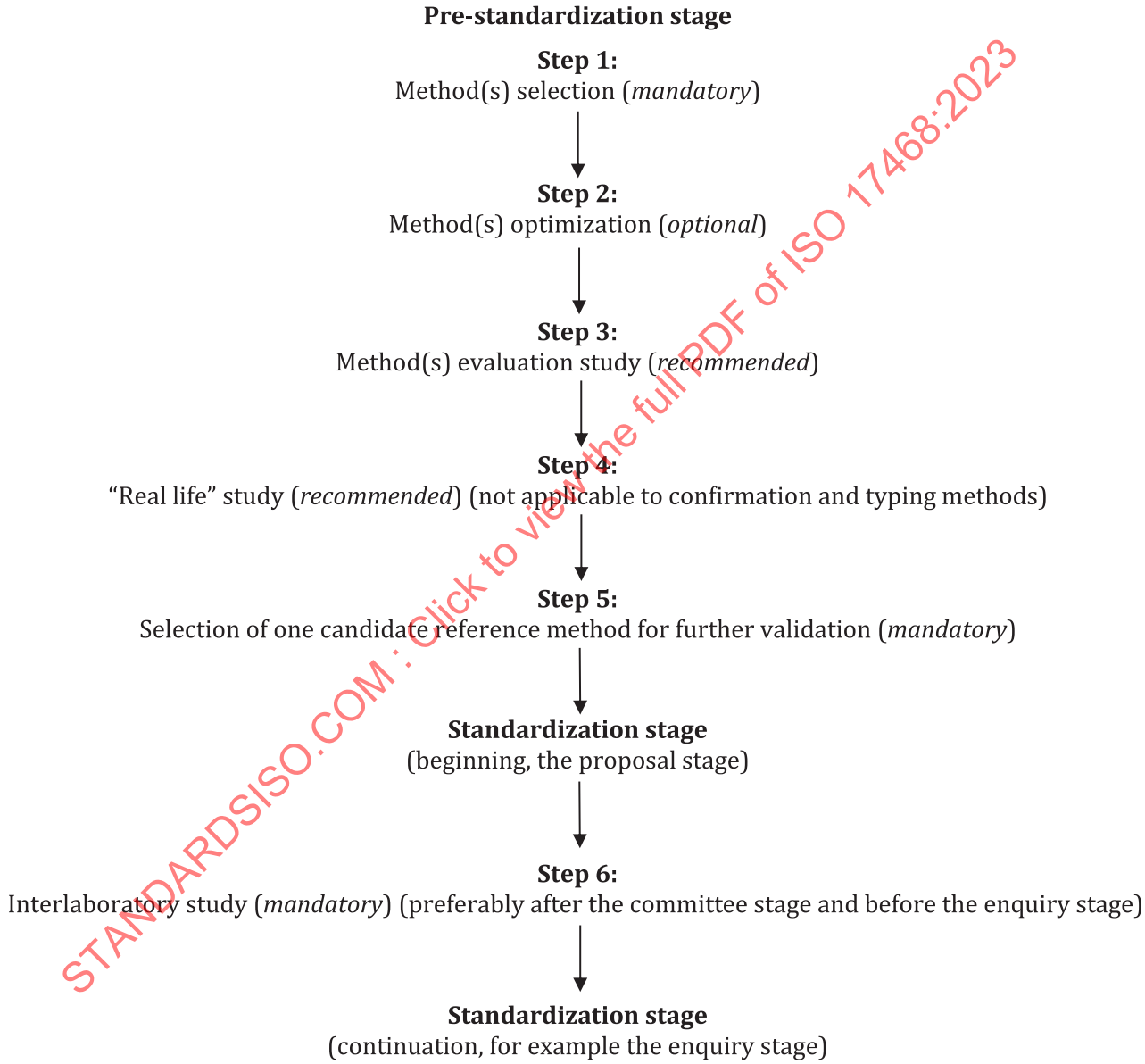
  — conventional approach (in accordance with ISO 16140-4:2020, 6.2.1): relative trueness, accuracy profile, in-house precision study (repeatability and in-house reproducibility), inclusivity/exclusivity, and if applicable, limit of quantification.

Step 3 “method(s) evaluation study of confirmation and typing methods” shall fulfil the requirements of the method comparison study for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures, as stated in ISO 16140-6:2019, Clause 6: inclusivity and exclusivity. The new method is compared to a reference method (the existing standardized reference method).

The flow chart of the technical steps for the revision of a standardized reference method is given in Figure A.1.

Annex A
(informative)

Flow chart on technical steps for the establishment or revision of a standardized reference method



NOTE Step 4 can be conducted in parallel with step 1 or step 3.

Figure A.1 — Flow chart of the technical steps in the pre-standardization and the standardization stages

Annex B
(informative)

Guidance on optimization studies to compare two options in the development of a new standardized reference method or for its revision

B.1 General

This annex provides guidance to working groups on the experimental design and interpretation of studies to compare two options for one factor of the method (e.g. culture medium, incubation temperature, incubation time), in the development and/or optimization of methods before their validation and standardization.

The purpose of an optimization study described in this annex and corresponding to the optional step 2 "method(s) optimization" (see 4.2.2) is to compare two options and to determine the option allowing the method to perform "at best" to analyse (detect or quantify) the target microorganism in the intended scope of the method [(food) categories].

The performance characteristics to interpret the results of the optimization study were selected from the performance characteristics listed in step 3 "method(s) evaluation study" (see 4.2.3).

The study can be conducted in one laboratory or preferably in several laboratories. The participants analyse their own samples, representative of the products found in their country (as in step 4: "real life" study, see 4.2.4), and use culture media available in their country.

B.2 Qualitative methods

B.2.1 General aspects

The performance characteristic used in the optimization study is the relative level of detection (RLOD) between the two options and the performance criterion is the option out of the two giving the lowest LOD_{50} value. For the experimental design, samples should be artificially contaminated in the general case.

Choose categories to be the most relevant for the optimization study. If the method is expected to be applied to a broad range of food, then at least five food categories should be studied. If the method is expected to be validated for a restricted number of food categories, then only these categories need to be studied.

The cases of paired and unpaired studies (as described in ISO 16140-2:2016, 5.1.2) shall be distinguished.

B.2.2 Unpaired studies

The experimental design in terms of contamination levels and replicate test portions from one item should be as follows:

— 20 replicates at fractional positive level (e.g. the theoretical detection level of 0,7 cfu/test portion, resulting in 25 % to 75 % of positive replicates);

— 5 replicates at a higher level just above the theoretical detection level (e.g. 1 cfu to 1,5 cfu/test portion);

— 5 replicates at the negative control level.