INTERNATIONAL STANDARD

ISO 16140-5

First edition
2020-07

Microbiology of the food chain — Method validation —

Part 5: Protocol for factorial interlaboratory validation for non-proprietary methods

Microbiologie de la chaîne alimentaire — Validation des méthodes — Partie 5: Protocole pour la validation interlaboratoires de méthodes non commerciales par plan factoriel

Reference number
ISO 16140-5:2020(E)

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Contents

Page

Foreword

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

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

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

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

A list of all parts in the ISO 16140 series can be found on the ISO website.

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

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

— The second stage is method verification, where a laboratory demonstrates that it can satisfactorily perform a validated method. This is described in ISO 16140-3. Verification is only applicable to methods that have been validated using an interlaboratory study.

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

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

In the ISO 16140 series, reference methods include standardized reference (ISO and CEN) methods as defined in ISO 17468:2016, 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 ISO 16140-3) is not applicable. This document, 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 this document can be used for validation against a reference method. ISO 16140-4 (regarding qualitative and quantitative methods) and this document (regarding quantitative methods 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.

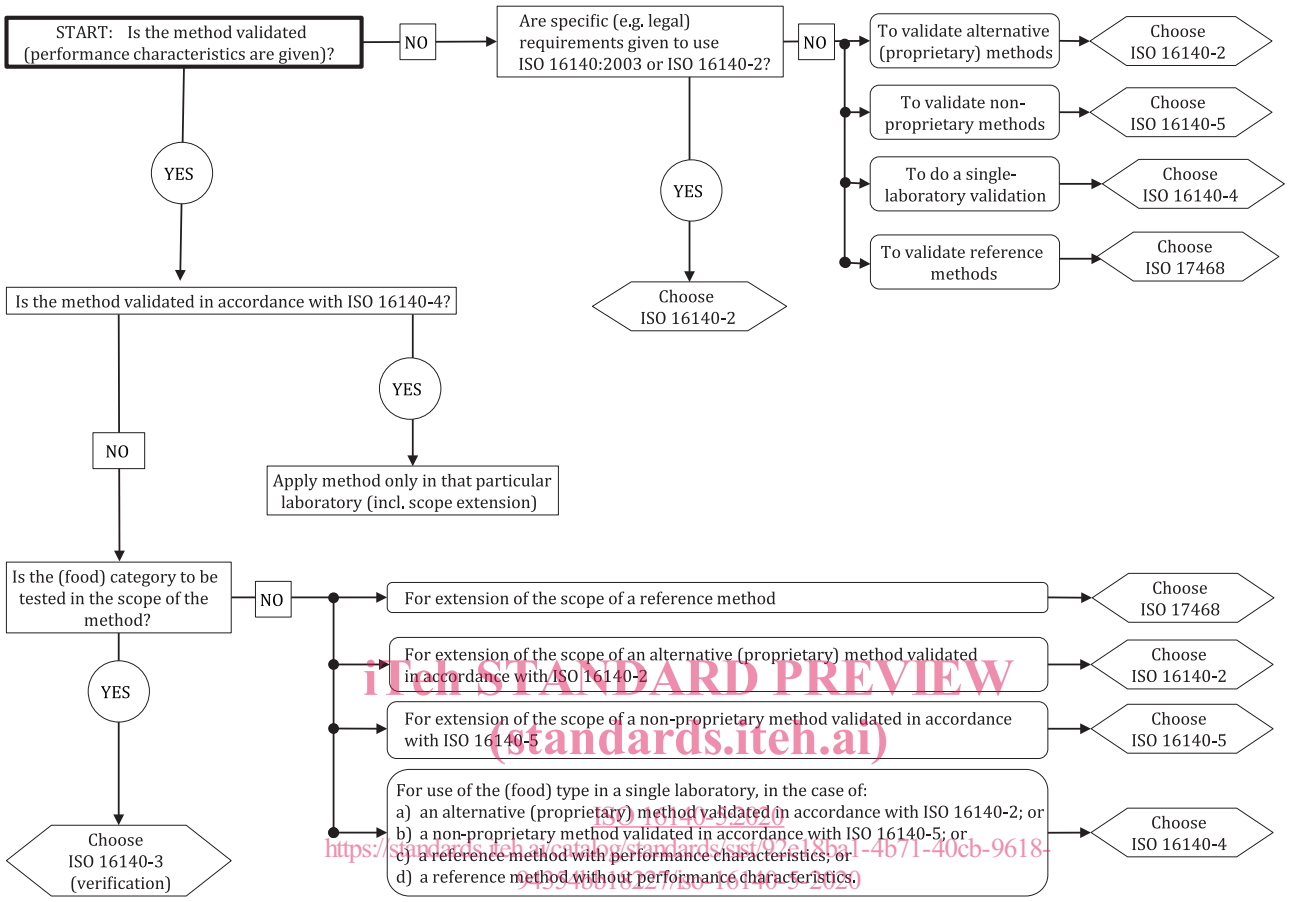

Figure 1 — Flow chart for application of the ISO 16140 series

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

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

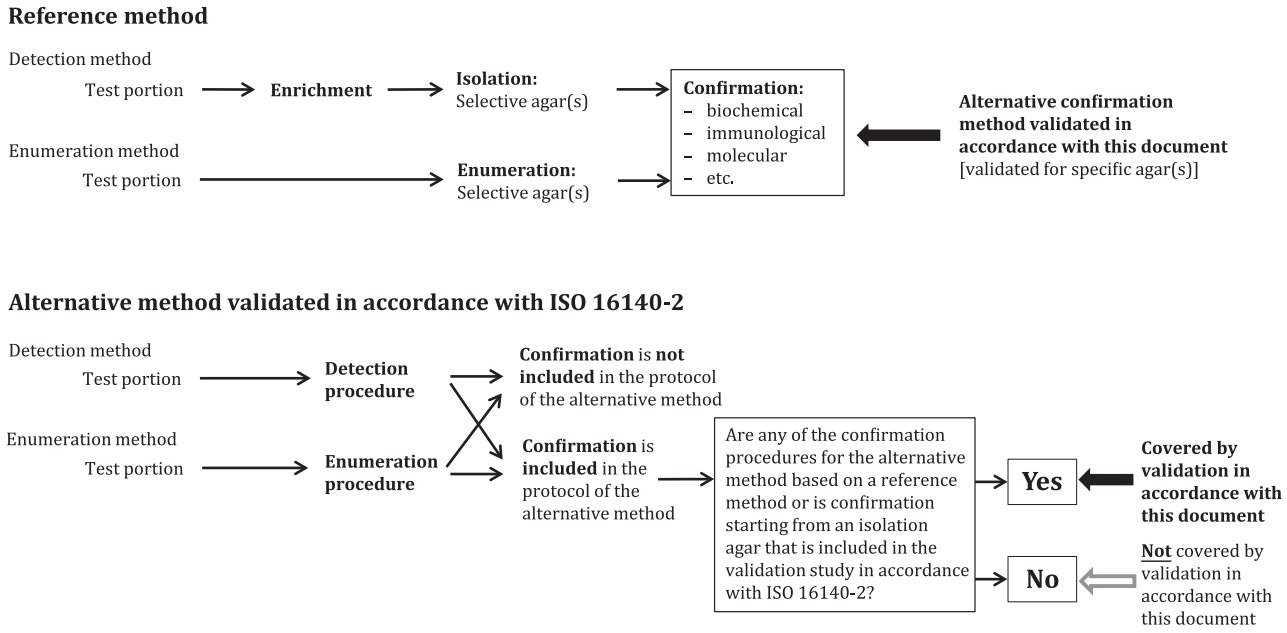

Figure 2 — Use of validated alternative confirmation methods (see ISO 16140-6)

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

An alternative confirmation method based on ELISA has been validated (in accordance with ISO 16140-6) to replace the biochemical confirmation for *Salmonella* as described in ISO 6579-1. In the validation study, XLD (mandatory agar in accordance with ISO 6579-1) plus BGA and a specified chromogenic agar (two optional agars for second plating in accordance with ISO 6579-1) were used as the agars to start the confirmation. The validated confirmation method can be used to replace the biochemical confirmation under the following conditions:

— by laboratories using 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 Validation protocols in the ISO 16140 series

An interlaboratory validation study, in accordance with ISO 16140-2, requires at least eight laboratories for quantitative methods and at least ten laboratories for qualitative methods.

This document provides a protocol that addresses the special case where the number of laboratories required in an interlaboratory validation of a method by ISO 16140-2 cannot be achieved. The protocol allows a method validation based on a minimum of four laboratories. It applies, for example, in situations where there is an urgent need for a validated method but the in-house and interlaboratory studies in accordance with ISO 16140-2 take too long to complete. This document also addresses the problem of method validation of highly specialized methods, for which only a few laboratories might be available for a validation study. This document can only be used for non-proprietary methods. Table 1 provides an overview of the different protocols.

Table 1 — Overview of different validation protocols described in the ISO 16140 series

Number of participating laboratories	With reference method	Without reference method
1	ISO 16140-4	ISO 16140-4
4 to 7 (quantitative method)/ 4 to 9 (qualitative method)	This document: for non-proprietary methods only	This document: for non-proprietary quantitative methods only
≥ 8 (quantitative method)/ ≥ 10 (qualitative method)	ISO 16140-2: for the interlaboratory study part	Not applicable

In order to reduce the number of required laboratories to a minimum of four, while maintaining the applicability of the validation to all laboratories, the protocol is based on a factorial experimental design. In the factorial design, factors such as the technician or culture medium are altered simultaneously, and the method is used in a range of different factor settings. This approach allows a more detailed analysis of the precision parameters of the method while, at the same time, requiring a smaller number of laboratories and tests.

Microbiology of the food chain — Method validation —

Part 5: Protocol for factorial interlaboratory validation for non-proprietary methods

1 Scope

This document specifies the general principles and the technical protocols (based on orthogonal, factorial studies) for the validation of non-proprietary methods for microbiology of the food chain.

This document is applicable to the validation of methods used for the analysis (detection or quantification) of microorganisms in:

— products intended for human consumption;

— products intended for animal feeding;

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

— 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.

This document specifies protocols for the validation against a reference method for both quantitative and qualitative methods. This document also provides a protocol for the validation of quantitative methods without a reference method. Qualitative methods cannot be validated without a reference method in accordance with this document.

NOTE ISO 16140-2 specifies the general principle and the technical protocol for the validation of alternative, mostly proprietary, methods against a reference method.

This document is only applicable to the validation of methods that are fully specified with regard to all relevant parameters (including tolerances on temperatures and specifications on culture media) and that have already been optimized.

Methods that have been validated in accordance with this document can be used by the laboratories of the specified population of laboratories.

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 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*

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*

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
factor
qualitative or quantitative parameter within the method that can be varied at two or more levels within the limits of the specified method

EXAMPLE Technician.

Note 1 to entry: In this document, only those factors that are in line with the protocol of the method are considered.

3.2
factor level
value of the *factors* (3.1) within the experimental design

EXAMPLE Technician "a", technician "b", etc.

Note 1 to entry: In this document, each factor is varied at two factor levels: "a" and "b".

Note 2 to entry: This definition is based on how ISO 3534-3:2013, 3.1.12, defines "factor level". In ISO 3534-3:2013, 3.1.12, the definition is more general, but the statistical meaning is the same.

3.3
orthogonal design

factorial design, in which for every pair of *factors* (3.1), each combination of *factor levels* (3.2) occurs the same number of times across the possible factor levels

Note 1 to entry: This definition is based on how ISO 3534-3:2013, 3.1.31, defines "orthogonal array", but for "orthogonal design", a more general and more theoretical definition is used.

3.4
reference value
<reference method is available> estimated concentration level obtained with the reference method

3.5
reference value
<no reference method is available, for artificially contaminated samples only> estimated concentration level obtained from the concentration level of the inoculum

3.6
setting
combination of *factor levels* (3.2)

EXAMPLE Technician "a" + culture media "b" + temperature "a" + etc.

Note 1 to entry: These conditions can be described by the combination of levels of factors varied within the study.

4 General principles for the factorial interlaboratory validation of non-proprietary methods

4.1 General

This document uses a protocol based on orthogonal, factorial studies. A high certainty of the determined method validation parameters is achieved by focusing on suitable factors (e.g. technician, culture media, sample preparation, temperature, test duration) that can influence the test result. This allows the number of required laboratories to be reduced to a minimum of four. General concepts and considerations given in ISO 16140-2 shall apply, unless explicitly excluded. The validation can be conducted against a reference method or, in the case of a quantitative method, without a reference method.

The outcome of the validation study applies to:

— any laboratory: if the four laboratories can be considered a "random sample" of independent laboratories from different organizations;

— all laboratories within an organization: if the four laboratories can be considered a "random sample" of laboratories at different sites from one organization.

4.2 Validation against a reference method

The validation protocol comprises two phases:

— an in-house validation study of the non-proprietary method against the reference method carried out in the organizing laboratory (see ISO 16140-1:2016, 2.45);

— an interlaboratory study of the non-proprietary method against the reference method carried out in different laboratories.

The technical protocol for performing the in-house validation study and the interlaboratory study are given in Clauses 5 and 6, depending upon whether the test method is qualitative or quantitative in nature.

The selected factors for the factorial interlaboratory study should be relevant and applied to both the reference and the alternative method.

4.3 Validation without a reference method

The validation protocol applies to the validation of quantitative methods only. It comprises two phases:

— an in-house validation study of the non-proprietary method against the reference value carried out in the organizing laboratory;

— an interlaboratory study of the non-proprietary method against the reference value carried out in different laboratories.

The technical protocol for performing the in-house validation study and the interlaboratory study for quantitative methods are given in Clause 6.

5 Qualitative methods — Technical protocol for factorial interlaboratory validation

5.1 In-house validation study

The in-house validation study is the part of the validation process that is performed in the organizing laboratory. It can be conducted in accordance with the conventional approach or the factorial approach, as described in ISO 16140-4.

An in-house validation study can be used to demonstrate the performance of the method for the laboratory that conducted the study. It is the first step in the framework of general method validation. The in-house validation study assesses the performance of the method across (food) categories, (food) types and (food) items, whereas the interlaboratory study assesses the performance of the method across laboratories.

5.2 Interlaboratory validation study against a reference method

5.2.1 General considerations

The aim of the interlaboratory study is to compare the performance of the reference method to the alternative method in terms of the RLOD obtained by different laboratories. The results of the same set of samples, examined under reproducibility conditions, are compared with pre-set criteria for the acceptable difference between the reference method and the alternative method. Wherever possible, the study conditions should reflect the normal variation between laboratories.

The interlaboratory study is planned by the organizing laboratory. The organizing laboratory prepares the samples and a data sheet for the recording of all experimental data and critical experimental conditions used by each laboratory. Each laboratory shall demonstrate its competence in the use of the alternative and the reference method in accordance with ISO 7218 prior to participating in the study.

Technicians involved in the preparation of the samples used in the interlaboratory study shall not take part in the testing of the samples in the interlaboratory study.

5.2.2 Measurement protocol

A minimum of four independent laboratories are required to conduct the test series.

The laboratories shall belong to different organizations or shall be located at different sites.

If all laboratories belong to one organization or network, results of the validation study can only be used by laboratories belonging to this organization or network.

EXAMPLE 1 Laboratories belonging to one public or private organization.

EXAMPLE 2 Network of federal, state and/or provincial laboratories.

EXAMPLE 3 Network of national reference laboratories coordinated by a European Union reference laboratory.

If possible, more than four laboratories should participate, so that results from at least eight technicians are available for analysis even if, for some reason, certain data cannot be used.

The protocol is as follows.

— In cases where different enrichment protocols for the alternative method exist, the most challenging enrichment protocol shall be selected, e.g. the protocol having the shortest incubation time or the most selective enrichment conditions. The selected (food) type shall be relevant for the chosen enrichment protocol. The (food) item, which is used to prepare the test samples, should contain natural background microbiota.

— The selected (food) item shall be artificially contaminated with the target organism. The protocol for inoculation of the samples shall be appropriate for the selected (food) item. Samples shall be prepared by the organizing laboratory to ensure homogeneity between and within samples using preparation protocols contained in ISO 16140-2:2016, Annex C. In general, liquid samples (compared to solid samples) give greater assurance of homogeneity. The samples should be shown to be homogeneous by the organizing laboratory. Homogeneity tests and criteria for acceptance are described in ISO 22117.

— At least three different levels of contamination shall be used: a blank (L_0) and two levels (L_1 and L_2). Level L_1 should be between the LOD_{50} of the reference method ($LOD_{50,ref}$) and the LOD_{50} of the alternative method ($LOD_{50,alt} = RLOD \cdot LOD_{50,ref}$). Level L_2 should be 1 $\log_{10}$ above level L_1. Level L_1 shall produce fractional positive results.

— Inocula should be enumerated using a non-selective medium. Enumeration shall be performed as described in ISO 7218.

— For the selected (food) item, for each setting and for each laboratory, four replicates shall be conducted at level $L_{1.}$ One replicate is conducted at the two other levels L_0 and L_2.

— According to the factorial, orthogonal design, eight settings shall be conducted by each laboratory for both test methods (alternative and reference) and for all levels of contamination (L_0, L_1 and L_2). In total 96 tests (1 + 4 + 1 replicates × 8 settings × 2 methods) are conducted in each laboratory.

— All samples should be blind-coded to ensure that the technicians are not aware of their level of contamination.

— Settings shall be common to both test methods (paired and unpaired). Paired results are obtained when the primary enrichment is the same for the alternative and reference method. That is, one test portion is used to conduct tests according to the alternative and the reference method. Unpaired results are obtained when the alternative and reference methods use different primary enrichments. That is, two sets of test portions are required: one test portion is analysed by the alternative method and another test portion by the reference method.

— The data are reported in two tables, giving the results from the reference method and from the alternative method before and after confirmation of the results.

  — If the results for alternative and reference methods have been obtained from the same initial enrichment broth (paired data), there is no need to confirm the presumptive results of the alternative method if the results agree with that obtained with the reference method. However, confirmation of the positive result is required when the reference method gives a negative result and the alternative method gives a positive result.

  — If the results for alternative and reference methods have been obtained from different enrichments (unpaired data), then all enrichments obtained with the alternative method shall be taken forward for confirmation. Confirmation pathways are described in ISO 16140-2:2016, 5.1.3.3.

— The organizing laboratory can indicate that broths, plates and/or isolates shall be retained for a certain period of time to enable confirmation of results obtained by a laboratory, if needed.

— The analysis of samples shall be performed by each laboratory on the stipulated date.

5.2.3 Selection of the factors to be studied

Decisions on the most suitable factors for the particular study should be based on expert knowledge. For example, optimal conditions are specified in each method, e.g. incubation temperature and duration at 37° C and 24 h, and these will give the best results. However, ranges around these, due to inaccuracies of the instrument, but which provide still acceptable conditions (e.g. 37 °C ± 1 °C, 24 h ± 1 h), are permitted and the study design should test the extremes of these. Acceptable operating conditions for equipment are described in ISO 7218. Other influences such as stress conditions can also be taken into account.

To estimate the accuracy under routine conditions, relevant method factors that are difficult to control shall be selected and varied systematically, e.g. technicians, culture media and incubation conditions. The choice of these factors and factor levels is crucial to the reliability of the test result. For unpaired study designs, chosen settings biased against the reference method cannot be used.

Five relevant method factors shall be varied simultaneously, each on two levels.

