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Microbiology of the food chain — Method validation —

Part 6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures

Microbiologie de la chaîne alimentaire — Validation des méthodes —

Partie 6: Protocole pour la validation de méthodes alternatives (commerciales) pour la confirmation microbiologique et le typage

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Foreword

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

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

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*.

A list of all parts of 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 validated 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 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. 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.

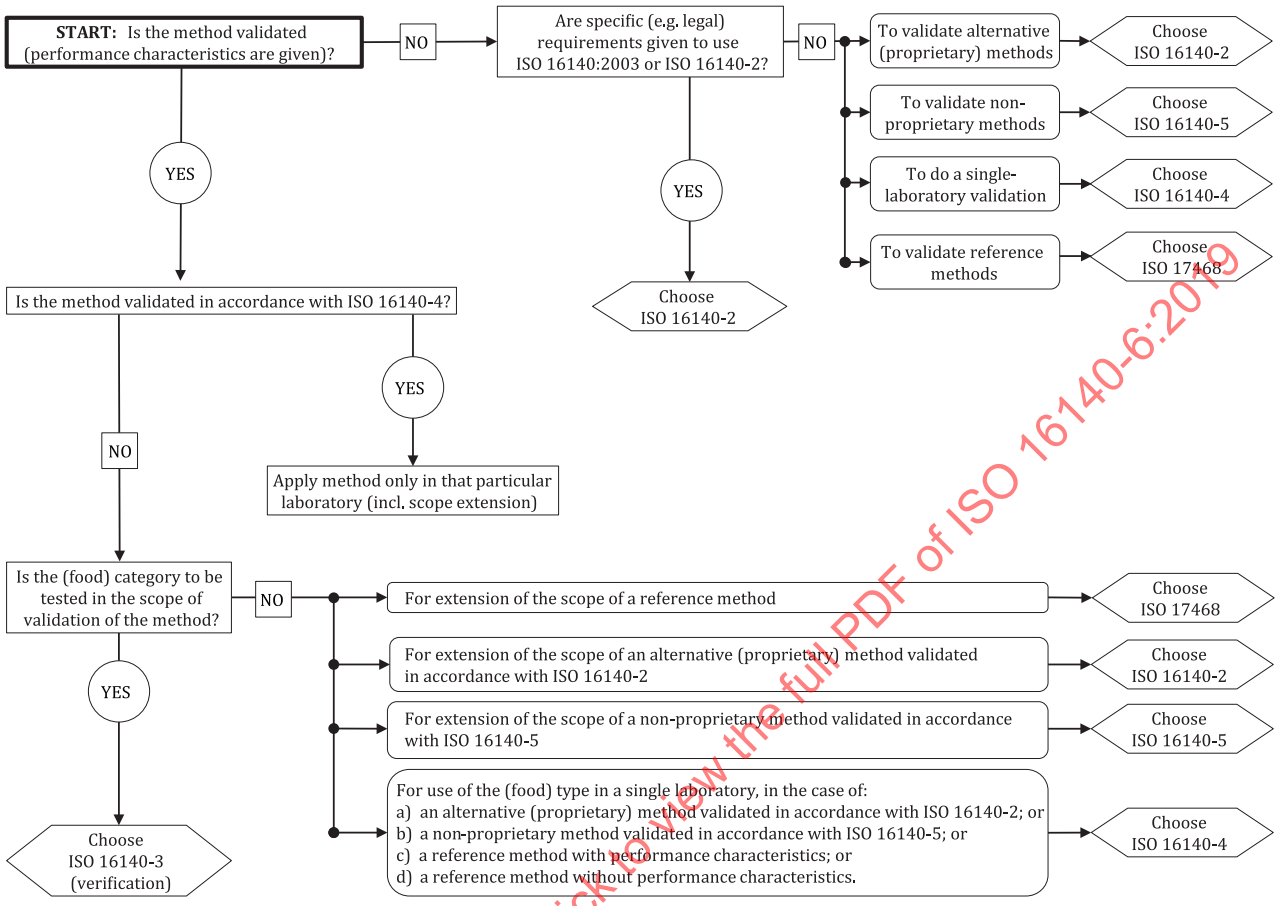

Figure 1 — Flow chart for application of the ISO 16140 series

NOTE 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.

This document, 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 this document. The validation study in this document 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 this document can be applied (see text in the boxes).

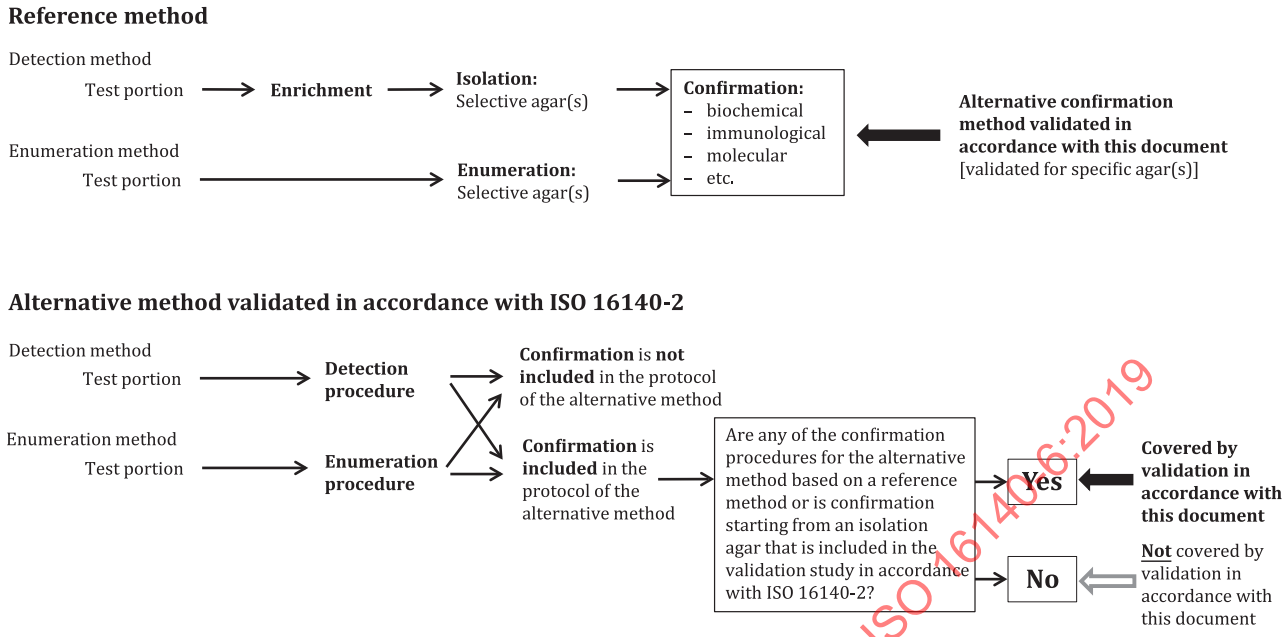

Figure 2 — Use of validated alternative confirmation methods (described in this document)

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 Validation and verification of methods for the microbiological confirmation and typing procedures

The procedure described in this document is intended for the "full" validation of alternative (proprietary) methods for microbiological confirmation and/or typing, hereafter referred to as "alternative confirmation methods".

During the validation study, the performance of the alternative confirmation method is compared to the performance of the reference confirmation procedure.

The procedure for verification of alternative confirmation methods in a single laboratory is described in ISO 16140-3.

Microbiology of the food chain — Method validation —

Part 6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures

1 Scope

This document specifies the general principle and the technical protocol for the validation of alternative confirmation methods for microbiology in the food chain. This document compares the result of the alternative confirmation method against the confirmation procedure of a reference method or, if needed, a reference confirmation method (e.g. whole genome sequencing).

This document is applicable to the validation of alternative confirmation methods used for the analysis (detection or quantification) of isolated 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.

Validated alternative confirmation methods can be used to replace (partly or completely) the confirmation procedure described in:

— the reference method;

— an alternative method validated in accordance with ISO 16140-2 only if one of the isolation agars specified in the validation study of the alternative confirmation method is used.

This document is also applicable to the validation of alternative typing methods, where the reference method can be, for example, a serological method (e.g. serotyping of *Salmonella*) or a molecular method (e.g. typing of Shiga toxin-producing *E. coli*).

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

Validation studies in accordance with this document are primarily intended to be performed by organizations or expert laboratories involved in method validation, but can also be used by a single laboratory, especially when performing in-house validation under certain conditions (see ISO 16140-4).

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 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
acceptability limit
AL
maximum positive or negative acceptable difference between the reference value (or if not known, the accepted reference value) of a sample and an individual result obtained when applying the operating procedure of an analytical method

Note 1 to entry: Annex D provides further information on the use of AL for this document.

[SOURCE: ISO 16140-1:2016, 2.1, modified — Note 1 to entry has been replaced.]

3.2
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.

Note 2 to entry: For clarity of reading, the text in this document generally describes validation of a confirmation method (detailed examples are given in Annex B). If applicable, this can be read as validation of a typing method (detailed examples are given in Annex C).

3.3
confirmation procedure
number of defined *confirmation tests* (3.4) that are performed on a strain, the combined results of which are used to definitively confirm the identity of that strain

3.4
confirmation test
single test which is carried out to verify a presumptive result

Note 1 to entry: The result of a single test may not on its own be able to definitively confirm the identity of the strain.

[SOURCE: ISO 16140-1:2016, 2.17, modified — In the term and definition, "procedure or" has been removed, "single" has been added and the Note 1 to entry has been replaced.]

3.5
microbial (sub)type
group of closely related microorganisms (within a species) distinguished by their shared specific characteristics as determined by, for example, serological testing (serotype) or molecular testing (genotype)

3.6
non-target strain
strain, defined according to the scope of the reference method, that would not reasonably be expected to be confirmed by the alternative method

[SOURCE: ISO 16140-1:2016, 2.44, modified — In the definition, "confirmed" has replaced "detected or enumerated".]

3.7
reference confirmation or typing procedure
combination of the confirmation or typing tests that are claimed to be replaced by the *alternative confirmation or typing method* (3.2)

Note 1 to entry: The number of *confirmation tests* (3.4) depends on the reference method for the specific microorganisms. The number of confirmation tests can also be one.

3.8
target strain
strain, defined according to the scope of the reference method, that is expected to be confirmed by the alternative method

[SOURCE: ISO 16140-1:2016, 2.74, modified — In the definition, "confirmed" has replaced "detected or enumerated".]

3.9
typing procedure
process of determining a particular *microbial (sub)type* (3.5)

4 General principles for the validation of confirmation and typing methods

In the validation study, the alternative confirmation method is compared to the confirmation procedure described in the reference method for the enumeration or detection of specific (groups of) microorganisms.

The validation protocol comprises two phases:

— a method comparison study, of the alternative confirmation method against the reference confirmation procedure, carried out in the organizing laboratory;

— an interlaboratory study.

NOTE It is possible, if relevant, to include inclusivity or exclusivity data obtained in an ISO 16140-2 validation study into a study related to this document.

The validation protocol shall clearly define the selective agar(s) from which strains can be confirmed using the alternative confirmation method. All inclusivity and exclusivity strains shall be tested. In cases where some strains are unable to grow on the specified selective agar(s), a non-selective agar plate shall also be included in the method comparison study and the interlaboratory study.

The technical rules for performing the method comparison study and the interlaboratory study are given in Clauses 6 and 7. The following six cases are covered; a distinction is made between the confirmation/typing of *Salmonella* and that of other microorganisms:

— validation of methods used for confirmation to the family level (non-*Salmonella*);

— validation of methods used for confirmation to the genus level (non-*Salmonella*);

— validation of methods used for confirmation to the species level (non-*Salmonella*);

— validation of methods used for confirmation/typing to the microbial (sub)type level (non-*Salmonella*);

— validation of methods used for confirmation/typing to the *Salmonella* genus or species level;

— validation of methods used for confirmation/typing to the *Salmonella* serovar level.

5 Strains

The pure strains used for determining the inclusivity and the exclusivity shall be well-characterized in line with the purpose of the validation study. The identification information of each strain will be used to (additionally) confirm the result in cases of discrepancies between the results of the reference confirmation procedure and the alternative confirmation method.

NOTE National, regional or international reference laboratories could be contacted during such investigations.

6 Method comparison study

6.1 General

The method comparison study is the part of the validation that is performed in one laboratory. It consists of an inclusivity and exclusivity study of the alternative confirmation method. The results are then compared to those of the reference confirmation procedure.

6.2 Selection of test strains

A range of strains shall be used. Criteria for selecting test strains are given in Annex A. The strains selected should take into account the measurement principle (e.g. culture-based, immunological, molecular-based) of the alternative method. Different measurement principles may require the use of panels of different test strains, representing the diversity of the studied microorganism(s). It is important to include non-target microorganisms that may grow on the media used for the reference and for the alternative method, including those that produce suspect colonies (i.e. look like those produced by the target strains).

The rationale for the choice of the strains and their characteristics shall be included in the validation study report.

Each strain shall be characterized biochemically and/or serologically and/or genetically in sufficient detail for its identity to be known. Strains should preferably have been isolated from foods, feed, the food-processing environment or from primary production; depending on the scope of the validation. However, clinical, environmental and culture collection strains can also be used. The original source of all strains should be known, and they should be held in a local (e.g. expert laboratory), national or international culture collection to enable them to be used in future testing if required. See ISO 11133 for guidance on the local maintenance of stock cultures.

Results generated by a specialized reference laboratory, using the reference method, can be used if the laboratory performing the validation study is not able to perform the confirmation/typing of rare strains according to the reference method. For example, the use of serotyping results of a *Salmonella* reference laboratory is allowed in cases of rare *Salmonella* serovars.

6.3 Inclusivity study

6.3.1 Testing of target strains

Pure cultures of all target strains shall be tested with both the reference confirmation procedure and the alternative confirmation method. It is not necessary to repeat the reference confirmation procedure along with the alternative confirmation method if the required data for the reference procedure are available. As all inclusivity strains shall be tested, subculture the strains on a non-selective agar plate, along with the clearly defined selective agar(s) from which strains can be confirmed using the alternative confirmation method. This will ensure that a viable strain is available for confirmation.

The number of strains to be tested under the various options (see 6.3.2 to 6.3.7) are summarized in Tables D.1 and D.2.

In cases where the required number of specific target strains are not available, the number of strains to be tested could be reconsidered, taking into account factors such as the frequency of occurrence and the public health or spoilage significance of these specific strains. There should be a minimum of 5 strains per microbial (sub)type.

6.3.2 Family level (non-*Salmonella*)

For inclusivity, a minimum of 200 different target strains shall be tested.

EXAMPLE The alternative method claims to confirm *Enterobacteriaceae*. A minimum of 200 different strains of *Enterobacteriaceae* are included in the inclusivity study.

6.3.3 Genus level (non-*Salmonella*)

For inclusivity, a minimum of 150 different target strains shall be tested.

EXAMPLE The alternative method claims to confirm *Listeria* spp. A minimum of 150 different strains of *Listeria* spp. are included in the inclusivity study.

6.3.4 Species level (non-*Salmonella*)

For inclusivity, a minimum of 100 different target strains per claimed species shall be tested.

EXAMPLE The alternative method claims to confirm *L. monocytogenes*. A minimum of 100 different strains of *L. monocytogenes* are included in the inclusivity study.

6.3.5 Microbial (sub)type level (non-*Salmonella*)

For inclusivity, a minimum of 25 different target strains per claimed microbial (sub)type shall be tested.

If 5 or more microbial (sub)types are claimed, then a minimum of 100 strains shall be tested. These should be proportionally divided according to the distribution and availability of the various microbial (sub)types under study, with a minimum of 5 strains per microbial (sub)type.

EXAMPLE The alternative method claims to confirm *E. coli* O157, *E. coli* O111, *E. coli* O26, *E. coli* O103 and *E. coli* O145. Therefore, at least 20 different strains of each of these 5 serogroups are included in the inclusivity study, taking into account the minimum of 100 strains in total.

6.3.6 *Salmonella* genus or species level

For inclusivity, a minimum of 150 different target strains shall be tested.

— If the alternative confirmation method claims confirmation of *Salmonella* spp., target strains include at least 2 strains each of *S. bongori, S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, *S. enterica* subsp. *indica*. Supplement these with strains of *S. enterica* subsp. *enterica,* covering common serovars. Preferably, include at least one representative of each (somatic) O-antigen described (see ISO/TR 6579-3).

— If the alternative confirmation method claims confirmation of *S. enterica*, target strains include at least 2 strains each of *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, *S. enterica* subsp. *indica*. Supplement these with strains of *S. enterica* subsp. *enterica* covering common serovars. Preferably, include at least one representative of each (somatic) O-antigen described (see ISO/TR 6579-3).

— If the alternative confirmation method claims confirmation of *S. enterica* subsp. *enterica*, target strains include different strains (and serovars) of *S. enterica* subsp. *enterica* only, covering common serovars. Preferably, include at least one representative of each (somatic) O-antigen described (see ISO/TR 6579-3).

NOTE More information on common serovars can be found on the following websites: www.cdc.gov/, www.ecdc.europa.eu/ and www.efsa.europa.eu/.

6.3.7 *Salmonella* serovar level

For inclusivity, a minimum of 25 different target strains per claimed *Salmonella* serovar shall be tested.

If 11 or more serovars are claimed, then a minimum of 250 strains shall be tested. These should be proportionally divided, according to the general serovar distribution.

In cases where the required number of specific target strains are not available, the number of strains to be tested could be reconsidered, taking into account factors such as the frequency of occurrence and the public health significance of these specific strains. There should be a minimum of 5 strains per serovar.

EXAMPLE The alternative method claims to confirm *Salmonella* Enteritidis and *Salmonella* Typhimurium. A minimum of 25 different strains of *Salmonella* Enteritidis and 25 different strains of *Salmonella* Typhimurium are included in the inclusivity study.

6.4 Exclusivity study

6.4.1 Testing of non-target strains

Pure cultures of all non-target strains shall be tested with both the reference confirmation procedure and the alternative confirmation method. It is not necessary to repeat the reference confirmation procedure along with the alternative confirmation method if the required data for the reference procedure are available. As all exclusivity strains shall be tested, subculture the strains on a non-selective agar plate, along with the clearly defined selective agar(s) from which strains can be confirmed using the alternative confirmation method. This will ensure that a viable strain is available for confirmation.

The number of strains to be tested under the various options (see 6.4.2 to 6.4.7) are summarized in Tables D.1 and D.2.

Preferably, select non-target strains that are able to grow on the selective agar(s) as used in the reference method.

6.4.2 Family level (non-*Salmonella*)

For exclusivity, a minimum of 100 different non-target strains shall be tested.

6.4.3 Genus level (non-*Salmonella*)

For exclusivity, a minimum of 100 different non-target strains shall be tested.

6.4.4 Species level (non-*Salmonella*)

For exclusivity, a minimum of 100 different non-target strains shall be tested, of which there are:

— 50 strains from non-target genus;

— 50 strains from non-target species within the target genus.

EXAMPLE The alternative method claims to confirm *L. monocytogenes*. A minimum of 50 different strains of non-*Listeria* spp. and 50 different strains of *Listeria* spp., but not *L. monocytogenes,* are included in the exclusivity study.

6.4.5 Microbial (sub)type level (non-*Salmonella*)

For exclusivity, a minimum of 100 different non-target strains shall be tested, of which there are:

— 25 strains from non-target family;

— 25 strains from target family or target genus (but not the target species);

— 50 strains from non-target microbial (sub)type within the target species.

EXAMPLE The alternative method claims separately to confirm *E. coli* O157, *E. coli* O111, *E. coli* O26, *E. coli* O103 and *E. coli* O145. A minimum of 25 different strains of non-*Enterobacteriaceae*, 25 different strains of *Enterobacteriaceae* (but non-*E. coli*) and 50 different strains of *E. coli* serogroups other than the 5 serogroups mentioned above are included in the exclusivity study.

6.4.6 *Salmonella* genus or species level

For exclusivity, a minimum of 100 different non-target strains shall be tested.

— If the alternative confirmation method claims confirmation of *S. enterica,* include:

  — at least 2 strains of *S. bongori*;

  — at least 75 (to make up to the total of 100) strains from the target family (*Enterobacteriaceae*).

— If the alternative confirmation method claims confirmation of *S. enterica* subsp. *enterica*, include:

  — at least 2 strains each of *S. bongori*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica*;

  — at least 75 (to make up to the total of 100) strains from the target family (*Enterobacteriaceae*).

6.4.7 *Salmonella* serovar level

For exclusivity, a minimum of 100 different non-target strains shall be tested.

If the alternative method claims typing of all *Salmonella* serovars, a total of 100 different strains from non-target genus, but within target family (*Enterobacteriaceae*), shall be tested.

If the alternative method claims typing of specific *Salmonella* serovars, include:

— at least 25 strains from non-target genus, but within target family (*Enterobacteriaceae*);

— at least 75 strains from non-target serovars within the target subspecies. These should include non-target serovars with partly the same O-antigens or H-antigens as the target *Salmonella* serovars.

EXAMPLE The alternative method claims to confirm *Salmonella* Enteritidis and *Salmonella* Typhimurium. A minimum of 25 different strains of *Enterobacteriaceae* (but non-*Salmonella* spp.) and 75 strains of 75 different *Salmonella* serovars other than *Salmonella* Enteritidis and *Salmonella* Typhimurium are included in the exclusivity study. These 75 strains include non-target serovars with partly the same O-antigens or H-antigens as *Salmonella* Enteritidis (1,9,12:g,m:-) and *Salmonella* Typhimurium (1,4,[5],12:i:1,2).

6.5 Expression and interpretation of results

Tabulate the results for inclusivity (see 6.3) as in Table 1 and include the final interpretation in accordance with Table 2. Tabulate the results for exclusivity (see 6.4) as in Table 3 and include the final interpretation in accordance with Table 4. Any discrepancy in results needs to be investigated by the organizing laboratory, using relevant identification or characterization methods (e.g. DNA sequencing). The outcome, preferably tabulated, with an explanation, shall be included in the validation study report.

NOTE Detailed examples are given in Annex B (validation of an alternative confirmation method) and Annex C (validation of an alternative typing method).

Table 1 — Presentation of the results for inclusivity

Target strain	Characteristics of the strain	Final result (+/−)		Final interpretation of results in accordance with Table 2 (IA/ID)	Comments
		Reference confirmation procedure	Alternative confirmation method		
1					
2					
3					
Etc.					

Key

+: positive result, indicating the strain is confirmed to be the target as defined by the procedure of the method

–: negative result, indicating the strain is not confirmed to be the target as defined by the procedure of the method

IA: inclusivity agreement, ID: inclusivity deviation

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.

Table 2 — Comparison and interpretation of results between the reference and alternative methods for the inclusivity study

Result per strain			First interpretation	Second interpretation (if applicable)	Final interpretation
Reference confirmation procedure	Alternative confirmation method	Identity of the (target) strain	Alternative confirmation method compared to reference confirmation procedure	Alternative confirmation method compared to identity of the strain	
+	+	+	PA	Not applicable	IA
+	–	+	FN	ID	ID
–	+	+	PD	IA	IA
–	–	+	NA	Not applicable	IA

Key

+: positive result, indicating the strain is confirmed to be the target as defined by the procedure of the method

–: negative result, indicating the strain is not confirmed to be the target as defined by the procedure of the method

PA: positive agreement, FN: false negative, PD: positive deviation, NA: negative agreement, IA: inclusivity agreement, ID: inclusivity deviation

NOTE The third and fourth rows of results in this table are unlikely outcomes in an inclusivity study.

Table 3 — Presentation of the results for exclusivity

Non-target strain	Characteristics of the strain	Final result (+/−)		Final interpretation of results in accordance with Table 4 (EA/ED)	Comments
		Reference confirmation procedure	Alternative confirmation method		
1					
2					
3					
Etc.					

Key

+: positive result, indicating the strain is confirmed to be the target as defined by the procedure of the method

−: negative result, indicating the strain is not confirmed to be the target as defined by the procedure of the method

EA: exclusivity agreement, ED: exclusivity deviation

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.

Table 4 — Comparison and interpretation of results between the reference and alternative methods for the exclusivity study

Result per strain			First interpretation	Second interpretation (if applicable)	Final interpretation
Reference confirmation procedure	Alternative confirmation method	Identity of the (non-target) strain	Alternative confirmation method compared to reference confirmation procedure	Alternative confirmation method compared to identity of the strain	
−	−	−	NA	Not applicable	EA
−	+	−	FP	ED	ED
+	+		PA	Not applicable	EA
+	−	−	FN	EA	EA

Key

+: positive result, indicating the strain is confirmed to be the target as defined by the procedure of the method

−: negative result, indicating the strain is not confirmed to be the target as defined by the procedure of the method

PA: positive agreement, FN: false negative, FP: false positive, NA: negative agreement, EA: exclusivity agreement, ED: exclusivity deviation

NOTE The third and fourth rows of results in this table are unlikely outcomes in an exclusivity study.

6.6 Evaluation

Summarize the results of the method comparison study, as in Table 5 (in total numbers of IA, ID, EA and ED found), based on the interpretations in accordance with Table 1 (inclusivity) and Table 3 (exclusivity).

Table 5 — Summary of the results in the method comparison study

	Number of strains	IA	ID	EA	ED
Inclusivity				Not applicable	Not applicable
Exclusivity		Not applicable	Not applicable		

Key

IA: inclusivity agreement, ID: inclusivity deviation, EA: exclusivity agreement, ED: exclusivity deviation

Use Table 6 to evaluate the results, as summarized in Table 5, in accordance with the acceptability limits (ALs) given in Table D.1 (non-*Salmonella*) or Table D.2 (*Salmonella*). The AL is not met when the observed value is higher than the AL. When the AL is not met, investigations should be conducted (e.g. root cause analysis) in order to provide an explanation for the observed results. The fitness for purpose of the alternative confirmation method is then determined, based on the AL and the additional information from the investigation. The reasons for acceptance of the alternative confirmation method, when the AL is not met, shall be stated in the validation study report.

NOTE Detailed examples are given in Annex B (validation of an alternative confirmation method) and Annex C (validation of an alternative typing method).

Table 6 — Evaluation of the method comparison study results

	AL	ID	ID ≤ AL	ED	ED ≤ AL	Evaluation
Inclusivity				Not applicable	Not applicable	
Exclusivity		Not applicable	Not applicable			
Key AL: acceptability limit, ID: inclusivity deviation, ED: exclusivity deviation NOTE The AL values are taken from Table D.1 (non-*Salmonella*) or Table D.2 (*Salmonella*).						

7 Interlaboratory study

7.1 General

The aim of the interlaboratory study is to determine the variability of the results obtained by different collaborators using the same strains (reproducibility conditions). Whenever possible, the study conditions should reflect the normal variation between laboratories.

7.2 Data sets to be obtained

The interlaboratory study shall produce 10 valid data sets from at least 10 collaborators. The collaborators shall come from a minimum of 5, but preferably 10, different organizations, excluding the organizing laboratory. Technicians, involved in the preparation of the strains used in the interlaboratory study, shall not take part in the testing of those strains within the interlaboratory study.

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

— A total of 24 strains shall be tested per collaborator and all strains shall be tested with the reference confirmation procedure and the alternative confirmation method.

— The interlaboratory study will generally use strains with a positive result in the reference confirmation procedure for the inclusivity study and strains with a negative result in the reference confirmation procedure in the exclusivity study.

— For inclusivity: a total of 16 different target strains.

— For exclusivity (in general): a total of 8 different non-target strains.

— For exclusivity (non-*Salmonella*):

  — in the case of confirmation to the species level: a total of 8 different non-target strains: 4 strains from non-target genus and 4 strains from non-target species within the target genus;

    EXAMPLE 1 The alternative method claims to confirm *L. monocytogenes*.

    — Inclusivity part: 16 different strains of *L. monocytogenes*.

— Exclusivity part: *Bacillus cereus*, *Enterococcus faecalis*, *Lactobacillus helveticus*, *Staphylococcus aureus*, *L. ivanovii*, *L. innocua*, *L. welshimeri* and *L. seeligeri*.

— in the case of confirmation to the microbial (sub)type level: a total of 8 different non-target strains; 4 strains from non-target species within the target family and 4 strains from non-target microbial (sub)types within the target species.

EXAMPLE 2 The alternative method claims to confirm *E. coli* O157, *E. coli* O111, *E. coli* O26, *E. coli* O103 and *E. coli* O145.

— Inclusivity part: 3 different strains each of *E. coli* O157, *E. coli* O111, *E. coli* O26, *E. coli* O103 and *E. coli* O145, plus 1 additional strain from the microbial (sub)types under study, making the total number of strains to 16.

— Exclusivity part: *Citrobacter freundii, Escherichia vulneris, Hafnia alvei, Salmonella enterica, E. coli* O6, *E. coli* O55, *E. coli* O78 and *E. coli* O128.

— For exclusivity (*Salmonella*):

— in the case of confirmation to the *Salmonella* genus or species level: a total of 8 different non-target strains: 7 strains from non-target genus, but within target family *Enterobacteriaceae* and 1 strain from non-target family;

EXAMPLE 3 The alternative method claims to confirm *Salmonella* spp.

— Inclusivity part: 16 different strains (and serovars) of *Salmonella* spp.

— Exclusivity part: *Citrobacter freundii, Cronobacter sakazakii, Enterobacter cloacae, Escherichia coli, Hafnia alvei*, *Serratia marescens, Yersinia enterocolitica* and *Aeromonas hydrophila*.

— in the case of confirmation to the *Salmonella* serovar level: a total of 8 different non-target strains: 4 strains from non-target genus, but within target family *Enterobacteriaceae* and 4 strains from non-target serovars within the target subspecies. These should be non-target serovars with partly the same O-antigens or H-antigens as the target *Salmonella* serovars.

EXAMPLE 4 The alternative method claims to confirm *Salmonella* Enteritidis (1,9,12:g,m:-) and *Salmonella* Typhimurium (1,4,[5],12:i:1,2).

— Inclusivity part: 8 different strains of *Salmonella* Enteritidis and 8 different strains of *Salmonella* Typhimurium.

— Exclusivity part: *Citrobacter freundii, Enterobacter cloacae, Escherichia coli, Hafnia alvei*, *Salmonella* Hato (1,4,[5],12:g,m,s:[1,2]), *Salmonella* Lagos (1,4,[5],12:i:1,5), *Salmonella* Mendoza (9,12:l,v:1,2) and *Salmonella* Seremban (9,12:i:1,5).

7.3 Protocol

Strains shall be prepared by the organizing laboratory to ensure homogeneity between shipments (originating from the one purified colony per test strain).

Depending on the characteristics of the strains to be sent, shipment of strains can take place on, for example, agar transport swabs, agar tubes or non-selective agar plates.

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

The organizing laboratory should provide the participants with adequate information on safe handling of the strains upon arrival and storage, and protocol(s) for the testing and reporting of results.

The analysis of the strains may start from the isolation of the strains on non-selective agar. The testing of the strains shall be performed by the reference confirmation procedure and by the alternative confirmation method.

If the alternative confirmation method claims to be able to directly confirm from selective agars, at least one of these selective agars shall be included in the interlaboratory study as the starting point of analysis.

The organizing laboratory can indicate that plates and/or strains shall be retained for a certain period to be able to confirm results obtained by a collaborator, if needed.

The analysis of strains shall be performed by each collaborator within a stipulated time frame.

A minimum of 480 results (240 by each method) are required for use in the interpretation.

When the interlaboratory study is completed, all the information on data sheets and the results shall be submitted to the organizing laboratory and examined. Disregard data from collaborators:

— who received strains/test kits, etc. that were damaged during transportation;

— using media formulations that are not in accordance with the method;

— if the reported technical results suggest that the laboratory has deviated from either the testing protocol(s) or the critical operating conditions.

7.4 Expression of results

Tabulate the results for the interlaboratory study as given in Table 7.

Any result discrepancies shall be included in the validation study report, accompanied by an explanation.

Table 7 — Presentation of the results for the interlaboratory study

Collaborators	Number of correctly confirmed strains in the inclusivity part		Number of correctly non-confirmed strains in the exclusivity part	
	Reference confirmation procedure	Alternative confirmation method	Reference confirmation procedure	Alternative confirmation method
Collaborator 1	/16	/16	/8	/8
Collaborator 2	/16	/16	/8	/8
Etc.	/16	/16	/8	/8
Collaborator 10	/16	/16	/8	/8
Total	/160	/160	/80	/80

7.5 Interpretation and evaluation

Summarize the results of the interlaboratory study as in Table 8 (in total numbers of IA, ID, EA and ED found), based on the interpretations in accordance with Table 2 (inclusivity) and Table 4 (exclusivity).

Table 8 — Summary of the results in the interlaboratory study

	Number of strains	IA	ID	EA	ED
Inclusivity				Not applicable	Not applicable
Exclusivity		Not applicable	Not applicable		
Key IA: inclusivity agreement, ID: inclusivity deviation, EA: exclusivity agreement, ED: exclusivity deviation					

Use Table 9 to evaluate the results as summarized in Table 8.

The AL is not met when the observed value is higher than the AL listed in Table D.3. When the AL is not met, investigations should be conducted (e.g. root cause analysis) in order to provide an explanation for the observed results. The fitness for purpose of the alternative confirmation method is then determined, based on the AL, and the additional information from the investigation. The reasons for acceptance of the alternative confirmation method, when the AL is not met, shall be stated in the validation study report.

Table 9 — Evaluation of the interlaboratory study results

	AL	ID	ID ≤ AL	ED	ED ≤ AL	Evaluation
Inclusivity				Not applicable	Not applicable	
Exclusivity		Not applicable	Not applicable			
Key AL: acceptability limit, ID: inclusivity deviation, ED: exclusivity deviation NOTE The AL values are taken from Table D.3.						

Annex A
(normative)

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

A.1 General

This annex outlines the minimum test requirements for general use. In the selection of test strains, the majority shall originate from foods, feed, the food-processing environment or the primary production, and should cover the recognized range of the target analyte with respect to the following: diversity in identification characteristics (e.g. biochemical, serovar, phage type), geographical distribution, incidence, and any other claims made by the producers of the alternative method. In addition, (food-borne disease related) clinical, environmental and culture collection strains can also be used.

A.2 Target group categories

a) Undefined group, e.g. total aerobic count, coliform, yeast, lactic acid bacteria.

b) Family, e.g. *Enterobacteriaceae*.

c) Genus, e.g. *Salmonella*, *Pseudomonas*, *Listeria*.

d) Species, e.g. *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli*.

e) Microbial (sub)type, e.g. *E. coli* O157, *Salmonella* Enteritidis.

A.3 Target group selection in inclusivity study

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

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

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

d) For species: use a range of strains from that species. For the selection of strains, more precise methods for subtyping and information on diversity of strains need to be considered. For example, *Salmonella* and *Listeria* are serotyped and/or typed with other (genetic) typing methods. In defining the positive strains to be used, organizing laboratories should use up-to-date information to ensure that strains are relevant to the target (food) categories, at the time of testing.

e) For microbial (sub)type: use a range of origins (such as different foods, different feed, etc.) of that microbial (sub)type.

A.4 Non-target groups selection in exclusivity study

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

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

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

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

e) When the target group is a microbial (sub)type: non-target strains shall include other microbial (sub)types.

Annex B
(informative)

Example of the validation of an alternative confirmation method to the species level (*Listeria monocytogenes*)

This annex shows an example of the validation of an alternative confirmation method to the species level for *Listeria monocytogenes* (see Tables B.1 to B.7 for further information). The reference confirmation procedure 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 alternative confirmation method is a commercially available PCR-test.

Method comparison study, inclusivity: 100 target strains that have been confirmed as *Listeria monocytogenes* by isolation 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 (see Annex A for further information on the selection of target strains).

Method comparison study, exclusivity: 100 non-target strains that have not been confirmed as *Listeria monocytogenes* as described for target strains that are likely to be relevant because of the development of colonies of similar appearance or the potential to cross react (see Table D.1 and Annex A for further information on non-target strains and their selection).

Interlaboratory study, inclusivity: 16 different target strains (16 different strains of *L. monocytogenes*).

Interlaboratory study, exclusivity: 8 different non-target strains: 4 strains from non-target genus and 4 strains from non-target species within the target genus (*Bacillus cereus*, *Enterococcus faecalis*, *Lactobacillus helveticus*, *Staphylococcus aureus*, *L. ivanovii*, *L. innocua*, *L. welshimeri* and *L. seeligeri*).

All 13 participants to the interlaboratory study tested the 24 strains by both the reference confirmation procedure and the alternative confirmation method.

The alternative confirmation method was successfully validated based on the results presented in Tables B.1 to B.7.

Table B.1 — Results for inclusivity in the method comparison study

Number of target strains (total no. 100)	Characteristics of the strain	Final result (+/−)		Final interpretation of results in accordance with Table 2 (IA/ID)	Com-ments
		Reference confirmation procedure	Alternative confirmation method		
99	*L. monocytogenes*	+	+	IA	None
1	*L. monocytogenes*	+	−	ID	None
Key +: positive result, indicating the strain is confirmed to be the target as defined by the procedure of the method −: negative result, indicating the strain is not confirmed to be the target as defined by the procedure of the method IA: inclusivity agreement, ID: inclusivity deviation					

Table B.2 — Results for exclusivity in the method comparison study

Number of non-target strains (total no. 100)	Characteristics of the strain	Final result (+/−)		Final interpretation of results in accordance with Table 4 (EA/ED)	Comments
		Reference confirmation procedure	Alternative confirmation method		
50	A variety of non-*Listeria* genera, e.g. *Bacillus*, *Jonesia*, *Escherichia*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Micrococcus*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*	–	–	EA	38 strains were not able to grow on the selective agar under study and were tested from non-selective agar only.
6	*L. grayi*	–	–	EA	Growth[a]
14	*L. innocua*	–	–	EA	Growth[a]
11	*L. ivanovii*	–	–	EA	Growth[a]
7	*L. seeligeri*	–	–	EA	Growth[a]
10	*L. welshimeri*	–	–	EA	Growth[a]
2	*L. ivanovii*	–	+	ED	Growth[a]

Key

+: positive result, indicating the strain is confirmed to be the target as defined by the procedure of the method

−: negative result, indicating the strain is not confirmed to be the target as defined by the procedure of the method

EA: exclusivity agreement, ED: exclusivity deviation

[a] All strains were able to grow on the selective agar under study.

Table B.3 — Summary of the results in the method comparison study

	Number of strains	IA	ID	EA	ED
Inclusivity	100	99	1	Not applicable	Not applicable
Exclusivity	100	Not applicable	Not applicable	98	2

Key

IA: inclusivity agreement, ID: inclusivity deviation, EA: exclusivity agreement, ED: exclusivity deviation

Table B.4 — Evaluation of the method comparison study results

	AL	ID	ID ≤ AL	ED	ED ≤ AL	Evaluation
Inclusivity	2	1	1 ≤ 2	Not applicable	Not applicable	Accepted
Exclusivity	2	Not applicable	Not applicable	2	2 ≤ 2	Accepted

Key

AL: acceptability limit, ID: inclusivity deviation, ED: exclusivity deviation

NOTE The AL values are taken from Table D.1.

Table B.5 — Results for the interlaboratory study

Collaborators	Number of correctly confirmed strains in the inclusivity part		Number of correctly non-confirmed strains in the exclusivity part	
	Reference confirmation procedure	Alternative confirmation method	Reference confirmation procedure	Alternative confirmation method
Collaborator 1	16/16	16/16	8/8	8/8
Collaborator 2	15/16	15/16	8/8	8/8
Collaborator 4	16/16	16/16	8/8	8/8
Collaborator 5	16/16	16/16	8/8	8/8
Collaborator 6	16/16	16/16	8/8	8/8
Collaborator 7	16/16	16/16	8/8	8/8
Collaborator 8	16/16	16/16	8/8	7/8
Collaborator 9	16/16	16/16	8/8	8/8
Collaborator 10	16/16	16/16	8/8	7/8
Collaborator 11	16/16	16/16	8/8	8/8
Collaborator 12	16/16	15/16	8/8	8/8
Collaborator 13	16/16	16/16	8/8	8/8
Total	191/192	190/192	96/96	94/96

NOTE 1 Data from the inclusivity study for collaborator 2 is assessed in line with Table 2.

NOTE 2 Data from collaborator 3 was excluded from interpretation, because of technical deviations from the testing protocol(s).

Table B.6 — Summary of the results in the interlaboratory study

	Number of strains	IA	ID	EA	ED
Inclusivity	192	191	1	Not applicable	Not applicable
Exclusivity	96	Not applicable	Not applicable	94	2

Key

IA: inclusivity agreement, ID: inclusivity deviation, EA: exclusivity agreement, ED: exclusivity deviation

Table B.7 — Evaluation of the interlaboratory study results

	AL	ID	ID ≤ AL	ED	ED ≤ AL	Evaluation
Inclusivity	2	1	1 ≤ 2	Not applicable	Not applicable	Accepted
Exclusivity	2	Not applicable	Not applicable	2	2 ≤ 2	Accepted

Key

AL: acceptability limit, ID: inclusivity deviation, ED: exclusivity deviation

NOTE The AL values are taken from Table D.3.

Annex C
(informative)

Example of the validation of an alternative typing method to the *Salmonella* serovar level (15 different serovars claimed)

This annex shows an example of the validation of an alternative typing method to the *Salmonella* serovar level (15 different serovars claimed), see Tables C.1 to C.7 for further information. 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.

Method comparison study, inclusivity: 250 target strains (see Table C.1).

Method comparison study, exclusivity: 100 non-target strains (see Table C.2).

Interlaboratory study, inclusivity: 16 different target strains (the 15 different serovars as claimed and 1 of these represented by 2 strains).

Interlaboratory study, exclusivity: 8 different non-target strains: 4 strains from non-target genus, but within target family *Enterobacteriaceae*, and 4 strains from non-target serovars within the target subspecies. These should be non-target serovars with partly the same O-antigens or H-antigens as the target *Salmonella* serovars (*Citrobacter freundii, Enterobacter cloacae, Escherichia coli, Hafnia alvei*, *Salmonella* Colindale (6,7:r:1,7), *Salmonella* Glostrup (6,8:z_{10}:e,n,z_{15}), *Salmonella* Panama (1,9,12:l,v:1,5) and *Salmonella* Saintpaul (1,4,[5],12:e,h:1,2)).

All 10 participants to the interlaboratory study tested the 24 strains by both the reference typing procedure and the alternative typing method.

The alternative typing method was successfully validated based on the results presented in Tables C.1 to C.7.