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AMENDMENT 1
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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 method comparison study data evaluation, relative level of detection calculations in the interlaboratory study, calculation and interpretation of the relative trueness study, and inclusion of a commercial sterility testing protocol for specific products

Microbiologie de la chaîne alimentaire — Validation des méthodes —

Partie 2: Protocole pour la validation de méthodes alternatives (commerciales) par rapport à une méthode de référence

AMENDEMENT 1: Révision de l'évaluation des données des études de comparaison de méthodes qualitatives, des calculs du niveau de détection de l'étude interlaboratoires et de l'interprétation de l'étude de justesse relative, et ajout d'un protocole pour la détermination de la stérilité commerciale pour des produits spécifiques

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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).

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.

Microbiology of the food chain — Method validation —

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

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Introduction

Replace the text with the following:

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 this document, ISO 16140-5 and ISO 16140-6). In the case when a method is validated within one laboratory (see ISO 16140-4), no interlaboratory study is conducted.

— The second stage is method verification, where a laboratory demonstrates that it can satisfactorily perform a validated method. This is described in 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 17468:2023, 3.7, 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 this document cannot be reached. ISO 16140-4 and ISO 16140-5 can be used for validation against a reference method. ISO 16140-4 (regarding qualitative and quantitative methods) and ISO 16140-5 (regarding quantitative methods only) can also be used for validation without a reference method.

The flow chart in Figure 0.1 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.

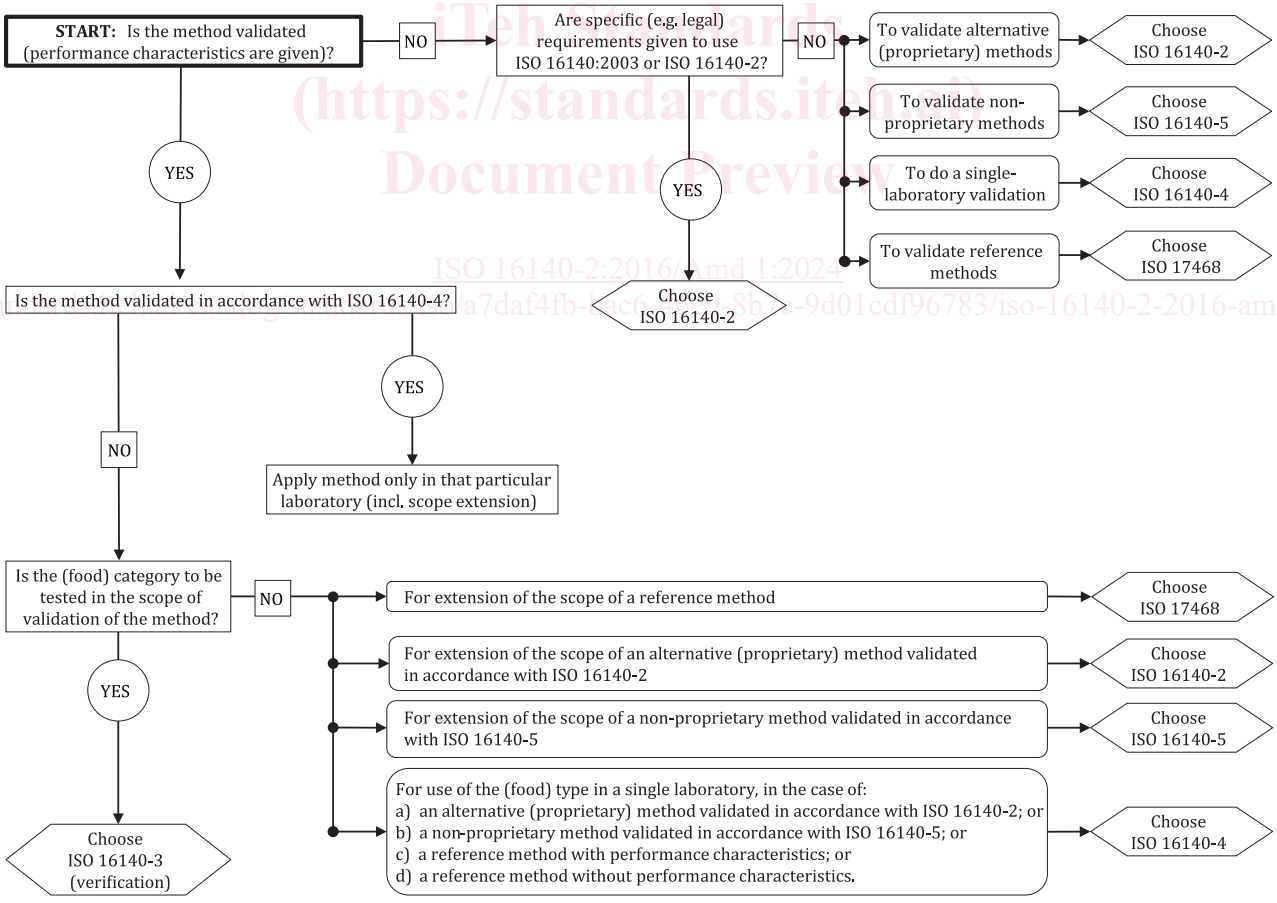

Figure 0.1 — Flow chart for application of the ISO 16140 series

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 0.2 shows the possibilities where an alternative confirmation method validated in accordance with ISO 16140-6 can be applied (see text in the boxes).

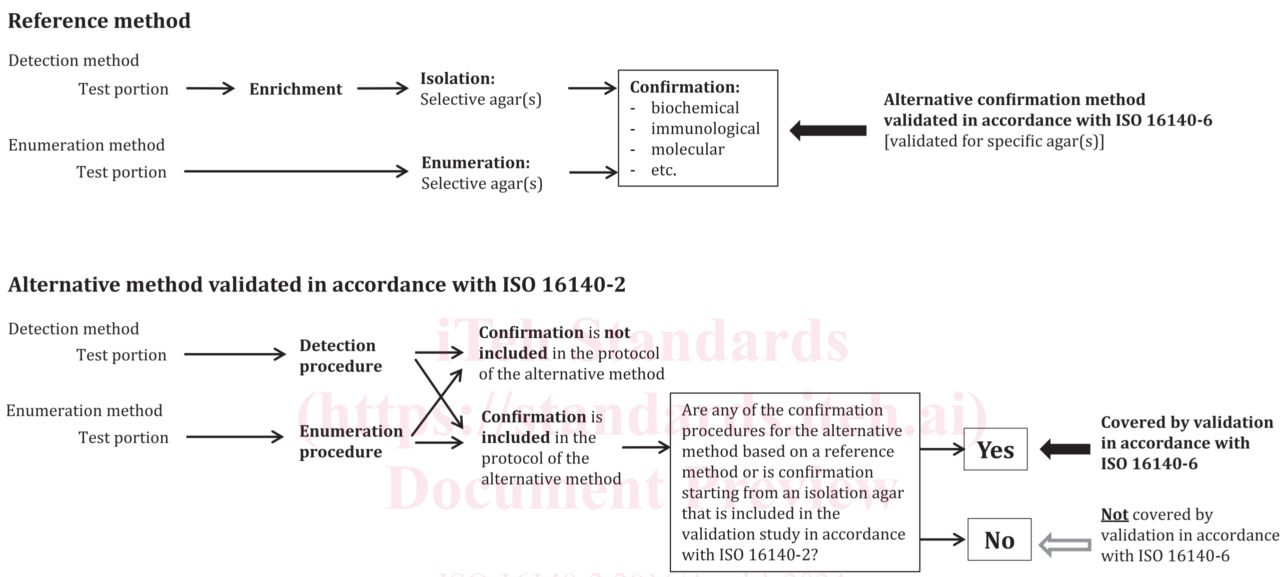

Figure 0.2 — Use of validated alternative confirmation methods (see ISO 16140-6)

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 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

This document describes the general approach to method validation in the field of microbiology of the food chain and serves as a fundamental basis to the other parts of the ISO 16140 series, which cross-reference to it. An understanding of the performance characteristics, the (food) categories, the technical protocol and data analysis as outlined in this document provides support in the application of the ISO 16140 series in general.

Clause 4

Add the following text at the end of the clause:

For the validation of an alternative qualitative method, a corresponding qualitative reference method is selected for carrying out the validation study. This is commonly done using test portions of 10 gram, 25 gram or higher. In some cases, it can be of interest to validate a qualitative alternative method against a quantitative reference method, using smaller test portion sizes.

EXAMPLE 1 *Enterobacteriaceae* criterion for pasteurized milk and other liquid pasteurized products in Regulation (EC) No 2073/2005 is < 10 cfu/ml and refers to the quantitative method ISO 21528-2.

In such situations, it is of interest to validate the performance of qualitative alternative methods against the specified (quantitative) reference method. To that end, the technical protocol for the validation of qualitative methods (see Clause 5) is to be used. For such a validation study, the quantitative results of the reference method have to be converted into qualitative results prior to interpretation according to Clause 5.

EXAMPLE 2 When one or more colonies are observed on a plate using 1 ml of a 10^{-2} dilution, this result corresponds to a positive detection in 0,01 gram.

NOTE Annex J provides the special case of validation of a method for commercial sterility testing for specific products [sterilized or ultra high temperature (UHT) dairy and plant-based liquid products].

If a technical change in a validated alternative (proprietary) method is evaluated as being major, a re-validation of this alternative method in accordance with this document is needed.

When the re-validation of the alternative 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 or minor 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 study shows that it has no significant impact on the performance characteristics or test results. A major (technical) change that, after re-validation, has a major impact on the performance characteristics of the alternative method, requires re-verification of the method by the user laboratory in accordance with ISO 16140-3.

5.1.1

Add the following text at the end of the subclause:

The organizing laboratory shall be competent to perform both the reference method as well as the alternative method.

NOTE Competence can be demonstrated in different ways (e.g. for the reference method, a documented proof of meeting the requirements of ISO/IEC 17025, and for the alternative method, a documented training).

5.1.2

Add the following text at the end of the subclause:

When the reference and alternative methods are based on two different principles and are performed with the same test portion, but do not share a common enrichment procedure, an unpaired data study is performed. For example, when a qualitative alternative method is validated against a quantitative reference method at a limit of 100 cfu/g. In this case, a suspension of the (food) item can be used to inoculate both culture media for the reference method and the alternative method before any enrichment/multiplication of the microorganism.

5.1.3.3

Add the following text at the end of the subclause:

The alternative method shall be evaluated for a defined test portion size (e.g. 25 g, 200 g, 375 g) during the validation study. The method is considered to be validated for any test portion size up to the validated test portion size if the testing protocol (dilution ratio, incubation time and incubation temperature) is the same as that used during the validation study.

EXAMPLE A reference method used in a validation study of this document was validated for a “broad range of foods” using a 25 g test portion and a 1:10 dilution ratio. The alternative method was validated for a “broad range of foods” using a 375 g test portion and a 1:5 dilution ratio at a determined incubation time. In practice, a user laboratory can use the alternative method for all food items (broad range of foods) using a test portion size up to 375 g test portion and a 1:5 dilution ratio at the validated incubation time (unless stated differently by the organization involved in the method validation).

5.1.3.4

Add the following text before the last sentence of the second paragraph:

The interpretation of the results (positive agreement, negative agreement, etc.) is based on a comparison of the reference method result (column 1 in Tables 1 and 2) and the alternative method result, including any confirmations as described in the alternative method protocol (column 2 in Tables 1 and 2). When positive or negative deviations are obtained, a footnote should be included at the end of each table to provide additional explanations for the interpretation of the deviations. The footnotes indicate if the result is due to a false positive or false negative result of the alternative method. The footnote is a comparison of the results of the alternative method (including any confirmations as described in the alternative-method protocol) (column 2 in Tables 1 and 2) and the confirmed alternative method (by any means) (column 3 in Tables 1 and 2).

5.1.3.4, after the second paragraph

Replace the text with the following:

Table 1 — Comparison and interpretation of sample results between the reference and alternative methods for a paired study

Result of the (reference or alternative) method per sample			
Reference method	**Alternative method** (including any confirmations as described in the alternative-method protocol)	**Confirmed alternative method** (by any means)[a]	**Interpretation** (based on the confirmed alternative-method result)
+	+	Not needed[b]	Positive Agreement (PA)
-	-	Not needed[b]	Negative Agreement (NA)
+	-	Not needed[b]	Negative Deviation due to false negative alternative-method result ($ND_{FN(alt)}$)
-	+	+	Positive Deviation (PD)
-	+	-	Positive Deviation due to false positive alternative-method result ($PD_{FP(alt)}$)[c]

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

[b] No need for additional confirmation test(s). Confirmed alternative-method result is the same as the alternative-method result.

[c] This false positive result (FP) shall also be used to calculate the false positive ratio.

Table 2 — Comparison and interpretation of sample results between the reference and alternative methods for an unpaired study

Result of the (reference or alternative) method per sample			
Reference method	**Alternative method** (including any confirmations as described in the alternative-method protocol)	**Confirmed alternative method** (by any means)[a,b]	**Interpretation** (based on the confirmed alternative-method result)
+	+	+	Positive Agreement (PA)
+	+	-	Positive Agreement due to false positive alternative-method result ($PA_{FP(alt)}$)[c]
-	-	-	Negative Agreement (NA)
-	-	+	Negative Agreement due to false negative alternative-method result ($NA_{FN(alt)}$)
+	-	-	Negative Deviation (ND)
+	-	+	Negative Deviation due to false negative alternative-method result ($ND_{FN(alt)}$)
-	+	+	Positive Deviation (PD)
-	+	-	Positive Deviation due to false positive alternative-method result ($PD_{FP(alt)}$)[c]

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

[b] Confirmation by any means is only required when the result of the alternative method does not produce viable organisms. This is used as the confirmed alternative method result in comparison to the reference method result.

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

Determine the Total Negative Deviation (TND) and Total Negative Agreement (TNA) for the validation study.

Paired evaluation: Total Negative Deviation: TND = $ND_{FN(alt)}$

Total Negative Agreement: TNA = NA + $PD_{FP(alt)}$

Unpaired evaluation: Total Negative Deviation: TND = ND + $ND_{FN(alt)}$ + $PA_{FP(alt)}$

Total Negative Agreement: TNA = NA + $NA_{FN(alt)}$ + $PD_{FP(alt)}$

Table 3 — Summary of results obtained with the reference and alternative methods (after confirmation) of all samples for each category

	Reference-method positive (R+)	**Reference-method negative** (R-)
Alternative-method positive (A+)	+/+ Positive Agreement (PA)	-/+ Positive Deviation (PD)
Alternative-method negative (A-)	+/– Total Negative Deviation (TND)	–/– Total Negative Agreement (TNA)

Based on data summarized in Table 3 for the combined categories per category and per type, calculate the values for sensitivity of the alternative method (see Formula (1)) and of the reference method (see Formula (2)), as well as the relative trueness (see Formula (3)) and false positive ratio and false negative ratio for the alternative method after the additional confirmation of the results (see Formula (4)).

Sensitivity for the alternative method:

$$SE_{alt} = \frac{(PA + PD)}{(PA + TND + PD)} \times 100\,\% \quad (1)$$

Sensitivity for the reference method:

$$SE_{ref} = \frac{(PA + TND)}{(PA + TND + PD)} \times 100\,\% \quad (2)$$

Relative trueness:

$$RT = \frac{(PA + TNA)}{N} \times 100\,\% \quad (3)$$

False positive ratio (FPR) and false negative ratio (FNR) for the alternative method:

$$\text{Paired evaluation: } FPR = \frac{PD_{FP(alt)}}{TNA} \times 100\,\%$$

$$\text{Unpaired evaluation: } FPR = \frac{PA_{FP(alt)} + PD_{FP(alt)}}{TNA} \times 100\,\% \quad (4)$$

$$\text{False negative ratio: } FNR = \frac{NA_{FN(alt)} + ND_{FN(alt)}}{PA + TND + PD}$$

where

N is the total number of samples (PA + PD + TND + TNA);

FP is the false positive results;

FN is the false negative results.

For explanation of the abbreviated terms used, see Tables 1 to 3.