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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

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

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO 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).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary Information

The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This first edition of ISO 16140-2, together with ISO 16140-1, cancels and replaces ISO 16140:2003, which has been technically revised. It also incorporates the Amendment ISO 16140:2003:Amd.1:2011.

ISO 16140 consists of the following parts, under 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*

The following parts are under preparation:

— *Part 3: Protocol for the verification of reference and validated alternative methods implemented in a single laboratory*

— *Part 4: Protocol for single-laboratory (in-house) method validation*

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

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

Introduction

Today, many alternative, mostly proprietary, methods exist that are used to assess the microbiological quality of raw materials and finished products and the microbiological status of manufacturing procedures. These methods are often faster and easier to perform than the corresponding standardized method. The developers, end users, and authorities need a reliable common protocol for the validation of such alternative methods. The data generated will also provide potential end users with performance data for a given method, thus, enabling them to make an informed choice on the adoption of a particular method. The data generated can also be the basis for the certification of a method by an independent organization.

This part of ISO 16140

— is intended to provide a specific protocol and guidelines for the validation of proprietary methods intended to be used as a rapid and/or easier method to perform than the corresponding reference method,

— can also be used for the validation of other non-proprietary methods that are used instead of the reference method,

— is intended as the successor of the validation protocol published in the first version of ISO 16140 (ISO 16140:2003), and

— is mainly written for the validation of methods that are capable of culturing the target microorganism, but can also be applied to methods for microorganisms that cannot be cultured such as viruses (e.g. Norovirus) and protozan parasites (e.g. *Cryptosporidium* or *Giardia*). In these cases, some wordings are to be interpreted so as to fit the situation for non-culturable organisms.

The use of this part of ISO 16140 involves expertise on relevant areas such as microbiology, statistical design, and analysis as indicated in the respective sections. The statistical expertise encompasses overview of sampling theory and design of experiments, statistical analysis of (qualitative and quantitative) microbiological data, and overview of statistical concepts on random sampling, sample heterogeneity, sample stability, design of experiments, and variance components.

When this part of ISO 16140 is next reviewed, account will be taken of all information then available regarding the extent to which the guidelines have been followed and the reasons for deviation from them in the case of particular products.

The harmonization of validation methods cannot be immediate and for certain groups of products, International Standards and/or national standards may already exist that do not comply with this part of ISO 16140. It is hoped that when such standards are reviewed, they will be changed to comply with ISO 16140 so that eventually, the only remaining departures from this part of ISO 16140 will be those necessary for well-established technical reasons. For example, ISO 16297[3] deals with a very specific validation for a specific subject (the hygienic status of raw milk samples) and will remain as a vertical standard besides ISO 16140. If such a validation is needed, the vertical standard is more important.

Microbiology of the food chain — Method validation —

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

1 Scope

This part of ISO 16140 specifies the general principle and the technical protocol for the validation of alternative, mostly proprietary, methods for microbiology in the food chain. Validation studies according to this part of ISO 16140 are intended to be performed by organizations involved in method validation.

This part of ISO 16140 is applicable to the validation of methods 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, and

— samples from the primary production stage.

This part of ISO 16140 is in particular applicable to bacteria and fungi. Some clauses of this part of ISO 16140 could be applicable to other (micro) organisms or their metabolites on a case-by-case-basis. In the future, guidance for other organisms (e.g. viruses and parasites) will be included in either this part or a separate part of ISO 16140.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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, *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 apply.

4 General principles for the validation of alternative methods

The validation protocol comprises two phases:

— a method comparison study of the alternative (proprietary) method against the reference method carried out in the organizing laboratory;

— an interlaboratory study of the alternative (proprietary) method against the reference method carried out in different laboratories.

The technical rules for performing the method comparison study and the interlaboratory study are given in Clause 5 and Clause 6, depending upon whether the alternative (proprietary) method is

qualitative or quantitative in nature. The data generated in some parts of the validation study are evaluated using the so-called Acceptability Limits (AL) and no statistical evaluation of the data are conducted. These AL are based on experts' opinion and data generated in existing validation studies.

5 Qualitative methods — Technical protocol for validation

5.1 Method comparison study

5.1.1 General considerations

The method comparison study is the part of the validation process that is performed in the organizing laboratory. It consists of three parts namely the following:

— a comparative study of the results of the reference method to the results of the alternative method in (naturally and/or artificially) contaminated samples (so-called sensitivity study);

— a comparative study to determine the relative level of detection (RLOD) in artificially contaminated samples (so-called RLOD study);

— an inclusivity/exclusivity study of the alternative method.

The results (tables and calculations) of the different parts and the interpretation of the results, including discrepant results, shall be given in a study report.

Test portions size shall be used as written in the reference method.

5.1.2 Paired or unpaired study

The reference and alternative methods shall be performed with, as far as possible, exactly the same sample (same test portion). However, a distinction is made between studies where the same test portion can be used for both the reference and the alternative method due to both methods having exactly the same first step in the (enrichment) procedure and those where different test portions need to be used for the reference and the alternative method (e.g. due to different enrichment broths). In the case where the same test portion is used for both methods, the results from both methods are highly related to each other. For example, when the sample is not contaminated, both methods should find the result of that sample negative. Due to this relationship, the data produced by the reference and the alternative method are named **paired** or matched. In this part of ISO 16140, the wording "paired study" will be used for this type of study.

The opposite situation where there is no shared initial (enrichment) step for both the reference and the alternative method is also possible. In this case, different test portions coming from the same batch or lot of product have to be used for the two methods and the resulting data are named **unpaired** or unmatched. In this part of ISO 16140, the word "unpaired study" will be used for this type of study. The choice of having a **paired** study or an **unpaired** study depends on the protocols of the reference and alternative method. If there is a common initial step in the (enrichment) procedures, a **paired** study design is mandatory.

This clause describes the method comparison study if the reference and alternative method have a joint initial step in the (enrichment) procedures (**paired** study) and if the reference and alternative method do not have a joint initial (enrichment) step (**unpaired** study). Differences between both types of studies are indicated in the text where appropriate.

5.1.3 Sensitivity study

The sensitivity study aims to determine the difference in sensitivity between the reference and the alternative method. This study is conducted using naturally and/or artificially contaminated samples. Different categories and types shall be tested for this. Acceptability Limits have been defined for the

maximum acceptable difference depending on the type of study (**paired/unpaired**) and the number of categories tested.

5.1.3.1 Selection of categories to be used

The selection of categories and types used within the validation will depend on the type or group of microorganism and the scope of the validation.

If the method is to be applied for a broad range of foods, then at least five categories of food shall be studied. The validation study report shall state the food categories used in the study. If the method is to be validated for a restricted number of food categories, e.g. “ready-to-eat, ready-to-reheat meat products”, and “heat-processed milk and dairy products”, then only these categories need to be studied. In addition to food, feed samples, environmental samples, and primary production stage samples can be included as additional categories. This will broaden the application of the use of the alternative method for these additional categories.

For all selected categories (food and others), at least three different types per category shall be included in the study. Annex A presents an overview of the relevant types and categories for specific microorganisms that might be relevant for the validation. Annex A should be used to facilitate the selection of categories, types, and items for the specific microorganism involved. It should not be regarded as a mandatory choice.

When selecting samples for the study, it is of the highest priority to find those that are naturally contaminated. If it is not possible to acquire a sufficient number of naturally contaminated samples, artificial contamination of samples is permissible (see Annex B and Annex C). Details on the preparation of the artificially inoculated samples should be given in the validation study report. It is desirable that food samples come from as wide a distribution as possible in order to reduce any bias from local food specialities and to broaden the range of validation.

It shall be ensured that with the selection of the different types, both high and low (natural) background microflora, different types of stresses due to processing, and raw (unprocessed) items are included in the study.

EXAMPLE For the validation of a method for detection of *Listeria monocytogenes* and the category “ready-to-eat, ready-to-reheat meat products”, the types can be (1) cooked meat products (lower background flora, heat stress), (2) fermented or dried meat products (high background flora, pH stress), and (3) raw cured (smoked) (a_w <0,92) (intermediate background flora, a_w stress).

In some cases, for example, for an alternative method that is applicable for a broad range of foods, it is possible to combine the “ready-to-eat” and “raw” categories from the same product group. For example, the categories raw and ready-to-eat meat (products) can be combined into one category having three types divided over relevant raw and ready-to-eat food types. The selection of (combined) food categories should be based on risk analysis.

5.1.3.2 Number of samples

For each category being examined, a minimum of 60 individual samples shall be tested made up of at least three types with at least 20 samples representative for each type (three types × 20 samples for each type = 60 samples). Fractional positive results by either the reference or alternative method (i.e. samples should not be all positive or all negative) shall be obtained for each type tested. In the ideal situation, 10 samples (50 %) tested per type should be positive and 10 negative, but should range between 25 % and 75 %. For each category, at least 30 samples shall have a positive result by the reference and/or the alternative method.

5.1.3.3 Alternative-method result and confirmation

Many alternative-method protocols contain two steps, the first being the enrichment and detection step and the second being the confirmation of the detection result from step one. The end result of the alternative method is the result after step two. The end result will be the same as the result

after enrichment and detection in case there is no confirmation step included in the protocol of the alternative method.

The (end) result of the alternative method shall be confirmed for the sensitivity study part. All results obtained with the alternative method in an **unpaired** study shall be confirmed. In a **paired** study, only the positive results obtained with the alternative method, for which the corresponding result with the reference method was negative, shall be confirmed. This confirmation is needed to determine whether the result is a true-positive or false-positive result. The confirmation test or tests shall be able to recover and confirm the identity of the isolate as being the target of the method. These test(s) can be based on the confirmation procedure of the reference method, the confirmation step of the alternative method in case this procedure is able to isolate and confirm the identity of the target analyte, a combination of both, or by any other means that is able to isolate and confirm the identity of the target analyte.

If the enrichments of the reference and alternative methods differ in terms of the number of enrichments (i.e. primary/non-selective and secondary/selective) or total duration of incubation, an additional confirmation pathway is necessary for the validation study. The first pathway shall be that to be used with the alternative method according to its procedure/instructions (regular testing conditions by the alternative method according to the kit insert procedure; this does not include the complementary tests which can be performed during the validation study). The second pathway shall divert a portion of the alternative method's incubated enrichment to that of the reference method such that at minimum, the total duration of incubation of the reference method enrichment(s) is/are respected. The results of the two confirmation pathways are to be reported separately.

5.1.3.4 Calculation and interpretation for sensitivity

In general, the data shall be presented in a report in order to have an overview of the raw data obtained. Information shall be given on the type of contamination (naturally contaminated or artificially contaminated) of the samples used, the type of study design that was used (e.g. **paired** study or **unpaired** study), and the confirmation test(s) used to confirm the alternative-method result. For artificially contaminated samples, the (reference to the) procedure used for preparation shall be specified (see also Annex C).

The results obtained for the reference and alternative methods originating from the same sample, meaning from one test portion in case of a **paired** study or two test portions in case of an **unpaired** study, shall be described for a **paired** study according to Table 1 and for an **unpaired** study according to Table 2. Table 3 is prepared for the summarized sample results for all categories per category (≥60 samples) and per type (≥20 samples) for both a **paired** and **unpaired** study.

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)
-	+	+	Positive Deviation (PD)
-	+	-	Negative Agreement due to false positive alternative-method result (NA)[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]	**Interpretation** (based on the confirmed alternative-method result)
+	+	+	Positive Agreement (PA)
+	+	-	Negative Deviation due to false positive alternative-method result (ND)[b]
-	-	-	Negative Agreement (NA)
-	-	+	Negative Agreement due to false negative alternative-method result (NA)
+	-	-	Negative Deviation (ND)
+	-	+	Negative Deviation due to false negative alternative-method result (ND)
-	+	+	Positive Deviation (PD)
-	+	-	Negative Agreement due to false positive alternative-method result (NA)[b]

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

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

Table 3 — Summary of results obtained with the reference and alternative methods 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-)	+/- Negative Deviation (ND)	−/− Negative Agreement (NA)

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 (1) and of the reference method (2), as well as the relative trueness (3) and false positive ratio for the alternative method after the additional confirmation of the results (4) as follows:

$$\text{Sensitivity for the alternative method: } SE_{alt} = \frac{(PA + PD)}{(PA + ND + PD)} \times 100\ \% \quad (1)$$

$$\text{Sensitivity for the reference method: } SE_{ref} = \frac{(PA + ND)}{(PA + ND + PD)} \times 100\ \% \quad (2)$$

$$\text{Relative trueness: } RT = \frac{(PA + NA)}{N} \times 100\ \% \quad (3)$$

$$\text{False positive ratio for the alternative method: } FPR = \frac{FP}{NA} \times 100\ \% \quad (4)$$

where *N* is the total number of samples (NA + PA + PD + ND) and FP is the false-positive results. For explanation of the abbreviations used, see Table 1 to Table 3.

The confirmed alternative-method results shall be used to determine whether the alternative method produces comparable results to the reference method.

Calculate the difference between (ND – PD) for both **paired** and **unpaired** studies and the sum of (ND + PD) for **paired** studies. Check whether the difference and/or sum of PD and ND conform to the Acceptability Limit (AL) stated in Table 4 with respect to the type of study (**paired** or **unpaired**) and the number of categories used in the evaluation.

NOTE Acceptability Limits (AL) are based on data and consensus expert opinion. The AL are not based on statistical analysis of the data.

The interpretation of results shall be done per category and for all categories used in the validation study. An interpretation of results shall also be done per enrichment protocol in case different protocols are used for different types of samples. The AL is not met when the observed value is higher than the AL. When the AL is not met, investigations should be made (e.g. root cause analysis) in order to provide an explanation of the observed results. Based on the AL and the additional information, it is decided whether the alternative method is regarded as not fit for purpose for the category or categories involved. The reasons for acceptance of the alternative method in case the AL is not met shall be stated in the study report.

Table 4 — Acceptability limit parameters and values for a paired and unpaired study design in relation to the number of categories used

Number of categories	Paired study		Unpaired study
	(ND[a] - PD[b])	(ND + PD)	(ND - PD)
1	3	6	3
2	4	8	4
3	5	10	5
4	5	12	5
5	5	14	5
6	6	16	6
7	6	18	7
8	6	20	7
[a] ND = number of samples with Negative Deviation results.			
[b] PD = number of samples with Positive Deviation results.			

NOTE Information on differences observed between results of the alternative method before and after confirmation of the results (step 1 and step 2) according to the alternative-method protocol should be presented in the validation report as additional information, but is not used in the overall assessment of the alternative-method performance.

5.1.4 Relative level of detection study

A comparative study is conducted to evaluate the level of detection (LOD) of the alternative method against the reference method. The evaluation is based on the calculation of the relative level of detection (RLOD). In the study, replicates of artificially contaminated samples are used at three or more levels of contamination. Preferably, the levels are known as it allows calculation of the LOD. However, this is not required.

5.1.4.1 Selection of categories, number of samples, and replicates tested

For the selection of categories and types, see 5.1.3.1. The same categories will be used as selected for the sensitivity study (see 5.1.3). For each category, one relevant type is selected. In order to have a better representation of the evaluated category, this type should be different from those used in the sensitivity study (if possible). The samples shall be artificially inoculated. Procedures for the preparation of artificially inoculated samples are presented in Annex C. Each type will be inoculated with a different strain.

A minimum of three levels per type will be prepared consisting of at least a negative control level, a low level, and a higher level. Ideally, the low level shall be the theoretical detection level (i.e. 0,7 cfu per test portion) and the higher level just above the theoretical detection level (e.g. 1 cfu to 1,5 cfu per test portion). At least the low level should have fractional recovery by the reference method (fractional recovery at the low level should be between 25 % and 75 % of the number of samples tested). An estimate for the level of contamination (except for the negative control) should be made. At the negative control level, at least five replicate samples should be tested by both methods. For the second (low) level (theoretical detection level), at least 20, and for the third (higher) level, at least five replicates samples should be tested by both methods. The negative control level shall not produce positive results. When positive results are obtained, the experiments have to be repeated for all levels.

Positive deviating test results obtained with the alternative method shall be additionally confirmed (see 5.1.3.3). The RLOD shall be evaluated after confirmation.

NOTE 1 In order to have a better assurance that fractional recovery will be obtained, more levels of contamination can be produced and tested.

NOTE 2 The level of contamination needed targets the LOD of the reference method if the alternative method has a lower LOD than the reference method.

5.1.4.2 Calculation and interpretation of the RLOD

The RLOD is defined as the ratio of the LODs of the alternative method and the reference method:

$$RLOD = \frac{LOD_{alt}}{LOD_{ref}} \quad (5)$$

For each category, at least the RLODs shall be estimated by fitting a complementary-log-log (CLL) model to the combined absence/presence data of both methods as a function of method. The contamination levels are not needed for the calculations of the RLOD since they are included in the model resulting in curves in a graph of probability of detection versus log dose (contamination level). The statistical model and the calculations are worked out in Annex D. Calculations can be performed with the Excel®[1] spreadsheet of this part of ISO 16140. The Excel® spreadsheet for calculating RLOD values is freely available for download at http://standards.iso.org/iso/16140 and then select the RLOD file. For calculations using this Excel® spreadsheet, the option of "unknown concentration" shall be used. Calculate for each item *i* the $RLOD_i$. Tabulate the results as indicated in Table 5.

Table 5 — Presentation of RLOD before and after confirmation of the alternative-method results

	RLOD using the alternative-method results	RLOD using the confirmed alternative-method results
Item (category) (*i*)	$RLOD_i$	$RLOD_i$
1		
2		
…		
k		
Combined		

An Acceptability Limit (AL) for the RLOD based on the confirmed alternative-method results specifies the maximum increase in LOD of the alternative versus the reference method that would not be considered as relevant in consideration of the fitness for purpose of the method. Consequently, AL will be a value >1. The interpretation should be made for each item.

The AL for **paired** study data are set at 1,5, meaning that the LOD for the alternative method shall not be higher than 1,5 times the LOD of the reference method. An LOD value for the alternative method smaller than the LOD value for the reference method is always accepted as this means that the alternative method is likely to detect lower levels of contamination than the reference method.

The AL for **unpaired** study data are set at 2,5, meaning that the LOD for the alternative method shall not be higher than 2,5 times the LOD of the reference method. An LOD value for the alternative method smaller than the LOD value for the reference method is always accepted as this means that the alternative method is likely to detect lower levels of contamination than the reference method.

The AL is not met when the observed value is higher than the AL. When the AL is not met, investigations should be made (e.g. root cause analysis) in order to provide an explanation of the observed results. Based on the AL and the additional information, it is decided whether the alternative method is regarded as not fit for purpose for the item or category involved. The reasons for acceptance of the alternative method in case the AL is not met shall be stated in the study report.

In addition to the calculation of the RLOD, the data may be evaluated using the AOAC probability of detection (POD) model described in Reference [14] and included in the AOAC validation guidelines.[6] The evaluation using the POD model can give additional information on the equivalence of the methods.

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

5.1.5 Inclusivity and exclusivity study

5.1.5.1 Selection and number of strains

A range of strains shall be used. Criteria for selecting test strains are given in Annex E. The strains used should take into account the measurement principle of the alternative method (e.g. culture-based, immunoassay-based, and molecular). Different measurement principles may require the use of different test panels of strains.

Each strain used shall be characterized biochemically and/or serologically and/or genetically in sufficient detail for its identity to be known. Strains used should preferentially have been isolated from foods, feeds, the food-processing environment, or primary production taking into account the scope of the validation. However, clinical, environmental, and culture collection strains can be used. The original source of all isolates 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.

For inclusivity testing, at least 50 pure cultures of (target) microorganisms shall be tested. For testing the inclusivity for *Salmonella* methods, at least 100 pure cultures of different serotypes of *Salmonella* shall be tested.

For exclusivity testing, at least 30 pure cultures of (non-target) microorganisms shall be tested.

Some microorganisms will be difficult or impossible to culture like viruses or protozan parasites. Where the target organism cannot be cultured, pure suspensions of the test strains should be used for spiking at the earliest appropriate step of the method.

NOTE 1 For some microorganisms, it will be difficult to obtain the required number of strains for inclusivity and exclusivity. In these cases, an agreed set of test strains should be selected by the parties involved in the validation study.

NOTE 2 Guidelines for the preservation and maintenance of strains in (local) collections can be found in ISO 11133.[2]

5.1.5.2 Inoculation of target strains (inclusivity)

Each test is performed once and only with the alternative method (including a confirmation step if prescribed in the alternative-method protocol). Inoculation of a suitable growth medium is carried out with a dilution of a pure culture of each test strain. This culture is used for testing the inclusivity. No sample is added.

The pure culture should be grown in a non-selective broth under optimal conditions of growth to provide high cell populations in a stationary phase. The inoculum level shall be 10 times to 100 times greater than the minimum detection level of the alternative method being validated and the protocol of the alternative method shall be used including all (enrichments) detailed in the instructions of the alternative method. If the alternative method includes more than one (enrichment) protocol (e.g. for different sample types), then use the most challenging one with the complete panel of test strains. When negative or doubtful results are obtained, the test should be repeated and with the reference method included, checking that the strain could be detected with the appropriate reference method. If results are negative, consideration could be given to repeat the test with the addition of a food item. If the alternative protocol includes a confirmation step, the confirmation tests shall be included in testing the selected strains.

5.1.5.3 Inoculation of non-target strains (exclusivity)

Each test is performed once and only with the alternative method (including a confirmation step if prescribed in the alternative-method protocol). Inoculation of a suitable growth medium is carried out with a dilution of a pure culture of each test strain. This culture is used for testing the exclusivity. No sample is added.