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Validation of alternative analysis methods

Application to the food industry

Requirements of validation studies implemented according to the protocol of ISO 16140-2

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Summary

FOREWORD

Purpose

This document is applied in addition to the ISO 16140-2 standard, which it does not replace. It is partly a guide to the application of this standard and contains additional specific provisions required by the NF VALIDATION mark.

Update/Distribution

This document is updated by AFNOR Certification whenever changes are made by the Agri-Food Technical Board and/or by AFNOR Certification.
It is distributed by AFNOR Certification in a controlled manner with each update, to qualified expert laboratories and to members of the Technical Board.
It is distributed in an uncontrolled manner to any applicant.

Note 1: Criteria for acceptability of results

ISO 16140-2 introduced limits on the acceptability of results for the main endpoints.
However, it is up to the Technical Board to examine and evaluate the results for each of the criteria, and to give an opinion on all the detailed results.

Note 2: Acceptance of External Outcomes

External results previously obtained under another validation program may be accepted. Within the limits of its competence, and in the absence of official documents stating this, it is up to the Technical Board to judge the importance of the differences between the reference methods and/or validation protocols used.

Note 3: Gross results

The Expert Laboratory must be able to communicate all the raw data (which are not included in the report) to the reviewers of the file, to AFNOR Certification or to the members of the Technical Board if necessary.

Note 4: Compliance with these requirements

The Expert Laboratory must report throughout the study any deviations from the manufacturer's protocol and/or from this document. If no deviation is mentioned, compliance with the manufacturer's protocol and this document is implied and under the manufacturer's responsibility.

Note 5: ISO 16140-2 transition arrangements

The Validation Commission and its Technical Board have defined the transition modalities applicable to the transition to the new ISO 16140-2/A1 validation protocol, published in September 2024. These are specified in Appendix 1.

QUALITATIVE METHODS (§ 5 OF ISO 16140-2)

As part of the validation of alternative methods to the XP CEN ISO/TS 13136 technical specification, specific additional requirements have been defined by the Technical Board and the Validation Commission, and are specified in Appendix 2 to this document.

Conditions for confirmation of positive results

Within the framework of the NF VALIDATION mark, all alternative methods for the **detection of pathogenic microorganisms** (*Salmonella*, *Listeria monocytogenes*, *Listeria* spp., *Campylobacter*, *Cronobacter*, *E. coli* STEC, *Vibrio*), candidates for validation, must systematically include a step of confirmation of positive results in their routine protocol. This requirement does not apply to families including different genus, only some of which include pathogenic strains (e.g. ***Enterobacteriaceae***).

In the context of the validation study, only the results obtained after confirmation are considered definitive (tables, calculations, etc.). The results before confirmation are used for information. Differences in results between the different confirmation options should be documented in the report.

The Validation Commission and its Technical Board have defined the following "cases" of confirmation:

NOTE: The steps prior to confirmation must be explained.

Case 1: Implementation of the conventional tests described in the methods standardized by CEN or ISO from colonies (including the purification step).

Case 2: Implementation **of one or more method(s)** presenting **a different principle** from that of the validated method, and **described by the manufacturer** at the time of the initial application for validation, in a confirmation protocol.
NOTE: In this case, the validation study must relate to this method(s) and the appropriateness of implementing this method(s) is left to the discretion of the Technical Board, which may refuse this option. These specific confirmatory protocols should be documented in the study report.
As part of this "case 2" confirmation, the references of Latex Tests and other proprietary tests proposed are to be specified in the study report.

Case 3: Use of **any other method certified NF VALIDATION**, with a **different principle** from that of the validated method whose result is sought to be confirmed. The detection protocol of the second validated method must be respected as a whole, i.e. all the steps prior to the intermediate step from which we start again for confirmation must be common to both methods.
NOTE: The two validated methods (one used in detection and the other in confirmation) must therefore have a common core (e.g. common enrichment with the same environment).

Case 4: By the use **of a PCR test or isothermal amplification or any molecular biology methods that are part of an NF VALIDATION certified method** different from those of the detection method whose result is sought to be confirmed and from isolated colonies (with or without purification).
Case 5: any method certified NF VALIDATION by AFNOR Certification according to ISO 16140-6.

"Case 1" must be part of the protocol of the alternative method to be validated. "**Cases 2, 3, 4 and 5"** can complete it.

In the event of discordant results (presumptive positive by the alternative method, not confirmed by conventional tests or by the method(s) described by the manufacturer, in particular *(if applicable)* by the Latex test(s)*, or by another method certified NF VALIDATION), **the laboratory must implement sufficient means to ensure the validity of the result returned.**
***Additional clarification to be added in case Latex tests and other proprietary tests are offered for confirmation.*

Additional details in the case of *Listeria*:
Confirmation of *Listeria monocytogenes*: subject to satisfactory validation results on chromogenic agar, the following "case 2" confirmation can be proposed: "By isolation on chromogenic agar in accordance with the definition of the NF EN ISO 11290 standard (OAA formulation type) or as part of a method certified NF VALIDATION. The presence of characteristic colonies is sufficient to confirm the presence of *Listeria monocytogenes* regardless of the screening method used.

Confirmation of the genus *Listeria* spp.: subject to prior opinion of the Technical Office, the following "case 2" of confirmation may be proposed: "By isolation on PALCAM agar or chromogenic agar forming part of an NF Validation certified method for the detection of the genus *Listeria*. The presence of characteristic colonies is sufficient to confirm the presence of *Listeria* spp."

For alternative methods, validated for both the detection and enumeration of *Listeria monocytogenes* or *Listeria* spp., and using the same agar for detection and enumeration (same commercial reference of medium), it is possible to propose the following methods:
Detection part: "If a positive result is obtained for <*Listeria monocytogenes* or *Listeria* spp.> during the detection step with the [name of method validated for detection] method, it is not necessary to confirm this result if it has already been confirmed after the enumeration of the [name of method validated for enumeration] method."
Enumeration part: "If a positive result is obtained for <Listeria *monocytogenes* or *Listeria* spp.> during the enumeration step with the [name of the method validated for detection] method, it is not necessary to confirm this result if it has already been confirmed at the end of the detection step with the method [name of the method validated for enumeration]. Failure to confirm 5 colonies in enumeration implies a risk of giving an overestimated result due to the possible presence of characteristic colonies that would not be <*Listeria monocytogenes* or *Listeria* spp.>. »
Further details are available in Appendix 7 to this document.

Comparative study of methods (§ 5.1)

General considerations (§ 5.1.1)

The objective of this study is to compare the performance of the alternative method and the reference method, for the following parameters:

- Sensitivity study
- Relative level of detection

And to determine the following parameters for the alternative method:

- Inclusivity and exclusivity study
- Practicability

The **same test portions** may be tested for the alternative method and the reference method provided that the expert laboratory demonstrates that the RLOD 10 or 25 g vs x g (large test portions) are less than 2.5 (AL for unpaired methods). This must be confirmed for each of the categories concerned by the large test portions.
Tests with test intakes of x g (upper limit tested) allow for the inclusion of weighings of 1g to x g for a given enrichment protocol.

NOTE: Be careful to follow the recommendations of the ISO 22964 standard for the detection of Cronobacter spp.

Sensitivity study (§ 5.1.3)

Selection of categories to be used (§ 5.1.3.1)

The analyses will be carried out on samples naturally or artificially contaminated or not by the target microorganism, belonging to different food categories, representative of the products usually subjected to this type of analysis (see Annex A of the ISO 16140-2 standard). It may be acceptable to combine certain categories subject to the opinion of the Technical Board. This is in order to increase the diversity

of the matrices tested, especially in the context of validation on a wide range of foods. The origin of the samples must be as varied as possible in order to limit biases related to food specialties.

The types and categories listed in Appendix 3 to this document are given for illustrative purposes only and may be defined differently depending on the manufacturer's needs. If the sprouted seed analysis is to be claimed, 3 positive samples must be tested.

In the event that **"mixed" categories** are proposed for validation, they will be accepted subject to proposing at least a total of 5 categories of human food products.

The different **contamination options**, to be applied in order of priority, are (see Annex B of ISO 16140-2):

- ➔ Option 1: Naturally contaminated samples
- ➔ Option 2: Contamination by mixing
- ➔ Option 3: Artificial contamination ("seeding" or "spiking")
- ➔ Option 4: Reference Material

If it is not possible to obtain a sufficient number of naturally contaminated foods in each of the categories, it will be possible to resort to artificial contamination. The expert laboratory will have **to justify** the difficulty of using naturally contaminated samples.

NOTE: For Listeria spp. and Salmonella spp., the Technical Board examines on a case-by-case basis, when presenting the comparative study project, the number of samples naturally contaminated in the light of the updated scientific literature (state and official agencies).

The protocols for cross-contamination and artificial contamination ("spiking" and "seeding") are specified below (see Annex C of ISO 16140-2):

C.1 Cross-contamination

Applicable to homogenizable matrices.

Experimental design:

- Naturally contaminated product diluted at least 1/5 if the product is of the same nature (raw beef in raw beef)
- Naturally contaminated product diluted at least 1/10 if the product is of the same type (raw pork in raw beef)
- Product of the same type (with consistency in type)
- A naturally contaminated product should not be used more than 6 times in the same study
- Mixing to allow good homogenization (without grinding)

Store the cross-contaminated sample for at least 1 day at the usual storage temperature.

– **C.2 Artificial Seeding Contamination**

To be preferred except in cases of stress specified for spiking (see C3 below).

Contamination should be **≤ 3 CFUs/test portion** if possible. However, this contamination rate can be adjusted for difficult matrices and/or germs to allow positive results to be obtained. The Expert Laboratory will be required to document these cases in the study report.

– **C.3 Artificial contamination by spiking**

To be maintained in particular in the case of processed products (stress by Heat Treatments (HT) or pH), and in the case of samples from the production environment.

Contamination should be **≤ 5 CFU/test portion** if possible. However, this contamination rate can be adjusted for difficult matrices and/or germs to allow positive results to be obtained. The Expert Laboratory will be required to document these cases in the study report.

The Expert Laboratory must describe and then demonstrate the stress of the strain at the time of inoculation (by comparing the counts obtained on selective and non-selective media): a difference of at least 0.5 log is expected. The Expert Laboratory must specify the medium(s) used.

- **C.4 Artificial Seeding and Spiking Contamination**

The contamination rate between 3 CFU/test portion (seeding) or 5 CFU/test portion (spiking) and 10 CFU/test portion must not exceed 20% of the positive samples in all categories. In the case of a renewal, a maximum of 2 samples per type and 3 samples per category may be stored with a contamination rate > 10 CFU/test portion and ≤ 3 CFU/test portion.

In general, the Technical Board recommends that the sources of artificial contamination of samples should be varied. The same strain and stress cannot be used for all products in the same category. A single strain may not be used more than 6 times in total. The strains must mainly be of food origin. The origin of the strains must be known and informed. The strains must be representative of those most commonly present in the categories tested and their origin must be adapted to the type of contaminated product.

The **method** of contamination and the levels of contamination should be such as to obtain samples whose behaviour is identical to that of naturally contaminated samples.

- **Incubation time**

In the case of **"unpaired" studies** (separate broths) when the incubation times are different between the 2 methods, the simultaneous start of the studies should be preferred as far as possible.

The **incubation periods** of the broths used to carry out the tests for the alternative method are the minimum durations specified in the protocol. The minimum incubation period will also be used for the reference method to the extent possible. If the limits are extended, upper-limit trials may be discussed. Changes in results between minimum and maximum time will need to be documented for wide incubation ranges.

For protocols with a broth incubation time of less than 24 hours (low limit):

- In the case of methods in agar media: the 2 lower and upper limits will be tested above 6 hours apart.
- Methods based on another principle including immuno-magnetic separation (IMS): the 2 lower and upper limits will be tested beyond 8 hours apart.

If the protocol for the alternative method provides for a **cold storage step** (duration defined by the manufacturer), the sensitivity study will have to be redone after refrigeration. In the case of broth storage, all positive samples (artificially and naturally contaminated), as well as samples with questionable results, will have to be retested. In the case of the storage of the boxes, all positive and negative samples will have to be retested.

NOTE: The following retention cases do not require validation testing to be validated:

- *Plates with DNA extracts can be stored at 4°C for a maximum of 24 hours before proceeding with the test;*
- *Possibility of storing DNA extracts at -20°C for several months;*
- *Possibility of storing a broth or agar provided that their formulation and protocol correspond to those described in the reference method (data are available in the standards for agar plates and broths of the same formula).*

The use of a neutralizer and the method of preparation of environmental samples must be specified as far as possible by the expert laboratory in the study report (type of sampling medium, surface area sampled, mode and rate of dilution of the support, etc.).

Case of *Salmonella* detection methods:

According to the requirements of ISO 6579-1 (2017) and its amendment (2020), it is possible to include

a temperature range of 34°C to 38°C for the incubation of selective and non-selective enrichment broths if the targeted temperature is included in this range (e.g. 35±1°C, 37±1°C or 36±2°C). Data based on a protocol suggested by the ISO/TC34/SC9 committee showed that there is no impact on the growth of *Salmonella* at 35±1°C or 37±1°C. The ISO 6579-1 (2017) standard prescribes a temperature range of 34-38°C for MKTTn subculture, and selective agar media (proprietary or not). Several agars were tested during the tests because each laboratory used the second agar according to its own selection. Based on this information, these requirements are applicable to alternative methods of detecting *Salmonella* if manufacturers so wish.

Number of samples (§ 5.1.3.2)

For **each category**, a minimum of 60 samples (positive and negative) are to be tested. Each category must consist of 3 types, with at least 20 samples (positive and negative) for each type. For each type, a minimum of 7 positive samples must be tested.

For each **specific enrichment protocol** of the alternative method, the same number of samples as for a category will be tested.

For ***Listeria* genus studies**, it is requested to respect a proportion of at least 15 to 25 samples contaminated with *Listeria* spp. excluding *Listeria monocytogenes* (alone and in mixture with *Listeria monocytogenes*) in order to best mimic the conditions of natural contamination.

NOTE: In the case of co-inoculation, the level of contamination must correspond to the requirements of the paragraph of this document.

Alternative method results and confirmation (§ 5.1.3.3)

The Expert Laboratory will **systematically carry out the following confirmation steps to** the alternative method:

➔ As a 1st confirmation: For **all positive and negative** samples (except in the case of agar media methods), by applying the confirmatory tests of the reference method and those proposed by the manufacturer. In the case of validation of the cold preservation step, one of the specific tests proposed by the manufacturer will be applied. This step must be detailed by the expert laboratory in the project.

➔ For 2nd confirmation: For **negative** samples only (including agar media methods):

- If the alternative method does not have a secondary enrichment broth, the broth from the alternative method must be subcultured in the secondary broth of the reference method (e.g., *Listeria*). Special case of *Salmonella* studies: the secondary broth used will only be SVR.
- If the alternative method has a secondary enrichment broth, its incubation time should be extended, if necessary, to that of the secondary broth of the reference method.
- If the 2 methods have a single enrichment step, and the incubation time of the alternative method is shorter, the incubation will have to be extended to the minimum time of the reference method.

Calculation and interpretation for sensitivity (§ 5.1.3.4)

In the context of validation studies of alternative methods in the context of the NF VALIDATION mark, false positive (FP) samples are defined as follows:

$PD_{FP(alt)}$ = Positive Deviation due to false positive alternative-method results

$PA_{FP(alt)}$ = Positive Agreement due to false positive alternative-method results

For the validation of methods based on the principle of growth on agar medium, the following elements must be provided in the raw results:

- level of ancillary flora: absence / low / high
- Appearance of the target colonies if atypical: micro-colony, size, colour, shape, halo, etc.
- need for re-isolation or not

- presence of less than 5 characteristic colonies (fill in the number)

For the other principles of methods, it is recommended to provide details on the signal level of the target germ (Ct, internal control, Tm and inhibitions for PCR methods, agglutination for Latex tests, intensity of stains for immunochromatographic tests on strips - "lateral flow", etc.).

Acceptability criteria are set by the ISO 16140-2 standard. If the criteria are not met for all the categories tested, it is possible to retest a category or type. The 2 sets of results must be presented in the file, for discussion in the Technical Board (data not to be consolidated). If the number of categories and/or samples tested exceeds the values set in ISO 16140-2, the expert laboratory will propose an interpretation to the Technical Board.

In the case of a validation study involving paired and unpaired protocols, interpretation for all categories is made using the limit of acceptability for unpaired methods (TND-PD).

Relative level of detection study (§ 5.1.4)

The goal is to determine the minimum detectable contamination in a food.

Selection of categories, number of samples, and replicates tested (§ 5.1.4.1)

Refer to Annex A of ISO 16140-2 for the selection of recommended product categories and types by target germ. It may be acceptable to combine certain categories subject to the opinion of the Technical Board. This is in order to increase the diversity of the matrices tested, especially in the context of validation on a wide range of foods. Appendix 3 of this document is given by way of example only. Other types/categories may be defined according to the manufacturer's needs.

The samples must be artificially contaminated. Sample preparation procedures are specified in Annex C of ISO 16140-2. In particular, it is recommended to favor in-mass inoculations ("Bulk inoculation" **in the case of a combined AOAC study**). It is also possible to carry out contamination in bags ("Individual Bag"). The associated flora level of the matrix will be determined.
One of the following inoculation protocols must be applied (defined according to the industrial processes used in the manufacture of the products):

- Raw products: Inoculation in "seeding" with a minimum of 48 hours at 4°C (do not leave too long). In the case of raw milk, inoculation by "seeding" with a minimum of 24 hours at 4°C is accepted.
- Frozen: Inoculation in seeding for 2 weeks at -20°C
- Cooked products (pasteurized egg flows, terrines): Either inoculation by "seeding" with storage at 4°C, or by "spiking" with heat treatment of the strain and stress assessment.
- Dehydrated or freeze-dried powders/products: Preferably inoculations in "seeding" with a freeze-dried or desiccated strain with storage for a minimum of 2 weeks (depending on the product tested) at the product's storage temperature (usually room temperature). It is also possible to carry out "spiking" with heat treatment of the strain and stress assessment.
- Primary production: Inoculation in the matrix and storage for 24 hours at room temperature.
- Process water or surface: Inoculation in seeding for 48 hours at 4°C.

The medium used for calibration, as well as all the contamination protocols, must be submitted to the Technical Board for its opinion when the comparative study project is presented. They should also be specified in the study report and in the summary report of the validation study.
Three levels of contamination are to be tested. The high rate must correspond to 3 to 5 times the low rate.

Calculation and interpretation of RLOD (§ 5.1.4.2)

Refer to ISO 16140-2. The validation report shall specify the LOD50 values calculated according to the PODLOD_version spreadsheet in effect on the ISO Standards Maintenance Portal website.

Inclusivity and exclusivity study (§ 5.1.5)

NOTE: Published data on the alternative method, obtained in accordance with the requirements of ISO 16140-2, may be used by the Expert Laboratory to inform this criterion.

In the case of an extension request that concerns the addition of confirmatory tests (Case 2 of a validated alternative method) and which does not fall within the scope of an ISO 16140-6 and 7 validation, only this parameter will be filled in, subject to prior advice from the Technical Board.

Selection and number of strains (§ 5.1.5.1)

Refer to Annex E of ISO 16140-2 for criteria for the selection of microorganisms.

For *the Salmonella* spp. studies, the Technical Board has established a minimum list of target and non-target strains to be tested cf. Appendix 5). The list must be completed by the Expert Laboratory to meet the required requirements and submitted to the Technical Board for its opinion. The same *Salmonella* serovar should not be tested more than 2 times, unless specifically advised by the Technical Board.

For *Listeria* spp. studies, it is mandatory to test *Listeria* sensu stricto as a priority, including two strains of *Listeria marthii* in the inclusivity study. It is also possible to add up to 20% *of Listeria* sensu lato as an inclusivity, if the manufacturer wishes. It is recommended that *Listeria marthii* be added to the exclusivity study of *Listeria monocytogenes* detection and/or enumeration methods.

Testing for Group N *Salmonella* exclusively for *E. coli* O157 methods.

To test *Enterococcus mundtii* exclusively for detection and enumeration methods based on the principle of isolation on Ottaviani and Agosti agar media followed by confirmation using tests involving rhamnose and PIPLC activity.

Inoculation of target strains (inclusivity) (§ 5.1.5.2)

For trials, the most selective protocol of the alternative method should be implemented.

In the case of alternative methods based on an ELISA test, offering a Latex test as a confirmatory test, the Expert Laboratory will have to test 150 target strains. The Technical Board reserves the right to request additional tests in other cases, depending on the principles proposed.

Inoculation of non-target strains (exclusivity) (§ 5.1.5.3)

For testing, a non-selective fortification broth will be used.
In the case of alternative methods based on an ELISA test, offering a Latex test as confirmation, the Expert Laboratory will have to test 100 non-target strains. The Technical Board reserves the right to request additional tests in other cases, depending on the principles proposed.

Expression and interpretation of the results (§ 5.1.5.4)

Refer to ISO 16140-2.

The results obtained for each of the confirmatory tests provided for in the routine protocol of the alternative method must be provided in the study report:

- Appearance of non-target colonies
- Appearance of the target colonies if atypical: micro-colony, size, colour, shape, halo, etc.
- Presence of less than 5 characteristic colonies (number).

For the other principles of methods, it is recommended to provide details on the signal level of the target germ (Ct, internal control, Tm and inhibitions for PCR methods, agglutination for Latex tests, intensity of stains for immunochromatographic tests on strips - "lateral flow", etc.).

Study of the practicability of the alternative method (specific requirement)

This is a special requirement of the Technical Board, not required by the ISO 16140-2 standard.

A practicability/adaptability study, including 4 criteria, will be carried out.
For each of these criteria, the method of communicating this criterion to the user and the method of checking this criterion have been defined.
Indeed, some criteria require communication on the packaging or the IFU, while others require communication in the summary report.

The data resulting from this feasibility study will be incorporated into the comparative study report.

	Criteria to be checked	How the criterion is communicated to users	Control Mode of the criterion by the Expert Laboratory
1	Storage conditions of the elements (+ expiry of unopened products)	Packaging or IFU	Verify that the conditions exist
2	Modalities of use after first use (in particular existence of expiry dates)	Packaging or IFU	Verification that the terms and conditions exist
3	Time to Results	Study and synthesis report	Establishment of 2 cycles describing each step of the method only in terms of time: -1st cycle: negative samples - 2nd cycle: positive samples
4	Common steps with the reference method	Study and synthesis report	Identifying Steps

Interlaboratory study (§ 5.2)

General considerations (§ 5.2.1)

The objective of the interlaboratory study is to determine the sensitivity gap between the two methods under conditions of reproducibility.

Measurement protocol (§ 5.2.2)

Laboratories will need to be sufficient in number to present at least 10 usable datasets, with at least 2 countries represented and a maximum of 3 collaborators per laboratory with independent testing. The list will be at the discretion of the Technical Board.

Two interlaboratory studies carried out on the same product may be compiled only after the opinion of the Technical Board, and under the following conditions:

- That there are 10 different collaborators (in certain special cases to be justified, and with the prior opinion of the Technical Board, it may be authorized that the Expert Laboratory make its premises available to geographically close collaborators for the performance of the tests. It may be agreed to involve an independent team from the Expert Laboratory, provided that it has not participated in the validation study)
- That the analysis protocols are exactly the same between the studies,
- That all the studies provided for in the project have been carried out,
- That the sets of results obtained by each collaborating laboratory are complete.
- The number of sets of results from each study is statistically acceptable.

Refer to Appendix 6 of this document for the general arrangements for the organization of the interlaboratory study.
The following clarifications are made to the measurement protocol described in the ISO 16140-2 standard: All configurations (instrument, PLC, kit format, etc.) previously validated in MCS can be used during the interlaboratory study, alone or in combination.

It is recommended to select a food matrix that is relevant to the test analysis protocol for the alternative method and the target microorganism.

NOTE: Liquid products should be avoided as much as possible as they are a source of intercontamination (risk of leaks and transfer from bottle to bag).

In the case of a study conducted on a "wide variety of foods", it is recommended to test one of the following matrices:

- *Salmonella* and *E. coli* O157 :H7: Hamburger
- *Cronobacter*: Milk powder with probiotics
- *Listeria*: Fresh cheese (goat, sheep, etc.)
- *Campylobacter*: Ground meat of poultry or pork.

NOTE: To limit the problems of natural contamination in the case of poultry meat, it is recommended to freeze the meat before use.

The sample should contain a representative ancillary microflora, which should also remain stable during transport. The laboratory will need to identify and report the levels of associated flora in the matrix.

The level of ancillary flora must be at least 10^3 CFU per mL or per g, unless otherwise advised by the Technical Board.

In the special case of a milk sample test, the flora must be naturally of dairy origin. In practice, the expert laboratory will have to find milk that is naturally contaminated at the level required above. If the level of contamination is below the required level, the sample will either have to evolve the sample to that level of contamination, or it will have to add raw milk (in reasonable proportions).

- Three levels of contamination are to be tested:
  - A **level 0** (L0) which corresponds to the negative control: 0 CFU/test portion.
  - An **intermediate level** (L1) which must be at the level of the LOD50 so as to preferably obtain a fractional overlap. Contamination rates of less than < 3 CFU/test portion are recommended. The Technical Board will decide in the event of a deviation.
  - A **higher level** (L2)

Calculations and synthesis of data (§ 5.2.3)

Refer to ISO 16140-2.

The results of employees for whom cases of intercontamination are proven at the L0 level are to be excluded from interpretation, on the basis of the following criterion: 1 positive (1 confirmed positive or 1 false positive) maximum acceptable at L0, per laboratory and per method.

Exception: as part of the validation study of an alternative method for the detection of *E. coli* O157:H7 or STEC (requiring an IMS), the results of the study will be examined by the Technical Board in order to define their acceptability. A laboratory with more than one contamination at the L0 level for the reference method could be retained for interpretation.

Interpretation of data (§ 5.2.4)

Refer to ISO 16140-2.

QUANTITATIVE METHODS (§ 6 OF ISO 16140-2)

Method comparison study (§ 6.1)

General considerations (§ 6.1.1)

The objective of this study is to compare the performance of the alternative method and the reference method, for the following parameters:

- Relative Accuracy Study
- Accuracy Profile

And to determine the following parameters for the alternative method:

- Limits of Quantification
- Inclusivity / Exclusivity
- Practicality

In general, the Technical Board recommends that the sources of artificial contamination of samples should be varied. The same strain and stress cannot be used for all products in the same category. The strains must mainly be of food origin. The origin of the strains must be known and informed. The strains must be representative of those most commonly present in the categories tested and their origin must be adapted to the type of contaminated product.

The **method** of contamination and the levels of contamination should be such as to obtain samples whose behaviour is identical to that of naturally contaminated samples.

The confirmation step is optional for enumeration methods with the exception of *Listeria monocytogenes*.

Relative trueness study (§ 6.1.2)

Selection of categories to be used (§ 6.1.2.1)

For the selection of grades and matrices, refer to Annex A of ISO 16140-2. It may be acceptable to combine certain categories subject to the opinion of the Technical Board. This is in order to increase the diversity of the matrices tested, especially in the context of validation on a wide range of foods. The types and categories listed in Appendix 3 of this document are given by way of example only and may be defined differently depending on the manufacturer's needs.

Number of samples (§ 6.1.2.2)

The samples must cover the usual measuring range in all categories.

Calculations and interpretation of relative trueness (§ 6.1.2.3)

All data that cannot be interpreted (by either method) must be included in an independent table.

The use of a neutralizer and the method of preparation of environmental samples must be specified by the expert laboratory in the study report.

Accuracy profile study (§ 6.1.3)

Selection of categories to be used (§ 6.1.3.1)

For the selection of grades and matrices, refer to Annex A of ISO 16140-2. It may be acceptable to combine certain categories subject to the opinion of the Technical Board. This is in order to increase the

diversity of the matrices tested, especially in the context of validation on a wide range of foods. The types and categories listed in Appendix 3 to this document are given for illustrative purposes only and may be defined differently depending on the manufacturer's needs.
There is no need to stress the strains for contamination.

Number of samples (§ 6.1.3.2)

At a minimum, test 2 batches of the same matrix inoculated with the same strain at 3 inoculation rates (low, medium, high).

For reasons of dispersion of results and statistical interpretation (Poisson distribution) with low counts, it is recommended to start the low inoculation rate from 100 CFU/g for pathogens and 300 CFU/g for other germs.

As far as possible, the high level should reflect the contamination rates of the regulatory criteria or have a high value of:

- *E. coli* / *Staphylococcus* / *B. cereus* / Coliforms and Enterococci: 10^5 CFU/g higher
- Total flora: 10^6 CFU/g
- *Listeria monocytogenes* : 3000 CFU/g
- *Listeria* spp.: 30000 CFU/g
- *Pseudomonas*: 10^6 CFU/g
- *Campylobacter*: 10^4 CFU/g
- Yeast and mold: 10^5 CFU/g

Calculation and interpretation of accuracy profile study (§ 6.1.3.3)

Refer to ISO 16140-2.

Limit of quantification study (§ 6.1.4)

General considerations (§ 6.1.4.1)

The study is only applicable in the case of instrumental methods (i.e. methods not based on colony counting).

Selection of categories to be used (Section 6.1.4.2)

For the selection of grades and matrices, refer to ISO 16140-2.

Number of samples (§ 6.1.4.3)

Refer to ISO 16140-2.

Calculation and interpretation of limit of quantification study (§ 6.1.4.4)

Refer to ISO 16140-2.

Inclusivity and exclusivity study (§ 6.1.5)

Follow the procedures described in ISO 16140-2.

Study of the practicability of the alternative method (specific requirement)

This is a special requirement of the Technical Office and not required by ISO 16140-2.

A **practicability/adaptability study**, including 4 criteria, will be carried out **by the Expert Laboratory.**
For each of these criteria, the method of communicating this criterion to the user and the method of checking this criterion have been defined.
Indeed, some criteria require communication on the packaging or the technical leaflet, while others

require communication in the summary report.

The data resulting from this feasibility study will be incorporated into the comparative study report.

	Criteria to be checked	Communication on the criterion	Control Method of the criterion by the Expert Laboratory
1	Storage conditions of the elements (+ expiry of unopened products)	Packaging or IFU	verification by the Expert Laboratory that the conditions exist
2	Modalities of use after first use (in particular existence of expiry dates)	Packaging or IFU	verification by the Expert Laboratory that the terms and conditions exist
3	Time to Results	Report and attestation	establishment of 2 cycles describing each step of the method only in terms of time: - 1st cycle: negative samples - 2nd cycle: positive samples
4	Common steps with the reference method	Report	verification by the Expert Laboratory

Interlaboratory study (§ 6.2)

General considerations (§ 6.2.1)

The objective of the interlaboratory study is to determine the sensitivity gap between the two methods under conditions of reproducibility.

Measurement protocol (§ 6.2.2)

There should be enough laboratories to present at least 8 usable datasets with at least 2 countries represented and a maximum of 3 collaborators per laboratory with independent testing. The list will be at the discretion of the Technical Board.

Refer to Appendix 5 of this document for the general arrangements for the organization of the interlaboratory study.

The following clarifications are made to the protocol described in ISO 16140-2:

- All configurations (instrument, PLC, kit format, etc.) previously validated in MCS can be used during the interlaboratory study, alone or in combination.

- It is recommended to select a food matrix that is relevant to the test analysis protocol for the alternative method and the target microorganism.

In the case of a study on a "wide variety of foods", it is recommended to test the following matrices:

- *Pseudomonas*: cottage cheese
- Staphylococci: fish terrine, milk
- Enterococci / Coliforms: pâté or cooked ham or pasteurized milk with ancillary flora
- *E. coli*: pan-fried
- *B. cereus*: puree or soup (for babies to be reconstituted / or for adults)

The sample should contain a representative ancillary microflora, which should also remain stable during

transport. The laboratory will need to identify and report the associated flora levels in the matrix.
The level of ancillary flora must be at least 10^3 CFU per mL or per g, unless otherwise advised by the Technical Board.

In the special case of a milk sample test, the flora must be naturally of dairy origin. In practice, the expert laboratory will have to find milk that is naturally contaminated at the level required above. If "non-contamination" is proven, he will either have to change the sample to reach this level of contamination, or he will have to add raw milk (in reasonable proportions).

- Three levels of contamination are to be tested:
  - A **level 0** (L0) which corresponds to the negative control: CFU/test portion.
  - A **minimum rate** for which it is recommended to start at 500 CFU/test portion (except for *Listeria*: start at 300 CFU/test portion), for reasons of dispersion of results and statistical interpretation (Poisson distribution) with low counts.
  - An **intermediate level.**
  - A **higher level** where the high level must reflect as much as possible the contamination rates of the regulatory criteria or have a high value of:
    - *E. coli* / Staphylococcus / *B. cereus* / Coliforms and Enterococci: 10^5 CFU/g higher
    - Total flora: 10^6 CFU/g
    - *Listeria monocytogenes*: 3000 UFC/g
    - *Listeria* spp.: 30000 UFC/g
    - *Pseudomonas*: 10^6 UFC/g
    - *Campylobacter*: 10^4 UFC/g
    - Yeast and mold: 10^5 CFU/g

Calculations, summary and interpretation of data (§ 6.2.3)

Refer to ISO 16140-2.

PROCEDURES FOR THE EXAMINATION BY THE TECHNICAL BOARD OF THE STUDIES CARRIED OUT BY THE EXPERT LABORATORY

The Expert Laboratory is chosen by the applicant from the list of qualified laboratories. He must inform the applicant at each stage of the studies and in the event of changes to the previously established protocol.
It must be qualified by AFNOR Certification (ACE), after consultation with the Technical Board. The qualification procedures for expert laboratories are set out in the NF102 certification rules (§ 2.4.1).

General Notes
The Expert Laboratory should present **finalized** studies and not hesitate to delay the submission of study results, if this is not the case.

ACE may only include on the agenda of each meeting those files (projects or results) for which the complete written reports are available on the date set in advance. This implies in particular that only completed studies - the results of which are known at the date of drafting of the agenda - can be presented at the corresponding meeting. Any study not completed by the date of drafting of the agenda will not be presented at the next session and will be postponed to a later session.

1 Presentation of a comparative study project

The Expert Laboratory must draw up a comparative study project. This is sent to AFNOR Certification by the Expert Laboratory, before the deadline set by ACE (generally 3 to 4 weeks before the meeting).

The Expert Laboratory is convened with the applicant to the meeting of the Technical Board by AFNOR Certification. He must present, with a visual support, the comparative study project he has established.
During this first stage, the Technical Board gives its opinion on:
the possibility of applying the NF VALIDATION mark to the proposed alternative method,
the method taken as a reference,
the comparative study project.
Two **reviewers** have been appointed: they are chosen from among the members of the Technical Board and will study more particularly the files for which they have been charged.

After the meeting, AFNOR Certification communicates the opinion of the Technical Board by e-mail to the applicant, with a copy to the Expert Laboratory, the reviewers and the Presidency.
Where applicable, all modifications relating to the comparative study project must be taken into account by the Expert Laboratory, which sends AFNOR Certification a modified project, if requested by the Technical Board. At the request of the Technical Board, a presentation of the project at the meeting may be requested, which will reconstitute the starting point of the study in terms of execution time.

2 Realization of the comparative study and presentation of the results

Important: the period between the presentation of the comparative study project and the presentation of the comparative study results must not exceed **1 year** (except in exceptional cases to be validated by AFNOR Certification).

The Expert Laboratory must notify AFNOR Certification on the agreed date (generally 4 weeks before the date of the Technical Board meeting), if it is ready to present the results of the comparative study.
The comparative study report must be drawn up in accordance with the template available from AFNOR Certification, where applicable.

The Expert Laboratory must send the **comparative study report** (and any additions), as well as **any accompanying documents** (draft IFU, etc.) to AFNOR Certification before the deadline set by ACE (generally 3 to 4 weeks before the meeting so that it can ensure that it is distributed to the members of the Technical Board).

AFNOR Certification convenes the Expert Laboratory with the applicant to the Technical Board. The Expert Laboratory must present, with a visual support, the comparative study report that it has prepared. During this 2nd stage, the Technical Board gives its opinion on the results obtained during the comparative study.

To do this, at the end of the presentation, a discussion takes place in the absence of the applicant and in the presence of the Expert Laboratory. Then **a vote is carried out** in the absence of the applicant and the Expert Laboratory, taking into account all the results of the comparative study.
This vote gives the opinion of the Technical Board. The result of this vote determines whether the results of the benchmarking study are accepted.
The results of the vote shall be communicated to the applicant and to the Expert Laboratory at the meeting.

Note: The results are accepted by a relative majority (the President having a casting vote in the event of a tie). The votes "For", "Against" and "Abstentions" are counted. Votes"Against" and "Abstentions" must be systematically justified during a round table. The number of abstentions must not exceed 50% of the voters present. Otherwise, a second round will be held, during which only "For" votes are cast. The second round is decided by a relative majority.

The Technical Board may request additional comparative studies on one or more of the criteria. This may delay the start of the interlaboratory study depending on the importance of these supplements.
If the full results of the comparative study (including additions if necessary) are accepted, the interlaboratory study can be performed.

In this case, the Expert Laboratory must therefore also present, at this meeting, a draft interlaboratory study. The Technical Board gives its opinion on the interlaboratory study project. The list of collaborating laboratories is included in the project or is distributed to AFNOR Certification after the meeting to submit it to the Technical Board for its opinion before the start of the study.
All changes relating to the interlaboratory study project must be taken into account by the Expert Laboratory, which subsequently sends AFNOR Certification a modified project, if requested by the Technical Board.

After the meeting, AFNOR Certification communicates all the opinions taken at the meeting, by email, to the applicant, with a copy to the Expert Laboratory, the reviewers and the Presidency.

3 Carrying out the interlaboratory study and presentation of the results

Important: The period between the presentation of the interlaboratory study project and the presentation of the results of the interlaboratory study must not exceed **1 year** (except in exceptional cases to be validated by AFNOR Certification).

The Expert Laboratory must notify AFNOR Certification on the agreed date (generally 4 weeks before the date of the Technical Board meeting), if it is ready to present the results of the interlaboratory study. The interlaboratory study report must be drawn up in accordance with the template available from AFNOR Certification.

The Expert Laboratory must send the **interlaboratory study report** (and any additions), as well as **any additional documents** (draft technical notices, etc.) to AFNOR Certification before the deadline set by ACE (generally 3 to 4 weeks before the meeting so that it can ensure that they are distributed to the members of the Technical Board).

AFNOR Certification convenes the Expert Laboratory with the applicant to the Technical Board.
The Expert Laboratory presents, with visual support, the interlaboratory study report that it has prepared.
During this 3rd stage, the Technical Board gives its opinion on the results of the interlaboratory study.
To do this, at the end of the presentation, a discussion takes place in the absence of the applicant and in the presence of the Expert Laboratory. Then **a vote is carried out** in the absence of the applicant and the expert laboratory, taking into account all the results of the study (preliminary and interlaboratory). This vote gives the final opinion of the Technical Board and takes into account all the results presented (comparative study and interlaboratory). The result of this vote determines whether the method can be validated or not.
The results of the vote shall be communicated to the applicant at the meeting.

Note: The results are accepted by a relative majority (the President having a casting vote in the event of a tie). The votes "For", "Against" and "Abstentions" are counted. Votes "Against" and "Absenteeism" must be systematically justified during a round table.
The number of abstentions must not exceed 50% of the voters present. Otherwise, a second round will be held, during which only "For" votes are cast. The second round is decided by a relative majority.

After the meeting, AFNOR Certification communicates all the opinions taken at the meeting, by email, to the applicant, with a copy to the Expert Laboratory, the reviewers and the Presidency.

4 Preparation of the NF VALIDATION certificate

AFNOR Certification takes the decision to certify the alternative method, upon final opinion issued by the Technical Board.

A letter of decision notice is issued by AFNOR Certification following the corresponding meeting, officially attesting to the certification of the method. This letter is issued, subject to any additions that may be requested by the Technical Board within the deadlines specified by ACE.
AFNOR Certification then drafts the certificate on the basis of the model defined by the Technical Board, if applicable. The various sections are completed according to the recommendations that the latter may have issued, during the various stages of presentation of the file (scope, possible restriction(s)...). If necessary, AFNOR Certification may consult the Technical Board to ensure that the elements reported are suitable.

The references of the technical instructions necessary for the implementation of the alternative method are reported on the NF VALIDATION certificate. The specific instruments and associated calculation software are also referenced on the NF VALIDATION certificate.
The certificate is published in French and English. Each certificate is signed by AFNOR Certification's Legal Officer. The French version of the certificate is authentic.
The holder receives the original certificate. A copy is available to the public on the http://nf-validation.afnor.org website.

5 Synthesis report of studies

Following the decision to certify the initial validation, renewal or extension of an alternative method, the Expert Laboratory must draw up a summary report of the study (comparative and interlaboratory). This document summarizes the important elements of these studies.

The Expert Laboratory must take into account, in the drafting of the summary report, the comments made during the meetings of the Technical Board on the interim study reports (comparative and interlaboratory).

Its objective is to be able to be distributed to any person who requests it. A template is available from AFNOR Certification, if applicable. All published summary reports are made available to the public on the http://nf-validation.afnor.org website. The holder must also validate the content of the agreement, as to the confidentiality of the elements contained therein.

This document must be sent by the Expert Laboratory to AFNOR Certification no later than **2 months following** the final opinion of the Technical Board.

6 Duration of certification

The duration of certification under the NF VALIDATION mark is 4 years, unless a sanction is taken against it.

If changes are made to the alternative method and require testing, this will be an extension study.

In the event of a change in the reference method or validation protocol during the certification period, the decision shall remain valid until the original scheduled expiry date. The file must be updated at the latest at the next renewal.

7 Extension/modification case

In the event that an additional study is to be carried out, two reviewers are appointed at a meeting of the Technical Board following the presentation of the corresponding study project.

The initial summary report should be supplemented by a summary of the additional studies carried out.

8 Cases of renewal

The terms and conditions of the renewal study and the content of the renewal file are specified in the certification rules (§ 5.3.2).

In the event that no further study is required, the two reviewers are ideally appointed before the corresponding meeting, at a previous session or via electronic consultation.

In the event that an additional study is to be carried out, the two reviewers shall be appointed at the latest at the meeting of the Technical Board at which the corresponding draft study was presented.

The synthesis report should include a reminder of the main results obtained during the previous validation studies, and the synthesis of the additional studies carried out if necessary.

Appendix 1

Transition arrangements for the transition to ISO 16140-2/A1 (2024)

For all applications (validation / extension / renewal):

- After publication of the amendment at the French level (September 2024):
  - Mandatory application of the new protocol for all files*
  - Issuing certificates according to NF EN ISO 16140-2:2016, no change necessary as an amendment cannot be used alone. The amendment may be present at the request of the manufacturer.

Additional tests required for the update according to ISO 16140-2 of previously validated dossiers (*Expected at the latest at the next renewal of the validation of the alternative method, early updating of the dossier also being possible)*

*Except for the following points of ISO 16140-2/A1: 2024 (these decisions are the responsibility of the Technical Board):

- **Form (4):** Report of False Negative Results

$$\text{Rapport des résultats faux négatifs : FNR} = \frac{\text{NA}_{\text{FN(alt)}} + \text{ND}_{\text{FN(alt)}}}{\text{PA} + \text{TND} + \text{PD}}$$

Put the result as a percentage (%) to be consistent with the other criteria and to make it easier for users to read.

- **Table 4 — Parameters and acceptability limit values for paired and unpaired method studies as a function of the number of positive samples obtained**

Do not take into account in the column entitled "Mixed study" the criterion "NDT + PD". When a study is unmatched, it is normal to have discordances and this criterion is only needed for matched studies.

- **Form 15 (Quantitative Methods):**

$$\left[\bar{D} \pm T \cdot \sqrt{\frac{s_D^2}{n}}\right]$$

The above wording of amendment 1 is false (communication from ISO which justifies the use of the old 2016 formula) present below:

$$\left[\bar{D} \pm T \cdot s_D \sqrt{1 + \frac{1}{n}}\right]$$

- **5.2.2, third bullet (sentence greyed out)**

"Replace the text with the following:
— at least three different levels of contamination must be used: one negative control (L0) and two levels (L1 and L2). At least one of the two must produce partial positive results. The level of contamination required to obtain partial positive results should be based on the reference method RLOD study data obtained from the comparative method study. In theory, an average contamination level of 1 CFU/sample is adequate to obtain partial positive results. The level of contamination must be determined. This allows the LOD50 of the alternative method, which is required in order to verify the performance of the alternative method when implementing the validated method in a laboratory in accordance with ISO 16140-3. The level of contamination is determined by performing an MPN analysis at the beginning of the interlaboratory study, see 5.2.4.4. »

Appendix 2

Specific provisions for the validation of alternative methods for the detection of STEC compared to the technical specification XP CEN ISO/TS 13136

Confirmation of positive specimens (case 2)

Conventional tests of the XP CEN ISO/TS 13136 technical specification cannot be applied for the confirmation of positive PCR samples by the alternative method. The manufacturer must therefore propose at least a "case 2" of confirmation (see definition in § Conditions for confirmation of positive results) and specify in the technical sheet the reference of the reagents used for the confirmation stage (to be provided as soon as the comparative study project is presented).

The text to be inserted in the technical sheet is as follows:

"Under the NF VALIDATION mark, samples that have been tested positive at the end of the detection stage must be confirmed in one of the following ways:

- "Case 2" (protocol(s) provided by the manufacturer to be described)

In the event of a discordant result (presumptive positive by the alternative method, not confirmed by the tests described above), the laboratory must implement sufficient means to ensure the validity of the result returned. In particular, the following protocol (if applicable) was tested during the NF VALIDATION study: <protocol applied by the Expert Laboratory> (and/or) The following protocol is recommended: <manufacturer's protocol>".

Comparative study (§ 5.1)

- **Sensitivity study (§ 5.1.3)**

Selecting the categories to be used (§ 5.1.3.1):

The following "matrix/serogroup" associations are preferred:

- O103, O26 in dairy products
- O157, O145 in meat products
- O111 for plants

Tests should be carried out as far as possible on naturally contaminated samples. For STEC, the Technical Office has not set a minimum threshold for naturally contaminated samples. The expert laboratory will have to justify the difficulty of using naturally contaminated samples. It is therefore possible to carry out artificial contamination. The different contamination options to be applied are specified in § Selection of categories to be used (§ 5.1.3.1) of this document.

Calculations and interpretation (§ 5.1.3.4):

For the reference method, add in the results tables an intermediate column with the results of the "*stx*/eae" targets, in order to provide an interpretation as described in the technical specification XP CEN ISO/TS 13136 in its chapter 10 (an example of a table is provided in the study report template available on request from AFNOR Certification).

- **RLOD Study (§ 5.1.4)**

Selection of categories, number of samples, and replicates tested (Section 5.1.4.1):

It is necessary to test 1 "matrix/strain" pair per category and serogroup, with the 5 serogroups represented, favouring the following associations:

- O103, O26 in dairy products
- O157, O145 in meat products
- O111 for plants

NOTE: All 5 serogroups must be considered in the case of initial validation, even if the application is for a food matrix only. In the case of an extension of the field of application, the serogroup most relevant to

the food matrix tested shall be selected for the tests.

- **Inclusiveness and exclusivity study (§ 5.1.5)**

The Technical Office has established a minimum list of target and non-target strains to be tested, which is presented below. The list must be completed by the Expert Laboratory to meet the required requirements and submitted to the Technical Office for its opinion.

List table of target and non-target strains.

Refer to the corresponding Excel file, available on request from AFNOR Certification.

Inclusivity:
Among the 50 strains to be tested in total (as required by EN ISO 16140-2), test:
- At least 5 strains for minor serogroups: O111 and O145
- At least 10 strains for major serogroups: O26, O157 and O103 Of all positive strains:
- Target the 2 *stx1* and *stx2* genes and at least the 3 most common variants (*stx2a, stx*2b, *stx2c)*
- Vary the origins of strains

Exclusivity
Among the 30 strains to be tested, test:
- A mix of *stx*+/eae$^-$, *stx*$^-$/eae+ strains (non-pathogenic strains but with one of the virulence genes)
- *Citrobacter rodentium*

- **Interlaboratory study (§ 5.2)**

"Matrix/strain" pair to be tested: Minced steak (minimum 15% fat) contaminated with *E. coli* O26.

Regarding the transport of samples, it must be adapted to this pathogen and carried out in compliance with the regulations in force in the territory concerned.

NOTE: Specimens containing confirmed verotoxin-producing E. coli are to be considered as specimens classified as "UN2814 - Human Infectious Material", while those for which the presence is not confirmed (not tested or presumptive positive not confirmed) are to be classified as "UN3373 - Diagnostic specimens".

For the choice of collaborating laboratories, it is possible to facilitate the implementation of the study to base itself on the list of "laboratories approved for *E. coli* STEC analyses within the framework of surveillance and control plans" by the French Ministry of Agriculture, Agri-Food and Forestry for official *E. coli* STEC analyses (available on the website http://agriculture.gouv.fr/laboratoires-agrees-et-reconnus-methodes-officielles-en-alimentation).

Conduct of the study:

- Expert laboratory: Sending contaminated samples to employees.
- Collaborators:
  - Reference method adapted from XP CEN ISO/TS 13136 of the Expert Laboratory (special case in the absence of an internal reference method within the participating laboratory):
    - Carrying out enrichments
    - Extraction step + Implementation of confirmations by performing an IMS (O26) followed by isolation on selective agar and a latex test on colony after purification step on non-selective agar
    - Sending the DNA extracts obtained to the Expert Laboratory + confirmatory results
  - Alternative method:
    - Protocol to be applied in its entirety
    - PCR results + Confirmatory results to be returned to the Expert Laboratory.

- Expert laboratory:
  - Implementation of the 2 methods (complete protocols to be applied)
  - PCR test on extracts returned by employees
  - Exploitation of the results of the 2 methods (before/after confirmation)

Appendix 3

Product categories and product types by target germ, stress and associated contamination patterns (informative list)

Refer to the corresponding Excel file, available on request from AFNOR Certification.

The types and categories listed in this Annex are for illustrative purposes only and may be defined differently depending on the needs of the Manufacturer.

Appendix 4

Acceptability limit for n+1 categories – Number of positive samples required for category(ies) n to apply the AL

Number of Category	Number of N+	Number of positive samples (N+)	Paired Study		Unpaired study
			TND-PD	TND + PD	TND-PD
1	30	31 to 59	3	6	3
2	60	60 to 89	4	8	4
3	90	90 to 119	5	10	5
4	120	120 to 149	5	12	5
5	150	150 to 179	5	14	5
6	180	180 to 209	6	16	6
7	210	210 to 239	6	18	7
8	240	240 to 269	6	20	7
9	270	270 to 299	7	22	8
10	300	300 to 329	7	24	8
11	330	330 to 359	7	26	9
12	360	360 to 389	8	28	9
13	390	390 to 419	8	30	10
14	420	420 to 449	8	32	10
15	450	450 to 479	9	34	11
16	480	480 to 509	9	36	11
17	510	510 to 539	9	38	12
18	540	540 to 569	10	40	12
19	570	570 to 599	10	42	13
20	600	600 to 629	10	44	13
21	630	630 to 659	11	46	14
22	660	660 to 689	11	48	14
23	690	690 to 719	11	50	15
24	720	720 to 749	12	52	15
25	750	750 to 779	12	54	16
26	780	780 to 809	12	56	16
27	810	810 to 839	13	58	17
28	840	840 to 869	13	60	17
29	870	870 to 899	13	62	18
30	900	900 to 929	14	64	18
31	930	930 to 959	14	66	19
32	960	960 to 969	14	68	19
33	970	970 to 1019	15	70	20
34	1020	1020 to 1049	15	72	20

Note: This requirement is an alternative to the initial interpretation of the standard's acceptability limits and when the number of positive samples obtained during the sensitivity study will allow such exploitation. As a first step, the results of the sensitivity study will always be analyzed taking into account the acceptability limit criteria as defined by the ISO 16140-2 standard.

Appendix 5

Salmonella *Selectivity Study* - Mandatory Strain Lists

Target and non-target strains to be included in the list of strains to be tested for the validation of alternative methods for the detection of salmonella. The lists must be supplemented to meet the specific requirements of this document (see section Inclusiveness and exclusivity study (§ 5.1.5)).

1 **Inclusivity**

Souches *Salmonella*

	GROUP "O"	SPECIES	SUBSPECIES	SEROVAR	FORMULA
1	**2 (A)**	S. enterica	*enteric (I)*	Paratyphi A	1,2,12 : a : 1,5
2	**4 (B)**	S. enterica	*enteric (I)*	Paratyphi B	1,4,[5],12 : b : 1,2
3		S. enterica	*enteric (I)*	Typhimurium	1,4,[5],12 : i : 1,2
4		S. enterica	*enteric (I)*	Bredeney	1,4,12,27 : L,V : 1,7
5		S. enterica	*enteric (I)*	Heidelberg	1,4,[5],12 : r : 1,2
6		S. enterica	*enteric (I)*	Indiana	1,4,12 : z : 1,7
7		S. enterica	*enteric (I)*	Saintpaul	1,4,[5],12 : e,h : 1,2
8		S. enterica	*enteric (I)*	Derby	1,4,[5],12 : f,g : [1,2]
9	**6,7 (C)**	S. enterica	*enteric (I)*	Paratyphi C	6,7,[Vi] : c : 1,5
10		S. enterica	*enteric (I)*	Livingstone	6,7,14 : d : l,w
11		S. enterica	*enteric (I)*	Mbandaka	6,7,14 : Z10 : E,N,Z15
12		S. enterica	*enteric (I)*	Virchow	6,7,14 : r : 1,2
13		S. enterica	*enteric (I)*	Children	6,7,14 : r : 1,5
14		S. enterica	*enteric (I)*	Cracks	6,7,14 : f,g : -
15		S. enterica	*enteric (I)*	Montevideo	6,7,14 : g,m,[p],s : [1,2,7]
16	**8 (C)**	S. enterica	*enteric (I)*	Manhattan	6.8 : d : 1.5
17		S. enterica	*enteric (I)*	Hadar	6.8: Z10: E,N,X
18		S. enterica	*enteric (I)*	Blockley	6,8 : k : 1,5
19		S. enterica	*enteric (I)*	Kottbus	6.8 : E, H : 1.5
20	**9 (D)**	S. enterica	*enteric (I)*	Typhi	9,12,[Vi] : d : -
21		S. enterica	*enteric (I)*	Naples	1,9,12 : l,z13: e,n,x
22		S. enterica	*enteric (I)*	Enteritidis	1,9,12 : [f],g,m,[p] : [1,7]
23		S. enterica	*enteric (I)*	Dublin	1,9,12,[vi] : g,p : -
24		S. enterica	*enteric (I)*	Gallinarum	1,9,12 : - : -
25	**3.10 (E)**	S. enterica	*enteric (I)*	London	3.10[15] : L.V : 1.6
26		S. enterica	*enteric (I)*	Anatum	3.10[15][15.34] : e,h : 1.6
27		S. enterica	*enteric (I)*	Regent	3,10 : f,g,[s] : [1,6]
28	**1,3,19 (E)**	S. enterica	*enteric (I)*	Senftenberg	1,3,19 : g,[s],t : -
29	**13 (G)**	S. enterica	*enteric (I)*	Kedougou	1,13,23 : i : l,w
30		S. enterica	*enteric (I)*	Havana	1,13,23 : f,g,[s] : -
31	**18 (K)**	S. enterica	*enteric (I)*	Hill	6,14,18 : z4,z23 : [1,5]
32	**48 (Y)**	S. enterica	*arizonae (IIIa)*	S.III a	48 : z4,z23 : -
33	**51**	S. enterica	*arizonae (IIIa)*	S.III a	51 : z4,z23 : -
34	**38**	S. enterica	*diarizonae (IIIb)*	S.III b	38 : L,V : Z53
35	**61**	S. enterica	*diarizonae (IIIb)*	S.III b	61 : k : 1,5,7

	GROUP "O"	SPECIES	SUBSPECIES	SEROVAR	FORMULA
36	**Variants de *Salmonella* Typhimurium**	S. enterica	*enteric (I)*	S.I	1,4,[5],12 : i : -
37		S. enterica	*enteric (I)*	S.I	1,4,[5],12 : - : 1,2
38		S. enterica	*enteric (I)*	S.I	1,4,[5],12 : - : -
39	**4 (B)**	S. enterica	*enteric (I)*	Agona	1,4,[5],12 : f,g,s : [1,2]
40		S. enterica	*enteric (I)*	Chester	1,4,[5],12 : e,h : e,n,x
41		S. enterica	*enteric (I)*	Stanley	1,4,[5],12,[27] : d : 1,2
42		S. enterica	*enteric (I)*	Schwarzengrund	1,4,12,27 : d : 1,7
43		S. enterica	*enteric (I)*	Abort	4.12 : - : e,n,x
44		S. enterica	*enteric (I)*	Abortusovis	4,12 : c : 1,6
45	**7 (C1)**	S. enterica	*enteric (I)*	Braenderup	6,7,14 : e,h : e,n,z15
46		S. enterica	*enteric (I)*	Bareilly	6,7,14 : y : e,n,x
47		S. enterica	*enteric (I)*	Lille	6,7,14 : z38 : -
48		S. enterica	*enteric (I)*	Oranienburg	6,7,14 : m,t : [z57]
49		S. enterica	*enteric (I)*	Thompson	6,7,14 : k : 1,5
50		S. enterica	*enteric (I)*	Tennessee	6,7,14 : z29 : [1,2,7]
51	**8 (C2-C3)**	S. enterica	*enteric (I)*	Munich	6.8:D: 1.2
52		S. enterica	*enteric (I)*	Newport	6,8,20:E,H:1,2
53		S. enterica	*enteric (I)*	Kentucky	8.20 : i : z6
54	**9 (D1)**	S. enterica	*enteric (I)*	Panama	1,9,12: L,V: 1,5
55		S. enterica	*enteric (I)*	Javiana	1,9,12 : l,z28 : 1,5
56	**3.10 (E1)**	S. enterica	*enteric (I)*	Give	3.{10}{15}{15.34}: l,v: 1.7
57		S. enterica	*enteric (I)*	Weltevreden	3.{10}{15} : r : z6
58		S. enterica	*enteric (I)*	Meleagridis	3,{10}{15}{15,34} : e,h : l,w
59	**11 (F)**	S. enterica	*enteric (I)*	Abaetetuba	11 : k : 1,5
60		S. enterica	*enteric (I)*	Venetian	11 : i : e,n,x
61		S. enterica	*enteric (I)*	Rubislaw	11 : r : e,n,x
62		S. enterica	*enteric (I)*	Aberdeen	11 : i : 1,2
63	**13 (O)**	S. enterica	*enteric (I)*	Poona	1,13,22 : z : 1,6
64		S. enterica	*enteric (I)*	Cuban	1,13,23 : z29 : -
65		S. enterica	*enteric (I)*	Mississippi	1,13,23 : b : 1,5
66		S. enterica	*enteric (I)*	Wells	13.23 : d : l,w
67	**6.14 (H)**	S. enterica	*enteric (I)*	Caracas	[1],6,14,[25] : g,m,s : -
68	**16 (I)**	S. enterica	*enteric (I)*	Hvittingfoss waterfall	16 : b : e,n,x
69		S. enterica	*enteric (I)*	Gaminara	16 : d : 1.7
70	**17 (J)**	S. enterica	*enteric (I)*	Michigan	17: L,V: 1,2
71	**21 (L)**	S. enterica	*enteric (I)*	Minnesota	21 : b : e,n,x
72	**30 (N)**	S. enterica	*enteric (I)*	Urban	30 : b : e,n,x
73	**35 (O)**	S. enterica	*enteric (I)*	Adelaide	35 : f,g : -
74	**39 (Q)**	S. enterica	*enteric (I)*	Wandsworth	39 : b : 1,2
75	**42 (T)**	S. enterica	*salamae (II)*	S. II	42 : b : e,n,x,z15
76	**40 (R)**	S. enterica	*houtenae (IV)*	S. IV	1,40 : z4,z23 : -
77	**6.14 (H)**	S. enterica	*indica (VI)*	S. VI	[1],6,14,[25] : a : e,n,x
78	**48 (Y)**	S. bongori	*(V)*	S. V	48 : z35 : -

2 Exclusivity

Souches not *Salmonella*

No.	GENDER	SPECIES
1	***Citrobacter*** *	*freundii, diversus, youngae, koseri, braaki,*
2	***Escherichia***	*coli, sibling*
3	***Proteus***	*mirabilis, vulgaris*
4	***Klebsiella***	*pneumoniae, oxytoca*
5	***Enterobacter***	*cloacae, sakazakii, agglomerans (ou Pantoea agglomerans)*
6	***Serratia***	*Marcescent*
7	***Hafnia***	*alvei*
8	***Shigella***	*flexneri*

* Choose three species from the five.

Appendix 6

General arrangements for the organisation of the interlaboratory study

Choice of collaborating laboratories:

The Expert Laboratory must propose to the Technical Office, when presenting the interlaboratory study project if possible, and in any case before the start of the interlaboratory study, a list of competent laboratories, public or private, preferably from several European countries. These laboratories are at least at the number required by the ISO 16140-2 standard, so as to obtain at least as many series of interpretable results. The Expert Laboratory and the Manufacturer's Laboratory are not counted among these laboratories.
The choice of collaborating laboratories is made in consultation between the manufacturer and the Expert Laboratory. Nevertheless, the final choice and monitoring of collaborating laboratories is the responsibility of the Expert Laboratory, which must ensure that they have implemented a quality assurance approach in the field concerned.

Tasks of the Expert Laboratory:

The Expert Laboratory prepares the samples for the collaborating laboratories and sends them the analysis protocol to be carried out for the alternative method.
The Expert Laboratory must ensure that it implements the means adapted to the important logistics due to this study. It is imperative that he have a temperature record during transport.

Regarding the preparation of samples for qualitative methods, the Technical Office considers that the homogeneity test is not necessary. The laboratory will have to check that the mixture is sufficiently stable over several days under the conditions of transport and storage.

Regarding the transport of samples, the Technical Office believes that expert laboratories should favor refrigeration rather than freezing samples. Indeed, freezing entails a risk of loss of target bacteria for samples with a low contamination rate. It is therefore the Technical Office that will decide for each study on the possibility of freezing the samples during transport or not. Regarding refrigeration, the following conditions (defined by the Technical Office) apply: the minimum and maximum temperature of the samples, during transport and on arrival at the laboratory, must be between 0°C and 8°C.

Concerning the organization of the interlaboratory study, the enumeration of the total bacterial flora must be done on a specific additional sample prepared by the Expert Laboratory.

Instructions to collaborating laboratories:

Employees have the option of analysing the samples on D1 or D2, provided that the same sample is taken on the same day for the alternative method and the reference method. Where appropriate, the instructions should be clear and precise.

The Expert Laboratory is advised to have each collaborating laboratory sign a certificate of acknowledgement of the instructions relating to the interlaboratory study.
In particular, the Expert Laboratory must set very clearly for collaborating laboratories the conditions for the disposal of a laboratory's results (at least the day of analysis and the maximum temperature at which samples are received). This allows for clear and non-debatable rules for the elimination of results, and also prevents a collaborating laboratory from carrying out unnecessary tests.

Storing fortification broths:

For interlaboratory studies, each laboratory must keep the enrichment broths of the various samples analysed, under the conditions set by the Expert Laboratory. If the Laboratory

After analysing the results, he or she may ask the collaborating laboratories to carry out additional tests to explain the discrepancy.

Appendix 7

Conditions for confirmation of positive results of alternative Listeria detection methods (supplement)

Step	*Listeria* spp.		*Listeria monocytogenes*	
Enrichment (including medium and conditions)	Reference Broth (Fraser 1/2)	Broth owner	Reference broth (Fraser 1/2)	Broth owner
Screening	Test ELISA, PCR	Chromogenic agar, ELISA, PCR	Test ELISA, PCR	Chromogenic agar, ELISA, PCR
Confirmation sur bouillon	• OAA and/or PALCAM agar: no validation test required • Proprietary agar: test for validation *	• OAA and/or PALCAM agar: test for validation • Proprietary agar: test for validation	• OAA agar: no validation test required • Proprietary agar: test for validation*	• OAA agar: test for validation • Proprietary agar: test for validation
Colony confirmation	• For ELISA and PCR tests, only the presence of a colony is sufficient to confirm the positive result. However, during validation, confirmation of the colony from OAA and/or PALCAM agar and proprietary agar should be performed using a biochemical assay (without purification step). • For chromogenic agars, the colony must be confirmed by a biochemical test during validation and during the application of the method in routine (user) analysis with or without a purification step according to the manufacturer's recommendations (concerns also the enumeration of colonies).			

* *In the absence of a proprietary medium proposed, the manufacturer must perform at least a confirmatory test on OAA or PALCAM agar.*

Appendix 8

Study and synthesis report templates for studies conducted according to ISO 16140-2

Refer to any corresponding models defined by the Technical Office, available on request from AFNOR Certification.