Validation of methods of analysis
Application in food microbiology

Requirements regarding comparison and interlaboratory studies for implementation of the standard EN ISO 16140-2

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(following approval by the Technical Board concerned)
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ESTABLISHED PRECEDENTS

- **Cronobacter studies:** Test a minimum of 10 positive samples with probiotics apportioned between "milk powders" and "liquid milks". Actual levels in the tested samples are also to be specified (see product labels or carry out tests to verify actual presence of probiotics).

- **For *L. monocytogenes* methods:** In the scope of validation it is permitted to initiate a confirmation after preservation of half Fraser broth for 48 hours, without storage tests (where the test has already been performed, it being permitted to preserve the broth in parallel in order to subsequently do the confirmation).

- **Renewal dossiers:** Systematically verify the protocol tested for inclusivity and if necessary repeat the tests with the most selective protocol.

- **Molecular methods:** Systematically document in the comparison study results reports:
  - The inhibition levels and, if necessary, describe any protocols applied during the study that enabled these inhibitions to be eliminated.
  - If applicable, the atypical curves obtained, and the curves for samples with discrepant results.

- **Cold storage tests:** Only test the positives in the case of broths and test all positives and negatives in the case of petri-dishes.

- **Alternative methods using petri-dishes for detection:** The tests must be conducted at the minimum and maximum incubation times, with an 8-hour interval between the incubation periods.

- **Alternative methods with subculture before isolation on selective agar (subject to the results obtained):** Direct confirmation by Latex test (reference to be provided) using an isolated colony is possible irrespective of the chromogenic agar used (reference tested during the validation study to be specified in the report, but subsequent openness to all chromogenic agars in the technical instructions.

- **Dossiers validated based on new version of validation protocol:** The validation protocol reference to be indicated in documentation relating to certification (conclusions, findings, certificate) is NF EN ISO 16140-2:2016 when new the version has been applied. For dossiers validated according to the previous validation protocol, the documentation shall still refer to NF EN ISO 16140:2003.

This instruction is also applicable to:
- alternative methods’ pack inserts issued by the requester/holder company’
- Study reports and summary reports prepared by the Expert laboratories;
- NF VALIDATION certificates.
PREFACE

Subject

This document applies in addition to EN ISO 16140-2:2016 for which it does not substitute itself. It constitutes partially an application guide for this standard and contains additional specific provisions required by the NF VALIDATION mark.

The French version of this document is the authentic one in legal terms.

Update/Circulation

This document is updated by AFNOR Certification each time a modification is made by the Microbiology Technical Board and/or by AFNOR Certification. After each update, it is distributed by AFNOR Certification to the qualified Expert laboratories and to the Technical Board members. The circulation of this document is unrestricted to anyone requesting it.

Note 1: Criteria of acceptability of results

Standard EN ISO 16140-2:2016 has introduced result acceptability limits for the main criteria. It falls to the relevant Technical Board however to examine and assess the results for each of these criteria, and supply an opinion on all the detailed results.

Note 2: Acceptance of external results

It is possible to accept external results obtained previously within the framework of another validation program. In the limit of its competence, and in the absence of official documents stating this competence, it falls to the Technical Board to judge the significance of the differences between the reference methods and/or the validation protocols used.

Note 3: Raw results

The Expert laboratory must be able to communicate all the raw data (not appearing in the report) to the reviewers who examine the dossiers, to AFNOR Certification or to the Technical Board members if necessary.

Note 5: Complying with present requirements

The Expert laboratory must state throughout the study any deviations from the alternative method’s protocol proposed by the applicant/holder company and/or from this document. If no deviation is mentioned, compliance with the alternative method’s protocol proposed by the applicant/holder company and with this document is implicit and is under its responsibility.

QUALITATIVE METHODS
Requirements relating to confirmation of positive results

In the context of the NF VALIDATION mark, all alternative methods for the detection of pathogenic microorganisms (Salmonella, Listeria monocytogenes, etc.), presented for validation, must systematically include a confirmation step involving confirmation of the positive results in their standard protocol. This requirement does not concern families that include different genera of which only some include pathogenic strains (e.g., enterobacteriaceae).

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

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

1. Using the conventional tests described in the standardized methods by CEN or ISO from colonies (including the purification step).
   
   **Note:** The steps prior to confirmation must be clearly explained.

2. Using one or more methods whose principle is different from that of the validated method, and described by the requester/holder company’s in a confirmation protocol at the time of the initial validation request.

   **NOTE:** In this case, the proposed confirmation method(s) shall be examined during the validation study and appraised by the Technical Board which may decide to reject this option. These specific confirmation protocols must be documented in the study report.

   In the context of this confirmation option 2, the Latex Test references proposed are to be specified in the study report.

3. Using any other NF VALIDATION certified method, the principle of which is different from that of the validated method the result of which it is intended to confirm. The detection protocol of the second validated method must be followed entirely, in other words all steps prior to the intermediate step which is the starting point for confirmation, must be common to both methods.

   **NOTE:** The two validated methods (one used for detection and the other for confirmation) must have common first steps (for instance, a common enrichment with the same medium).

4. Using nucleic probes different from those of the detection method the result of which it is intended to confirm and as described in NF EN ISO 7218 from isolated colonies (including or not the purification step).

   **Case 1** must be part of the protocol of the alternative method to validate. **Case 2, 3 and 4** can supplement it.

   **In the event of discordant results** (presumptive positive with the alternative method, not confirmed by one of the means described above [and in particular by the Latex test(s)]) the laboratory must employ adequate means to ensure validity of the result obtained.
Specific cases

- **Confirmation of *Listeria monocytogenes***:

  Subject to the validation results on at least two chromogenic agars, the following case 2 of confirmation can be proposed: "By isolation on chromogenic agar according to the definition of NF EN ISO 11290 (OAA formulation type) or forming part of a NF VALIDATION certified method. The presence of characteristic colonies is sufficient to confirm the presence of *Listeria monocytogenes*.

  For the alternative methods, validated both for detection and counting of *Listeria monocytogenes* and using the same agar in both cases (same commercial reference for the medium), the following procedures can be proposed:
  - Detection part: "If a positive result for *Listeria monocytogenes* is obtained at the detection step with the method [*name of validated detection method*], it is not necessary to confirm this result if it has already been confirmed on completion of counting for the method [*name of validated counting method*]."
  - Counting part: "If a positive result for *Listeria monocytogenes* is obtained at the counting step with the method [*name of validated counting method*], it is not necessary to confirm this result if it has already been confirmed on completion of the detection step with the method [*name of validated detection method*]. Lack of confirmation of 5 colonies when counting implies a risk of obtaining an overestimated result owing to the possible presence of characteristic colonies that are not *Listeria monocytogenes*.

- **Confirmation of the genus *Listeria* spp.**:

  Subject to a prior decision by the Technical Board, the following case 2 of confirmation can be proposed: "By isolation on PALCAM agar or on chromogenic agar forming part of a NF VALIDATION certified method for detection of the *Listeria* genus. The presence of characteristic colonies is sufficient to confirm the presence of *Listeria* spp.

  For the alternative methods, validated both for detection and counting of *Listeria* spp., and using the same agar in both cases (same commercial reference for the medium), the following procedures can be proposed:
  - Detection part: "If a positive result for *Listeria* spp. is obtained at the detection step with the method [*name of validated detection method*], it is not necessary to confirm this result if it has already been confirmed on completion of counting for the method [*name of validated counting method*]."
  - Counting part: "If a positive result for *Listeria* spp. is obtained at the counting step with the method [*name of validated counting method*], it is not necessary to confirm this result if it has already been confirmed on completion of the detection step with the method [*name of validated detection method*]. Lack of confirmation of 5 colonies when counting implies a risk of obtaining an overestimated result owing to the possible presence of characteristic colonies that are not *Listeria* spp."
Method comparison study (§ 5.1)

General considerations (§ 5.1.1)

The purpose of this study is to compare the performances of the alternative method against those of 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 are to be tested for the alternative method and the reference method.

- Tests with 25 g test portions enable inclusion of weighed quantities from 1 g to 25 g.
- For protocols with 1/4 dilution: Tests performed with test portions greater than 50 g (e.g., 375 g) enable inclusion of weighed quantities from 50 g to that tested in the study (e.g., 375 g). This means that there is a gap between 25 g and 50 g, but user interest is less within this range.
- For protocols with 1/10 dilution: Tests performed with test portions greater than 25 g (e.g., 375 g) enable inclusion of weighed quantities ranging from 1 g to that tested in the study (e.g., 375 g).

Sensitivity study (§ 5.1.3)

Selection of categories to be used (§ 5.1.3.1)

The analyses shall be carried out on samples naturally contaminated or artificially contaminated with a target microorganism, and not contaminated, belonging to different food categories, representative of products usually subjected to this type of analysis (see Annex A of EN ISO 16140-2). The origin of the samples must be the most varied possible so as to reduce instances of bias related to food specialties.

The types and categories listed in Annex 1 of this document are only given by way of example and may be defined differently depending on the requestor/holder company's need.

In cases where “combined” categories are proposed for validation, these will be accepted subject to proposal of at least a total of 5 food category products.

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

- 1st option: Naturally contaminated samples
- 2nd option: Contamination by mixture
- 3rd option: Artificially contaminated samples (“seeding” or “spiking”)
- 4th option: Reference material

If it is not possible to obtain a sufficient number of naturally contaminated foods in each of the categories, artificial contamination of food samples is permissible. The Expert laboratory must justify the difficulty in implementing naturally contaminated samples.
For *Listeria* and *Salmonella*, the Technical Board has set a strict threshold for artificially contaminated samples:

- **Listeria** (*spp and monocytogenes*):
  50% all categories of products combined

- **Salmonella**:
  
  - Food samples (under scope of EN/ISO 6579): 90% all categories of products combined (i.e. 10% minimum of naturally contaminated positive samples)
  
  - Environmental samples from primary production stage (under scope of NF EN ISO 6579): 75% all categories of products combined (i.e. 25% minimum of naturally contaminated samples, with at most 4 naturally contaminated samples from the same origin).

**NOTE:** If validation is requested for one category or one matrix only, the Technical Board will examine requirements concerning natural contamination levels on a case by case basis when the draft comparative study is presented. The same applies to the methods for detecting microorganisms other than *Listeria* and *Salmonella*.

The protocols for contamination by mixture and artificial contamination (“spiking” and “seeding”) are specified below (see Annex C of EN ISO 16140-2):

- **C.2 Contamination by mixture**

  Applicable to matrices that can be homogenized.

  Experimental design:
  - Naturally contaminated product diluted at least 1/5 if the product is of the same food item (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 (consistent with type characteristics)
  - A naturally contaminated product must not be used more than 6 times in the same study
  - Mixing should ensure good homogeneity (without milling)

  Store the sample contaminated by mixture for at least 1 day at the normal storage temperature.

- **C.3 Artificial contamination using “seeding”**

  Preferred option apart from stress cases specified for spiking (see C.4 below).

  If possible, contamination must be ≤ 3 CFU/test portion, with no more than 20% of contaminated samples between 3 and 10 CFU/test portion. This contamination level may however be adjusted for difficult matrices and/or microorganisms in order to obtain positive results. The Expert laboratory must document these cases in the study report.

- **C.4 Artificial contamination using “spiking”**

  To be used 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 must be ≤ 5 CFU/test portion, with no more than 20% of contaminated samples between 5 and 10 CFU/test portion (above this level is not accepted). This contamination level may however be adjusted for difficult matrices and/or microorganisms in order to obtain positive results. The Expert laboratory must document these cases in the study report.

  The Expert laboratory must describe 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 0,5 log minimum is expected. The Expert laboratory must specify the medium/media used.
In general, the Technical Board recommends that artificial contamination sources of samples are varied. In particular, a same strain and a same stress cannot be used for all the products of a same category. A same strain cannot be used more than 6 times in total. The strains used should mostly be of food origin. The real origin of strains must be known and recorded. The strains must be representative of those most commonly present in the tested categories and their origin must be appropriate to the type of contaminated product.

The contamination method and contamination levels must enable samples to be obtained with a behaviour that is identical to that of naturally contaminated samples.

In the case of “unpaired” studies (separate broths) when the incubation times are different for the 2 methods, simultaneous starting of the studies must be preferred as far as possible.

The incubation times chosen for performing the tests for the alternative method are the minimum times specified in the protocol. The minimum incubation time will also be used for the reference method as far as possible. If the limits are extended, tests at the higher limit may be discussed. Changes in results between minimum time and maximum time must be documented for long incubation periods. For protocols with an incubation time under 24 hours, the 2 lower and upper limits are to be tested above a 6 hour interval.

If the alternative-method protocol specifies a cold storage step (duration defined by the requester/holder company), the sensitivity study must be redone after applying cold. All positive samples (artificially and naturally contaminated), and samples for which the results are doubtful, must be retested.

**NOTE:** The following cases of storage do not require validation tests in order to be validated:

- The case of plates with DNA extracts stored at 4°C for 24 hours maximum before proceeding with the test;
- The case of storing DNA extracts at -20°C;
- For *L. monocytogenes* methods, the case of initiating a confirmation after preservation of half Fraser broth for 48 hours (where the test has already been performed and preservation of the broth in parallel with a view to confirmation).

Use of a neutralizing buffer and the preparation method for environmental samples must be specified as far as possible by the Expert laboratory in the study report (type of sampling medium, surface area sampled, medium dilution method and level, etc.).

**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 be made up of 3 types, with at least 20 samples (positive and negative) for each type. For each type, a minimum of 7 positive samples are to be tested.

Each specific alternative-method protocol is to be treated as a category.

For *Listeria* genus studies, compliance per category with a proportion of at least 15 to 25 *Listeria* spp contaminated samples (alone or combined with *Listeria monocytogenes*) is requested. In the case of a method validated both on *Listeria* genus and *Listeria monocytogenes* parameters, this proportion may be lower.

**Alternative-method result and confirmation (§ 5.1.3.3)**

The Expert laboratory must systematically carry out the following confirmation steps for the alternative method:

- As a 1st confirmation: For all positive and negative samples (except in the case of petri dish methods), applying the confirmation tests of the reference method and those proposed by the requester/holder company's. In the case of validation of the cold storage step, one of the specific tests proposed by the requester/holder company will be applied.
As a 2nd confirmation: for negative samples only (including petri dish methods).

- If the alternative method has no secondary enrichment broth, a subculture of the broth of the alternative method in the secondary broth of the reference method should be employed (example: *Listeria*). Special case of *Salmonella* studies: the secondary broth used shall only be RVS.
- If the alternative method has a secondary enrichment broth, its incubation time must be extended, if necessary, to reach that of the secondary broth of the reference method.
- If both the methods have a single enrichment step, and if the incubation time of the alternative method is shorter, incubation must be extended to the minimum time of the reference method.

**Calculation and interpretation for sensitivity (§ 5.1.3.4)**

Regarding validation of methods based on the principle of growing colonies on agar, the following items must be included in the raw results:

- level of background flora: absence / low / high
- morphology of target colonies if atypical: micro-colony, size, colour, shape, halo, etc.
- need to isolate again or not
- presence of less than 5 characteristic colonies (state number)

For methods based on a different principle, it is recommended to provide as much information as possible on the level of the target microorganism (CT, Tm and inhibition for PCR methods, agglutination for Latex tests, colour intensity for lateral flow tests, etc.).

Acceptability criteria are fixed by the standard. If the criteria are not met for all the categories tested, it is possible to retest a category or a type. The 2 sets of results must be presented in the dossier, for discussion in the Technical Board (data not to be consolidated).

**Relative level of detection study (§ 5.1.4)**

The aim of this study is to determine the smallest contamination that can be detected in a food.

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

Refer to Annex A of EN ISO 16140-2 for choice of suggested product categories and types by target microorganism. Annex 1 of this document is given only by way of example. Other types/categories may be defined depending on the requester/holder company's need.

The samples must be artificially contaminated. The preparation procedures for sample preparation are specified in Annex C of EN ISO 16140-2. In particular, it is recommended to give preference to bulk inoculation in the case of an AOAC combined study. It is also possible to perform Individual Bag contaminations. The background flora of the matrix shall be determined.

It is recommended to apply the following stresses (defined in accordance with the industrial processes applied in manufacture of the products):

- **Raw products:** Inoculation using seeding minimum 48 hours at 4°C (do not leave too long).
- **Frozen products:** Inoculation using seeding for 2 weeks at -20°C.
- **Cooked products (pasteurized liquid egg, terrines):** Either inoculation using seeding with storage at 4°C, or using spiking with heat treatment of the strain and stress evaluation (if AOAC collaborative study).
- ** Powders / products dehydrated by lyophilization:** It is preferable to employ the seeding method of inoculation with a lyophilized or desiccated strain with a minimum of 2 weeks storage (depending on the product tested) at the normal storage temperature for the product (generally ambient temperature). There is also the possibility of carrying out spiking with heat treatment of the strain and stress evaluation.
- **Primary production:** Inoculation in the matrix and storage 24 hours at ambient temperature.
- Process or surface water: Inoculation using seeding for 48 hours at 4°C.

The medium used for the calibration, together with all the contamination protocols, must be submitted for opinion by the Technical Board when presenting the draft comparative study. They must also be specified in the study report and in the summary report for the validation study.

Three contamination levels are to be tested. The high level must be 3 to 5 times higher than the low level.

**Calculation and interpretation of the RLOD (§ 5.1.4.2)**

Refer to EN ISO 16140-2.

**Inclusivity and exclusivity study (§ 5.1.5)**

*NOTE: Published data on the alternative method, obtained as per the requirements of EN ISO 16140-2, may be used by the Expert laboratory to provide further information on this criterion.*

In the case of a request for extension relating to additional confirmation tests, information would be provided only on this parameter, subject to prior decision by the Technical Board.

**Selection and number of strains (§ 5.1.5.1)**

Refer to Annex E of EN ISO 16140-2 for criteria for selecting microorganisms.

For Salmonella studies, the Technical Board has established a minimum list of target and non-target strains to be tested (see Annex 2). The list should be added to by the Expert laboratory in order to meet the specific requirements and submitted for Technical Board opinion. Any single *Salmonella* serotype shall be tested no more than twice, unless specifically informed otherwise by the Technical Board.

**Inoculation of target strains (inclusivity) (§ 5.1.5.2)**

For the tests, the most selective alternative-method protocol must be used.

In the case of alternative methods based on an ELISA test, proposing a Latex test as confirmation, the Expert laboratory must 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 the tests, a non-selective enrichment broth is to be used.

In the case of alternative methods based on an ELISA test, proposing a Latex test as confirmation, the Expert laboratory must test 100 non-target strains. The Technical Board reserves the right to request additional tests in other cases, depending on the principles proposed.

Exclusivity testing of salmonella group N for *E. coli* O157 methods.

**Expression and interpretation of the results (§ 5.1.5.4)**

Refer to EN ISO 16140-2.

For each of the confirmation tests specified in the alternative-method standard protocol, the results obtained are to be provided in the study report.

Regarding validation of methods based on the principle of growing colonies on agar, the following items must be included in the raw results:
- morphology of non-target colonies
- morphology of target colonies if atypical: micro-colony, size, colour, shape, halo, etc.
- presence of less than 5 characteristic colonies (state number)

For methods based on a different principle, it is recommended to provide as much information as possible on the level of the target microorganism (CT, Tm and inhibition for PCR methods, agglutination for Latex tests, colour intensity for lateral flow tests, etc.).

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

This is a specific Technical Board requirement, not required by EN ISO 16140-2.

A practicability / adaptability study shall be conducted, based on 4 criteria. For each of these criteria, the method of communicating this criterion to the user and the method of control of this criterion have been defined. Indeed, some criteria require communication on the packaging or technical instructions whereas others require communication in the summary report.

The data resulting from this practicability study shall be incorporated in the comparison study report.

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Interlaboratory study (§ 5.2)

General considerations (§ 5.2.1)

The aim of the interlaboratory study is to determine the difference in sensitivity between the two methods under interlaboratory reproducibility conditions.

Measurement protocol (§ 5.2.2)

There must be a sufficient number of laboratories to present 10 usable independent sets of data.

Two interlaboratory studies carried out on the same product can be compiled only after the go-ahead from the Technical Board, and only if the following conditions are met:
- there are 10 different laboratories (evidence to be provided in some particular cases, and subject to a prior decision by the Technical Board, the Expert laboratory may be authorized to make its premises available to geographically close collaborators for performance of the tests. Involvement of an independent team forming part of the Expert laboratory may be accepted, on condition that it has not participated in the validation study.)
- the analysis protocols are exactly the same from one study to the next
- all the planned studies in the project have been executed
- the sets of results obtained by each collaborative laboratory are complete
- the number of sets of results generated from each of the studies is statistically acceptable

Refer to Annex 3 of this document for the general procedures for organization of interlaboratory studies.

The following specific details are given as regards the measurement protocol described in EN ISO 16140-2:

- It is recommended to select a food matrix that is relevant as regards the analysis protocol tested for the alternative method and as regards the target microorganism. **Note:** Liquid products are to be avoided as far as possible since possible sources of cross contamination (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: Minced (ground) beef
- *Cronobacter*: Milk powder with probiotic or liquid milk reconstituted from milk powder with probiotic
- *Listeria*: “Fromage frais” (from goat’s milk, ewe’s milk, etc.)
- *Campylobacter*: Minced poultry meat or pork **Note:** To minimize natural contamination problems in poultry meat, it is recommended to freeze the meat before use.

The sample should contain a representative background microflora, which also must remain stable during transport. The laboratory shall determine and indicate the levels of background flora in the matrix.

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

In the particular case of testing a milk sample, the flora must be naturally of dairy origin. In practice, the Expert laboratory must find a naturally contaminated milk at the above required level. If the contamination level is below the required level, it must either develop the sample in order to reach this contamination level, or it must add raw milk (in reasonable proportions).
**Note:** For interlaboratory studies already validated, but conducted with samples of milk with contamination levels below $10^3$ CFU/ml, the studies need not be repeated.

- Three contamination levels are to be tested:
  - A level 0 ($L_0$) which corresponds to a negative control: 0 CFU/test portion.
  - An intermediate level ($L_1$) which must be at the relative level of detection so as to preferably obtain fractional recovery. Contamination levels under < 3 CFU/ test unit are recommended. The Technical Board will reach a decision in the case of any deviation.
  - A higher level ($L_2$) for which it is recommended to inoculate between $3 < x < 10$ CFU/ test portion.

A sample contamination and counting protocol for low levels is given in Annex 4 of this document.

**Calculations and summary of data (§ 5.2.3)**

Refer to EN ISO 16140-2.

The results of collaborators for which cases of cross contamination are found at level $L_0$ are to be excluded from the interpretation, on the basis of the following criterion: 1 positive (1 confirmed positive or 1 false positive) maximum acceptable at $L_0$, per laboratory and per method.

**Note:** The criterion is not applicable to dossiers already validated.

**Interpretation of data (§ 5.2.4)**

Refer to EN ISO 16140-2.
Method comparison study (§ 6.1)

General considerations (§ 6.1.1)

The purpose of this study is to compare the performances of the alternative method against those of the reference method, for the following parameters:
- Relative trueness study
- Accuracy profile

And to determine the following parameters for the alternative method:
- Limit of quantification
- Inclusivity / Exclusivity
- Practicability

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

The contamination method and contamination levels must enable samples to be obtained with a behaviour that is identical to that of naturally contaminated samples.

Relative trueness study (§ 6.1.2)

Selection of categories to be used (§ 6.1.2.1)

For choice of categories and matrices, refer to Annex A of EN ISO 16104-2. The types and categories listed in Annex 1 of this document are only given by way of example and may be defined differently depending on the requester/holder company's need.

Number of samples (§ 6.1.2.2)

The samples must enable coverage of the normal measuring range, all categories combined.

Calculation and interpretation (§ 6.1.2.3)

All non-interpretable data (by either of the methods) must appear in a separate table.

The use of a neutralizing buffer as well as the environmental sample preparation method must be described 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 choice of categories and matrices, refer to Annex A of EN ISO 16104-2. The types and categories listed in Annex 1 of this document are only given by way of example and may be defined differently depending on the requester/holder company's need.

It is not necessary to apply stress to strains for contaminations.
**Number of samples (§ 6.1.3.2)**

As a minimum, test 2 batches of the same matrix inoculated by the same strain at 3 inoculation levels (low, intermediate, high).

For reasons relative to scatter of results and statistical interpretation (Poisson's law) with low counts, it is recommended to start the low inoculation level from 100 CFU/g for pathogens and 300 CFU/g for other microorganisms.

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

- **E. coli / Staphylococci / B. cereus / Coliforms and Enterococci**: high level of $10^5$ CFU/g
- Total flora: $10^6$ CFU/g
- **Listeria monocytogenes**: 3 000 CFU/g
- **Listeria spp**: 30 000 CFU/g
- **Pseudomonas**: $10^6$ CFU/g
- **Campylobacter**: $10^4$ CFU/g
- Yeasts and moulds: $10^5$ CFU/g

**Calculation and interpretation of accuracy profile study (§ 6.1.3.3)**

Refer to EN 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 (in other words methods not based on counting colonies).

**Selection of categories to be used (§ 6.1.4.2)**

For choice of categories and matrices, refer to Annex 1 of this document (categories and type of food products recommended for each target microorganism).

**Number of samples (§ 6.1.4.3)**

Refer to EN ISO 16140-2.

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

Refer to EN ISO 16140-2.

**Inclusivity and exclusivity study (§ 6.1.5)**

Follow the procedures described in EN ISO 16140-2.

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

This is a specific Technical Board requirement not required by EN ISO 16140-2.

A **practicability / adaptability** study, based on 4 criteria, will be conducted by the Expert laboratory. For each of these criteria, the method of communicating the criterion to the user and the method of control of this criterion have been defined.

Indeed, some criteria require communication on the packaging or technical instructions whereas others require communication in the summary report.

The data resulting from this practicability study shall be incorporated in the comparison study report.
### Criteria to be controlled

<table>
<thead>
<tr>
<th></th>
<th>Communication on the criterion</th>
<th>Method of control of the criterion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Storage conditions of the elements (+ time limit for unopened products)</td>
<td>Packaging or instructions</td>
</tr>
<tr>
<td>2</td>
<td>Methods of use after first use (particularly existence of limit dates)</td>
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<tr>
<td>3</td>
<td>Time to obtain the results</td>
<td>Validation study report and summary study report</td>
</tr>
<tr>
<td>4</td>
<td>Common steps with the reference method</td>
<td>Validation study report and summary study report</td>
</tr>
</tbody>
</table>

### Interlaboratory study (§ 6.2)

#### General considerations (§ 6.2.1)

The aim of the interlaboratory study is to determine the difference in sensitivity between the two methods under interlaboratory reproducibility conditions.

#### Measurement protocol (§ 6.2.2)

There must be a sufficient number of laboratories to present 8 usable independent sets of data

Refer to Annex 3 of this document for the general procedures for organization of interlaboratory studies.

The following specific details are given as regards the measurement protocol described in EN ISO 16140-2:

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

In the case of a study on a “wide variety of foods”, it is recommended to test the following matrices:
- *Pseudomonas*: “fromage frais”
- Staphylococci: fish terrine, milk
- Enterococci / Coliforms: “pâté” or cooked ham or pasteurized milk with background flora.
- *E. coli*: stir fry mixes
- *B. cereus*: purée or soup (for infants reconstituted / or for adults)

The sample should contain a representative background microflora, which also must remain stable during transport. The laboratory shall determine and indicate the levels of background flora in the matrix.
The level of background flora must be at least $10^3$ CFU per mL or g, unless otherwise specifically informed by the Technical Board.

In the particular case of testing a milk sample, the flora must be naturally of dairy origin. In practice, the Expert laboratory must find a naturally contaminated milk at the above required level. If there is “non-contamination”, it must either develop the sample in order to reach this contamination level, or it must add raw milk (in reasonable proportions).

**Note:** For interlaboratory studies already validated, but conducted with samples of milk with contamination levels below $10^3$ CFU/ml, the studies need not be repeated.

- Three contamination levels are to be tested:
  - A **level 0** (Lo) which corresponds to a negative control: 0 CFU/test portion.
  - A **minimum level** for which it is recommended to start at 500 CFU/test portion (except for *Listeria*: start at 300 CFU/test portion), for reasons relative to scatter of results and statistical interpretation (Poisson's law) with low counts.
  - An **intermediate level**.
  - A **higher level** where the high level must reflect as far as possible the contamination levels of regulatory criteria or have a high value of:
    - *E. coli* / Staphylococci / *B. cereus* / Coliforms and Enterococci: high level of $10^5$ CFU/g
    - Total flora: $10^6$ CFU/g
    - *Listeria monocytogenes*: 3 000 CFU/g
    - *Listeria spp*: 30 000 CFU/g
    - *Pseudomonas*: $10^6$ CFU/g
    - *Campylobacter*: $10^4$ CFU/g
    - Yeasts and moulds: $10^5$ CFU/g

**Calculations, summary and interpretation of data (§ 6.2.3)**

Refer to EN ISO 16140-2.
The Expert laboratory is chosen by the requester/holder company from the list of qualified laboratories. It must inform the requester/holder company at each stage of study execution and also in the event of any changes relative to the initially set protocol. The Expert laboratory must be qualified by AFNOR Certification, following a decision by the Technical Board. Qualification procedures for Expert laboratories are set out in the Certification rules NF102 (§ 2.4.1).

General notes

- The Expert laboratory must present only finalized studies and must feel free to delay its presentation of results until this is the case.

- AFNOR Certification can only include on the next meeting agenda those dossiers (draft studies or results) for which the complete written reports are available on the prearranged date. Consequently only completed studies – the results of which are known when drafting the meeting agenda – can be presented at the corresponding meeting. Any study not completed when drafting the agenda cannot be presented at the next meeting and the presentation for the study will be deferred to a later session.

1 Presentation of a draft comparative study

The Expert laboratory must prepare a draft comparative study. The Expert laboratory should send it to AFNOR Certification, before the deadline decided by AFNOR Certification (generally 3 to 4 weeks before the meeting date).

The Expert laboratory and the requester/holder company are summoned by AFNOR Certification to the Technical Board meeting. The laboratory must present the draft comparative study it has prepared, using a visual medium.

During this first stage, the Technical Board gives its opinion on:

- whether or not the NF VALIDATION mark can be applied to the alternative method put forward
- the method taken as reference
- the draft comparative study

Two reviewers are appointed: they are selected from within the Technical Board and will study those dossiers for which they are responsible in more depth.

After the meeting, AFNOR Certification communicates the Technical Board's decision to the holder/requester by letter, with a copy to the Expert laboratory and to the reviewers.

As necessary, all modifications relating to the draft comparative study must be taken into account by the Expert laboratory, who sends AFNOR Certification a modified draft, if the Technical Board so requests.
2 Execution of the comparative study and presentation of results

**Important:** the time interval between presentation of the draft comparative study and presentation of the comparative study results must not exceed 1 year.

The Expert laboratory must inform AFNOR Certification by the agreed date (usually 4 weeks before the date of the Technical Board meeting) on whether it is ready to present the comparative study results.

The comparative study report must be drawn up as per the document outline available from AFNOR Certification.

The Expert laboratory must send AFNOR Certification the **comparative study report** (along with any addenda) together with the **appended documents** (draft technical instructions, etc.) before the **deadline set by AFNOR Certification** (usually 3 to 4 weeks before the meeting so that the latter can circulate this file to the Technical Board members).

AFNOR Certification summons the Expert laboratory and the requesting requester/holder company to the Technical Board meeting. The Expert laboratory must present the comparative study report it has drawn up, using visual media.

At this 2nd stage, the Technical Board gives its findings on the results obtained during the comparative study.
To do so, a discussion takes place at the end of the presentation without the requester company being present but in the presence of the Expert laboratory. Then **a vote is taken** in the absence of both the requester company and the Expert laboratory, taking account of all the results of the comparative study.
This vote determines the Technical Board's decision. The voting outcome determines whether the results of the comparative study are accepted.
The voting outcome is communicated to the manufacturing company and the Expert laboratory at the meeting.

**Note:** The results are accepted by a simple majority vote (the Chairperson having the casting vote in the event of a tied vote). The votes are counted as “For”, “Against” and “Abstentions”. Reasons for “Against” votes and “Abstentions” must be routinely given at a round table discussion.
The number of abstentions must not exceed 50% of those present and voting. If this is not the case, **a second ballot will take place** during which only “For” votes are given. The second ballot is based on a simple majority vote.

The Technical Board can ask for additions to the comparative study on one or more criteria. This might delay the start of the interlaboratory study depending on how sizeable these additions are.

If the full results of the comparative study (including additions where relevant) are accepted, the interlaboratory study can proceed.

If this is the case, the Expert laboratory must also present the draft interlaboratory study at this meeting. The Technical Board gives its opinion on the draft interlaboratory study. The list of collaborative laboratories can be included in the draft or is circulated to AFNOR Certification before the start of the study for a decision by the Technical Board.
The Expert laboratory must take account of any modifications relating to the interlaboratory study and subsequently send AFNOR Certification its modified draft, if the Technical Board so requests.

After the meeting, AFNOR Communication communicates all the decisions made at the meeting to the requester/holder company by letter, with a copy to the Expert laboratory and to the reviewers.

### 3 Execution of the interlaboratory study and presentation of results

**Important:** The time interval between presentation of the draft interlaboratory study and presentation of the interlaboratory study results must not exceed 1 year.

The Expert laboratory must inform AFNOR Certification by the agreed date (usually 4 weeks before the date of the Technical Board meeting) on whether it is ready to present the interlaboratory study results.

The interlaboratory study report must be drawn up as per the document outline available from AFNOR Certification.

The Expert laboratory must send AFNOR Certification the **interlaboratory study report** (along with any addenda) together with the **appended documents** (draft technical instructions, etc.) before the deadline set by AFNOR Certification (usually 3 to 4 weeks before the meeting so that the latter can circulate this file to the Technical Board members).

AFNOR Certification summons the Expert laboratory and the requesting manufacturer to the Technical Board meeting.

The Expert laboratory must present the interlaboratory study report it has drawn up, using visual media.

At this 3rd stage, the Technical Board gives its findings on the results obtained during the interlaboratory study.

To do so, a discussion takes place at the end of the presentation without the manufacturer/requester being present but in the presence of the Expert laboratory. Then a **vote is taken** in the absence of both the manufacturer/requester and the Expert laboratory, taking account of all the results of the study (comparative and interlaboratory).

This vote gives the final decision of the Technical Board and takes account of all results presented (comparative and interlaboratory studies). The result of this vote determines whether or not the method can be validated.

The manufacturer is notified of the voting outcome at the meeting.

**Note:** The results are accepted by a simple majority vote (the Chairperson having the casting vote in the event of a tied vote). The votes are counted as “For”, “Against” and “Abstentions”. Reasons for “Against” votes and “Abstentions” must be routinely given at a round table discussion.

The number of abstentions must not exceed 50% of those present and voting. If this is not the case, a **second ballot will take place**, during which only “For” votes are given. The second ballot is based on a simple majority vote.

After the meeting, AFNOR Communication communicates all the decisions made at the meeting to the requester/holder company by letter, with a copy to the Expert laboratory and to the reviewers.
4 Preparation of the certificate of validation

AFNOR Certification takes the decision to certify the analysis method, based on the final decision of the Technical Board.

AFNOR Certification issues a decision notification letter following the corresponding meeting, officially attesting to certification of the method. This letter is sent, subject to any additions that the Technical Board may have requested within timescales specified by AFNOR Certification.

AFNOR Certification then prepares the certificate based on the document outline defined by the Technical Board. The various headings are completed based on any recommendations that the Technical Board may have made during the various dossier presentation stages (application scope, any restrictions, etc.). AFNOR Certification may possibly consult the Technical Board to ensure that the reported information is appropriate.

The references for the technical instructions necessary for implementation of the alternative method are given on the certification of validation. The references for the confirmation tests are not given on it.

The certificate is written in French and in English. Each certificate is signed by AFNOR Certification's Legal Representative. The French version of the certificate is the authentic one in legal terms.

The manufacturer receives the original copy of the certificate. The certificate is available to the public on the http://nf-validation.afnor.com web site.

5 Summary study report

Following the decision to validate, renew, or extend validation of a method, the Expert laboratory must draw up a summary report for the studies (comparison and interlaboratory).

This document covers the major components of these studies. When preparing the summary report, the Expert laboratory must take account of the comments made at Technical Board meetings on the intermediate study reports (comparison and interlaboratory).

Its purpose is to allow circulation to anyone who so requests. A document outline is available from AFNOR Certification. All published summary reports are available to the public on the website http://nf-validation.afnor.com. The manufacturer must therefore validate the content with regard to confidentiality of the items therein.

The Expert laboratory must send AFNOR Certification this document no later than 2 months after the Technical Board has passed its favourable vote.

6 Duration of validity

NF VALIDATION certification is valid for 4 years, unless the alternative method is modified or measures taken against it.
If modifications are made to the alternative method requiring tests to be conducted, the study will be considered a new study.

If the reference method or the validation protocol are modified during the certification period, the decision remains valid until the original expiry date.

7 Extension/Modification

If a complementary study must be conducted, two reviewers are appointed at the Technical Board meeting following presentation of the corresponding draft study.

The summary of complementary studies conducted shall be appended to the initial summary report.

8 Renewal

The renewal study procedures and content of the renewal dossier are specified in the Certification rules (§ 5.3.2).

If no complementary study is necessary, the two reviewers are ideally appointed before the corresponding meeting, at a previous session or via email consultation.

If a complementary study must be conducted, the two reviewers are appointed at the Technical Board meeting following presentation of the corresponding draft study.

The summary report shall contain a recap of the main results obtained during the first validation study, and a summary of the complementary studies conducted if they exist.

9. Updates to dossiers based on EN ISO 16140-2

9.1. Applicable transition time

For ALL DOSSIERS (validation / extension / renewal):

At the start of the 1st ISO/FDIS voting (19th March 2015):
- Option to start instructions based on the ISO/FDIS 16140:2015 protocol or to retain validation studies based on EN ISO 16140:2003
- Depending on the protocol version applied, issue of certificates based on EN ISO 16140:2003 or ISO/FDIS 16140-2

After publication of the new standard at French level (17th September 2016):
- Obligatory application of the new protocol EN ISO 16140-2:2016 for all dossiers
- Issue of certificates based on the new NF EN ISO 16140-2:2016 standard

9.2. Update tests based on EN ISO 16140 for previously validated dossiers

9.2.1. Qualitative methods

- Sensitivity study
It is possible to retain the categories already tested, and to re-do the interpretation based on the new standard EN ISO 16140-2. Products may be classified based on the new categories recommended in EN ISO 16140-2 (after possible reclassification, retesting as necessary of additional categories in order to have 5 categories in total in the case of “all food products” validation).

For “all food products for human consumption” validation, it will be necessary to at least have tested 5 categories of foods for human consumption. The other categories tested will be additional categories. If matrices are missing for a category, they will have to be added. Combined categories will only be permitted for an “all food products for human consumption” scope, on condition that there are 5 categories of products for human consumption.

Dossiers will be dealt with on a case by case basis. If necessary, the expert laboratory must propose a new classification at presentation of the renewal draft study, optimizing where possible to avoid tests.

NOTE: You are reminded that the specific protocols are to be treated as a category (test 30 positives and 30 negatives). This should be taken into account when updating dossiers based on the EN ISO 16140-2 protocol (additional tests to be performed or otherwise).

- **RLOD**

Retain the relative LOD data, and re-do the interpretation based on the new standard EN ISO 16140-2 (note: protocol without stress in EN/ISO 16140:2003). To be able to cover “all food products for human consumption”, it will be necessary to at least have tested 5 categories of foods for human consumption.

NOTE: Possibility of recovering this data generated from other validations if obtained using the proper reference method, and in accordance with the applicable technical rules.

- **Inclusivity and exclusivity study**

For salmonella studies, it will also be necessary to complete the inclusivity study with 100 strains. It could be possible to use data from AOAC studies, if obtained in accordance with the Technical Board’s technical rules.

- **Interlaboratory study**

Retain previous data and reprocess this data based on interpretation of the new standard.

### 9.2.2. Quantitative methods

- **Relative trueness**

Complete in order to have 15 samples / categories, and as necessary add a category to be able to be validated for “all food products for human consumption”. The first data of the two repetitions will be retained for the existing data.

- **Accuracy profile**

To be redone in its entirety.

- **Inclusivity / Exclusivity**

Complete to obtain 50 positive strains and 30 negative strains.
➢ **Limit of quantification**

To be done for instrumental methods.

➢ **Interlaboratory study**

Retain previous data and reprocess this data based on interpretation of the new standard.
Annex 1

Categories of products and product types by target microorganism, stress and associated contamination methods (informative list)

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

The types and categories listed in Annex 1 are only given by way of example and may be defined differently depending on the requester/holder company's need. On this basis, the Expert laboratory has to establish a proposal of categories and types of food products to be tested. The proposal is subjected to the approval of the Technical Board who may ask any changes.
Annex 2

Salmonella selectivity study - Lists of mandatory strains

Target and non-target strains to be included obligatorily in the list of strains to be tested for the validation of Salmonella detection kits. The lists shall be completed to respect the specific requirements of this current document (see "Inclusivity and exclusivity study" (§ 5.1.5)).

1 Inclusivity

<table>
<thead>
<tr>
<th>&quot;O&quot; GROUP</th>
<th>SPECIES</th>
<th>SUBSPECIES</th>
<th>SEROTYPE</th>
<th>FORMULA</th>
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<tbody>
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<td>Urbana</td>
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<td>Adelaide</td>
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<td>Wandsworth</td>
<td>39 : b : 1.2</td>
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<td>S. II</td>
<td>42 : b : e,n,x,z_{15}</td>
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<td>S. IV</td>
<td>1,40 : z_{4},z_{39} : -</td>
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<td>S. VI</td>
<td>[1],6,14,[25] : a : e,n,x</td>
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<td>78</td>
<td>S. bongori</td>
<td>(V)</td>
<td>S. V</td>
<td>48 : z_{35} : -</td>
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## Exclusivity

**Non-Salmonella strains**

<table>
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<tr>
<th>No.</th>
<th>GENUS</th>
<th>SPECIES</th>
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<tbody>
<tr>
<td>1</td>
<td><em>Citrobacter</em></td>
<td>freundii, diversus, youngae, koseri, braaki,</td>
</tr>
<tr>
<td>2</td>
<td><em>Escherichia</em></td>
<td>coli, hermanii</td>
</tr>
<tr>
<td>3</td>
<td><em>Proteus</em></td>
<td>mirabilis, vulgaris</td>
</tr>
<tr>
<td>4</td>
<td><em>Klebsiella</em></td>
<td>pneumoniae, oxytoca</td>
</tr>
<tr>
<td>5</td>
<td><em>Enterobacter</em></td>
<td>cloaca, sakazakii, agglomerans (or Pantoea agglomerans)</td>
</tr>
<tr>
<td>6</td>
<td><em>Serratia</em></td>
<td>marcescens</td>
</tr>
<tr>
<td>7</td>
<td><em>Hafnia</em></td>
<td>alvei</td>
</tr>
<tr>
<td>8</td>
<td><em>Shigella</em></td>
<td>flexneri</td>
</tr>
</tbody>
</table>

*Choose 3 species from among the five.*
Annex 3

General procedures for organization of interlaboratory studies

Choice of collaborative laboratories:

If possible at presentation of the draft interlaboratory study, and in any event before the interlaboratory study begins, the Expert laboratory must submit a list of competent public or private laboratories to the Technical Board, preferably from several European countries. At least as many laboratories as specified in standard EN ISO 16140-2 are required so as to obtain no fewer than this number of sets of interpretable results. The Expert laboratory and requester/holder's own laboratory are not included in this number of laboratories. The collaborative laboratories are chosen after close discussion between the manufacturer and the Expert laboratory. The final choice and monitoring of the collaborative laboratories remains however the responsibility of the Expert laboratory which must ensure that they have implemented a quality assurance policy in the relevant field.

The Expert laboratory's duties:

The Expert laboratory prepares samples for the collaborative laboratories and sends them the analysis protocol to be used for the alternative method. The Expert laboratory must ensure methods are implemented to take account of the strict logistical requirements of this study. It is essential that it keeps a record of temperature during transport.

Concerning the preparation of food samples for qualitative methods, the Technical Board considers that the homogeneity and stability tests are not necessary. The laboratory must check that the mixture is sufficiently stable over several days in transport and storage conditions.

Concerning the transport of samples, the Technical Board considers that the Expert laboratories must give preference to refrigerating rather than freezing the samples. Indeed, freezing involves a risk of losing target bacteria for samples with a low contamination level. Hence, the Technical Board will decide for each study on whether or not the samples can be frozen for transport. Concerning refrigeration, the following conditions (defined by the Technical Board) 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 organization of the interlaboratory study, the count of the total bacterial flora must be performed on a specific additional sample prepared by the Expert laboratory.

Use of branded media and from different batches introduces further variability.

Instructions to collaborative laboratories:

It is possible to allow collaborators the possibility of analysing samples at D1 or D2, subject to use of the same sample on the same day for the alternative method and the reference method. If applicable, the instructions must be clear and precise.

The Expert laboratory is advised to have each collaborative laboratory sign an acknowledgement of their awareness of the instructions relating to the interlaboratory study. The Expert laboratory must lay down very clear conditions to the collaborative laboratories on elimination of results from a laboratory (including at least the day of analysis and maximum temperature of the samples on receipt). This provides clear, non-challengeable rules for the elimination of results and also allows collaborative laboratories to avoid carrying out unnecessary tests.

The Expert laboratory shall also advise collaborative laboratories to have a metrologically verified thermometer available to verify sample temperatures on arrival.
Preservation of enrichment broths:

For the interlaboratory studies, each laboratory must keep the enrichment broths for the different samples analysed in the conditions set by the Expert laboratory. If, after analysing the results, the Expert laboratory finds discordance in the data, it can ask collaborative laboratories to conduct extra tests to explain such discordance.
Annex 4
Sample contamination and counting protocol for low levels

1. Calibration of parent suspensions of microorganisms

   The calibration is performed using the formula: \( N = K \times DO \)

   The DO is measured at the indicative wavelength of 660 nm, to be reconfirmed by a spectrum of the strain so as to select the most suitable wavelength.

   \( N \): number of CFU/ml
   \( K \): calibration coefficient of the strain

   \( K \) is determined in the following manner:

   a) At least 5 calibration curves must be made for each strain after determining the DO and \( N \) (by counting) in parallel over several experiments, performed under the same culture conditions. The \( k \) factor is determined for each linear part of each calibration curve, in the following manner: \( k = N/DO \).

   b) The \( K \) factor is an average of the \( k \) factors obtained for each calibration curve.

2. Preparation of the parent suspension

   a) From a culture kept according to good laboratory practice, place the strain to inoculate in culture;

   b) Incubate for 24 hours at the optimum temperature for the strain;

   c) Replace in the broth, incubation 16 - 17 hours (overnight);

   d) Dilute in the broth so that the values obtained are in the linear part of the calibration curve;

   e) Measure the DO of the dilution;

   f) Calculate \( N \) (number of CFU/ml) using the formula \( N = K \times D.O. \).

3. Preparation of the inoculum suspensions

   Dilutions in “peptone-salt” are made to obtain one suspension at 125 bacteria/ml, one suspension at 25 bacteria/ml and one suspension at 5 bacteria/ml

   This proposal is to be adjusted depending on the final contamination level required.
4 Estimation of accuracy

The basic hypothesis is that the distribution of the contaminants follows Poisson's Law.

Example: estimation of the confidence interval on the count for the 125 bacteria/ml suspension:

- Inoculate 2 PCA petri-dishes with 1 ml of suspension in each.
- Count the total number of bacteria in the 2 dishes after incubation: m
- If m is greater than 200, the confidence interval on the suspension count will lay at most between:

  \[ m \times 0.434 \text{ and } m \times 0.575 \, \text{bacteria/ml} \]

Example: estimation of the confidence interval on the count for the 25 bacteria/ml suspension:

- Inoculate 10 PCA culture petri-dishes with 1 ml of suspension to count in each dish.
- Count the total number of bacteria in the 10 dishes after incubation: n
- If the suspension is contaminated in a homogeneous manner, no more than one dish out of the 10 must lay outside of the confidence interval given by Poisson's Law.

Example: for 20 bacteria, no more than one dish with less than 12 or over 30 bacteria.
- If n is greater than 200, the confidence interval for the suspension count will lay at the most between:

  \[ n \times 0.0868 \text{ and } n \times 0.115 \, \text{bacteria/ml} \]

<table>
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<tr>
<th>Theoretical level targeted (bacteria/25 ml)</th>
<th>Actual level targeted (bacteria/25 ml)</th>
<th>Concentration of the inoculum solution</th>
<th>Volume of inoculum (ml) per sample of 25 g</th>
<th>Estimation of the lower limit of the contamination per 25 g of sample</th>
<th>Estimation of the upper limit of the contamination per 25 g of sample</th>
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<td>10 to 100</td>
<td>50</td>
<td>125 b / ml</td>
<td>0.4</td>
<td>[ m \times 0.173 ]</td>
<td>[ m \times 0.23 ]</td>
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<tr>
<td>5 to 50</td>
<td>25</td>
<td>125 b / ml</td>
<td>0.2</td>
<td>[ m \times 0.0868 ]</td>
<td>[ m \times 0.115 ]</td>
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<tr>
<td>2 to 20</td>
<td>10</td>
<td>25 b / ml</td>
<td>0.4</td>
<td>[ n \times 0.035 ]</td>
<td>[ n \times 0.046 ]</td>
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<tr>
<td>1 to 10</td>
<td>5</td>
<td>25 b / ml</td>
<td>0.2</td>
<td>[ n \times 0.0173 ]</td>
<td>[ n \times 0.023 ]</td>
</tr>
</tbody>
</table>

\[ b = \text{bacteria} \]

Example: estimation of the confidence interval on the count for the 5 bacteria/ml suspension:

See the calculation table attached.
Calculation table
Factors for 95 Percent Confidence Limits for Mean of a Poisson-distributed Variable

<table>
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<tr>
<th>Observed number on which the estimation is based</th>
<th>Lower limit factor</th>
<th>Upper limit factor</th>
<th>Observed number on which the estimation is based</th>
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</table>
Annex 5

Study report and summary report document outlines for studies conducted based on EN ISO 16140-2

Refer to any corresponding document outlines defined by the Technical Board, available on request from AFNOR Certification.