Validation of alternative analytical methods

Application in food microbiology

Requirements regarding validation study (preliminary and interlaboratory) carried out by an expert laboratory

Version 4
Approved by the NF VALIDATION Technical Committee the 02.02.2012

SUMMARY

PREFACE

SUBJECT

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

UPDATE/CIRCULATION

This document is updated by AFNOR Certification each time a modification is made by the corresponding technical committee and/or by AFNOR Certification.
After each update, it is distributed by AFNOR Certification to the approved laboratories which have been qualified and the technical committee members.
The circulation of this document is unrestrected to anyone requesting it.

NOTE 1 : Criteria of acceptability of results

The standard EN ISO 16140 sets no result acceptability threshold for the different criteria tested. It falls to the third party validation organisations using this protocol to examine and assess the results for each of these criteria, and supply an opinion on their acceptability.

NOTE 2 : Acceptance of external results

The standard EN ISO 16140 provides rules for accepting external results obtained previously within the framework of another validation programme (see appendix A normative). In the limit of its competence, and in the absence of official documents stating this competence, it falls to the validation organisation to judge the significance of the differences between the reference methods or the validation protocols used.

NOTE 3 : Raw results

The expert laboratory must be able to communicate all the raw results of the studies (comparative and interlaboratory) to the rewievers that examine the dossiers, to AFNOR Certification or to the experts technical committee if necessary.

NOTE 4 : General procedure concerning the interlaboratory study

Choice of collaborative laboratories:
If possible during the presentation of the draft interlaboratory study, and in any event before the study begins, the expert laboratory must submit a list of competent public or private laboratories to the Technical Committee, preferably from several European countries.
At least as many laboratories as specified in standard EN ISO 16140 are required so as to obtain no fewer than this number of sets of interpretable results. The expert laboratory and manufacturer's own laboratory are not included in this number.
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 the responsibility of the expert laboratory, making sure 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 must above all keep a record of temperature during transport.

Instructions to collaborative laboratories:
The expert laboratory is advised to get each collaborative laboratory to 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 for eliminating the 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 tests for nothing.

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 be 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.

NOTE 5 : Complying with present requirements

In its study drafts, the expert laboratory must state any deviation from the manufacturer's protocol and/or the "Requirements for studies". If no such deviation is mentioned, compliance with the manufacturer's protocol and the "Requirements for studies" is implicit and is under its responsibility.

<u>QUALITATIVE METHODS</u> :

COMPARATIVE STUDY

(section § 5.1 of EN ISO 16140 standard)

The purpose of this study is to compare the performances of the alternative method against to those of the reference method, testing the following parameters :

- Relative accuracy, specificity and sensibility
- Relative detection level

And to determine the following parameters for the alternative method :

- Inclusivity and exclusivity
- Praticability

IMPORTANT : Requirements relating to confirmation of positive results

In the context of NF VALIDATION, and for all methods of **detecting pathogen microorganisms** (*Salmonella*, *Listeria*, …), all samples identified as positive by the alternative method **must be** systematically confirmed.

Only the results obtained after confirmation are considered as positive (tables, calculations,…). The results before confirmation are exploited for information.

The Validation Commission and its technical committee have defined modalities of confirmation of positive results as written below :

1. a/ Using the **conventional tests described in the standardized methods by CEN or ISO from colonies (including the purification step)**.
   The confirmation step must start from the enrichment broth or (in the case of chromogenic media) from typical colonies identified on selective media.

   b/ Using nucleic probes as described in EN ISO 7218 standard (including or not the purification step)**.**

2. Using **one (or more) specific methods** (to be described).
   The principle of these methods shall be different form the validated method's one, and their protocol shall be clearly described by the supplier when requesting Validation. The proposed confirmation methods shall be tested during the validation study and accepted by the technical committee which may decide to not accept them on principle.

3. Using any **other NF VALIDATION certified method**, **the principle of which is different from the validated method's one**. The protocol of the second validated method not including confirmation steps shall be followed entirely. All steps that are before the step from which the confirmation is done shall be common to both methods.
   So that the first validated method (used for detection) and the second validated method (used for confirmation) must have common first steps (for instance, a common enrichment with the same medium).

The first option (1a) is based on the use of classical tests issued from reference methods and must be part of the protocol of the alternative method to validate. **The other options** (1b, 2 and 3) can complete it.

In the event of discordant results (positive with the alternative method, non-confirmed by the classical tests or by the specific method proposed by the supplier - in particular by Latex test(s)* - or by another method certified NF VALIDATION) the laboratory must follow the necessary steps to ensure validity of the result obtained.

* Additional reference if Latex tests are proposed in confirmation.

Relative accuracy, relative specificity, relative sensitivity (section 5.1.1)

MEASUREMENT PROTOCOL : INTERPRETATION OF SECTION 5.1.1.2

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 appendix B – informative – of EN ISO 16140 standard: recommended categories).

The origin of the samples must be the most varied possible so as to reduce errors related to food specialities.

> **Specific requirements of the technical committee:** The times of incubation chosen for performing the tests are the **minimum times specified** in the protocol of the method to validate.

Interpretation of section 5.1.1.2.2 (Number of samples)

The foods to analyse are subdivided into **five categories**, themselves subdivided into **types**. There must be at least **4 human food categories**. In each category, there must be three types of food, where a single type contains several food **matrices**. Refer to the requirements below for the studies of *Salmonella* and *Listeria*.

For example: in the "*meats*" category, the "*heat-processed*" type can be found, and in this type the matrices "*cooked meals, blood sausages, pâté*, etc."

> **Specific requirements of the technical committee:**
>
> Below the types of food who have to appear at least in a validation study of method of detecting ***Salmonella***:
>
Raw poultry (raw or frozen state):	At least 20 samples (positive and negative)
Raw milk cheeses (type fermented dairy products in appendix B):	At least 20 samples (positive and negative)
Eggs and by-products, including mayonnaise:	At least 20 samples (positive and negative)
>
> Below the types of food who have to appear at least in a validation study of method of detecting ***Listeria***:
>
Smoked fish:	At least 20 samples (positive and negative)
Raw milk cheeses (= fermented dairy products):	At least 20 samples (positive and negative)
Meat:	3 types of products
>
> If the method studied includes a **protocol specific to a type or category of food**, this type or category must be tested.
>
> If the validation scope includes one single food type within a food category, this particular food type is to be considered as a full category.
>
> If the validation of the alternative method is requested for **animal feeding stuffs**, the samples of feeding stuffs for animals must be considered as a category and consequently tested.
>
> If the validation of the alternative method is also requested for **environmental samples**, they can be considered as a category and at least three types of environmental products must be tested among the following:
>
> - surface samples (these can be obtained using sponges),
> - dust, sweepings, residues (on production lines, for example, cutters, vacuum cleaners),
> - siphon brushes, drains,
> - various types of water (washing, rinsing, etc.)

The use of a neutralizing buffer as well as environmental sample collection protocol must be described by the expert lab in the study report.

The **veterinary samples** mentionned in appendix B.2 of the standard must be considered separately and not be included in the 5 categories defined above. These are samples for the detection of *Salmonell*a. If the request for validation includes them, the same criteria as for the other samples must be applied, but with the corresponding reference method.

Specific requirements of the technical committee:

Salmonella study: if validation is requested for "**environmental samples from primary production stage**", it shall be considered as an additional category and the 4 following types of products shall be tested:

Poultry faeces	At least 20 samples (positive and negative)
Non faeces of poultry*	At least 20 samples (positive and negative)
Pork faeces	At least 20 samples (positive and negative)
Non faeces of pork*	At least 20 samples (positive and negative)

** Example: internal organs, chipped eggs, water from trough, wipes, etc.*

If the validation of alternative method is restricted to certain categories, as the producer's request, it is possible to study only 1, 2, 3 or 4 categories.

Analyses shall be carried out singly **using both methods**. At least 60 products per category shall be analysed, with a minimum of 30 positive products.

If the protocol of the alternative method specifies a cold storage step (duration defined by the manufacturer), all samples which tested positive by both the alternative and reference methods during the preliminary accuracy study, must be re-tested after cold storage. This includes samples that previously showed discrepant results.

The expert laboratory must **systematically confirm** the **positive** samples of the alternative method, as well as all the **discordant** samples, and also all the samples of the reference method, even in the case of the validation of cold storage.

ARTIFICIAL CONTAMINATION (SECTION. 5.1.1.2.3 - TEST SAMPLE PREPARATION):

If it is not possible to acquire a sufficient number of naturally contaminated foods for each of the categories, artificial contamination of food samples is permissible. The expert laboratory must **justify** the difficulty in implementing naturally contaminated samples.

Specific requirements of the technical committee:

For the methods of detecting *Listeria* and *Salmonella*, the technical committee set a maximum rate of artificially contaminated samples:

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

"Food samples" (under scope of EN ISO 6579 :2002 Standard): 85% all categories of products (i.e. 15% minimum of naturally contaminated samples)

"Environmental samples from primary production stage" (under scope of EN ISO 6579/Appendix D : 2007): 75% all categories of products (i.e. 25% minimum of naturally contaminated samples, with at most 4 samples from the same breeding)

If validation is requested for one matrix only, the requirements concerning natural contamination will be examined on a case by case basis when the study project is presented. The same applies to the methods for detecting microorganisms other than *Listeria* and *Salmonella* (e.g. *E.coli* O157).

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

For *Listeria* and *Salmonella*, the maximum level is setted at 30 bacteria/25g, determined after applying stress on a non-selective medium.

For *E.coli* O157, the maximum level is set at 15 bacteria/25g, with at least 1/3 of samples contaminated with less than 5 bacteria/25g.

The technical committee recommends that artificial contamination sources of samples are **varied**. In particular, a same strain and a same stress should not be used for all the products of a same category. A same strain cannot be used more than 6 times in total.

The microbial strains used should mostly be food isolates. Their origin must be known and specified. The strains used for contamination must be representative of those most commonly associated with the tested food categories.

The three options of contamination are the following ones:

1. Contamination by mixture with naturally contaminated samples of similar type
2. Contamination with isolated strains of the same type of product. Artificial contaminations must include at least a physical or chemical stress, representative of natural conditions. The 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 mediums: a difference of at least 0.5 log must be observed for at least 90% of the positive results in artificially contaminated samples). The laboratory must specify the medium(s) used.

   Warning of the technical committee :

   - For some strains of *Listeria monocytogenes* and *Listeria spp*, **applying positive cold may not constitute a stress**.
   - In the case of *Salmonella* studies when validation is requested for the '' Environmental samples from primary production stage'' category, the samples must be contaminated and left for at least 24 hours at ambient temperature (chemical and/or physical stress not recommended) in order to reach matrix stability and mimic natural contamination conditions. The contamination rates must be adapted to remain within reference thresholds.

3. The use of reference materials

   Specific requirements of the technical committee:

   The technical committee specifies the methodology described in the standard (see appendix D) as follows:

   - **If at least 1 step is common** (see D1), inoculate the artificial contamination into the first common suspension.
   - **If there is no common step**:
     - For solid samples:
       - fully homogenize the sample and individually inoculate each test sample, or
       - if the concentration of the first enrichment broth can be adjusted, for the alternative method and the reference method, obtain a first half suspension before inoculation. Inoculate before separation (see D2).
     - For liquid or easily homogenizable samples: inoculate directly into the sample, without diluting beforehand.

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

- Level of contamination flora
- Morphology of targeted colonies, if not typical : micro-colony, size, shape, color, halo,…
- Need to isolate again or not
- Presence of less than 5 colonies (state number)

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

CALCULATION AND INTERPRETATION (SECTION 5.1.1.3)

The results shall be exploited so as to define:
- Relative accuracy
- Relative specificity (before and after confirmation step)
- Relative sensitivity

For calculation and interpretation, refer to the tables 1 and 2 of EN ISO 16140 standard.

Specific requirements of the technical committee:

Construct a 3rd table listing the results by food type (see table 2 with as many extra lines as there are products tested, retaining only the first 5 columns: PA, NA, ND, PD, Sum).

For the artificially contaminated samples, the expert laboratory must provide **all the positive and negative raw results** in an appendix to the report, specifying the source of the strains and the type of contamination, the type of stress applied and result of the stress, as well as the theoretical level of contamination.

The expert laboratory must show the results obtained **before** and **after confirmation** in the same table.

In order to interpret the difference in sensitivity between the alternative method and reference method, the laboratory must also show the following calculations (if the positive results are confirmed):

— (PA+PD) / (PA+ND+PD) for the alternative method
— (PA+ND) / (PA+ND+PD) for the reference method

In the presentation of the results, the expert laboratory must specify whether the **positive deviations** are considered to be additional true positives or whether they are false positives.

Relative detection level (section 5.1.2)

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

MEASUREMENT PROTOCOL (SECTION 5.1.2.2)

Different **"food product/strain" combinations** must be tested using both alternative and reference methods, for **five food categories**.
If the validation of the alternative method is also requested for **environmental samples**, they can be considered as a category.

Specific requirements of the technical committee:

At least the following **types** of products must be tested:

- in a ***Salmonella*** study:
  - □ Dairy products category: "raw milk"
  - □ Environmental samples from primary production stage : ''faeces (poultry or pork origin)''

  Note: The contamination levels will be determined similarly to food products and will be proposed by the expert laboratory. The expert laboratory will choose the test strains according to the documentation publicly available (European surveys, ANSES - French Food Health & Safety Agency- reports etc.) which is a source of information on the prevalence of strains per sample type. The strains will be chosen according to the sample chosen and its origin.
- in a ***Listeria*** study: "raw milk", "smoked fish"

For each “food product/strain” combination, at least **4 different levels of contamination** shall be used, including negative control.

— The first shall be the “level zero”.
— The second one shall be the level for which at least 50% of replicates will be positive for at least one of the methods.
— The third one shall be the level for which more than 50% but less than 100% of replicates will be positive for at least one of the methods.
— The fourth level will be such as 100 % of samples will be positive.

Remark: the laboratory may need to inoculate the inoculum to a level below 1 cell / 25g.

The background microflora contained in the matrix tested shall be evaluated.

INTERPRETATION OF SECTION 5.1.2.2 OF EN ISO 16140 STANDARD (MEASUREMENT PROTOCOL, 4TH PARAGRAPH)

Replicate each combination (food product, contamination level) **separately 6 times**. **Perform the division of each replication** at the level of separation of the two methods (see appendix D) **and analyse each one of them with the alternative method and the reference method**.

If the first step of each method is identical (for example, same pre-enrichment broth), perform the **division** at the second step (case 1, appendix D).

Refer to the diagram in appendix 1 of this document.

If the two methods do not have a common step (if the first culture mediums, methodology or dilutions are different), individually inoculate each test sample.

Refer to the diagram in appendix 1 of this document.

In section 5.1.2.3 (Test sample preparation) and appendix D : “*Replication*" must be replaced by "***division***" in the text and the diagrams, and "*duplication*" replaced by "***division***" in the title of the appendix D. It is indeed the term "*division*" that is used in the English version of the standard used as reference. It appears that the term "re*plication*" comes from an incorrect translation into French. This is the step in which one separates the sample into two equal parts so as to analyse one by the reference method and the other by the alternative method.

The command of low contaminations is obtained through accurate calibration and experience acquired on the strains studied.

Specific requirements of the technical committee:
The medium used for the calibration, together with all the contamination protocols, be subject to the opinions of the technical committee by the expert laboratory prior to performing the comparative study. They must also be specified in the study report.

Example given by the technical committee
An example of **contamination and counting protocol for low rates** is given in appendix 2 of this document.

INTERPRETATION OF RESULTS (SEE SECTION 5.1.2.4)

The interpretation shall be done comparing both methods at each level of contamination and for each “food/strain” combination.

The relative detection level shall be estimated by calculating the **50% detection limit (LOD50)**, with an associated confidence limit, according to the Spearman-Kärber method of calculation* (Excel software is available). This detection limit estimates the contamination level that would correspond to 50% recovery.

Remark: if 100% of the samples are detected at the 1 cell/25g level, the study must be continued by starting from the level 1 gradually diluted.

** FDA. 2006. Final Report and Executive Summaries from the AOAC International Presidential Task Force on Best Practices in Microbiological Methodology. Appendix K. Statistics Working Group (Tholen, D. W., D. S. Paulson, B. Jarvis, D. M. Mettler, B. Lombard, K. Newton, M. A. Mozola, and A. D. Hitchins.) Report Part 4a - LOD50. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/ucm124900.htm*

Inclusivity and exclusivity (selectivity) of the alternative method (section 5.1.3)

MEASUREMENT PROTOCOL (SECTION 5.1.3.2)

It must be defined by analysing at least of:

- 50 positive pure strains (target microorganisms)
- 30 negative pure strains (non-target microorganisms) famous to interfer with positive strains.

Specific requirements of the technical committee:

For ***Salmonella***, the standard EN ISO 16140 limits the number of positive pure strains to 30, but the technical committee estimates that this number is insufficient in the event of a difficulty in interpretation. The technical committee maintain the requirement to 50 positive pure strains.

In addition, the technical committee has established a minimum list of target and non-target strains to be tested for *Salmonella* studies (see appendix 3).

However, **for some bacteria**, it may be difficult or even impossible to find 50 positive pure strains and the technical committee will reach a decision on a case by case basis on the number of strains to test.

Studies ***Salmonella***: validation of "Environmental samples from primary production stage" category:

- If the protocol of the alternative method is the same as for other matrices, no specific test is required.
- If the protocol of the alternative method is specific to "environmental samples from primary production stage", test specifically for this category 50 pure positive strains for inclusivity study (comprising the strains listed in appendix 3) and 30 pure negative strains for exclusivity study (list to be proposed by the expert lab and submitted to to technical committee)

Criteria for selecting test strains are given in appendix G of EN ISO 16140 standard:

- the strains must be of food origin (or strains of faeces origin in case of "Environmental samples from primary production stage"). The real origin of a strain shall be known and recorded.
- the strains must be representative of those most commonly present, taking account of their geographical distribution and incidence.
- Strains shall be characterised biochemically, serologically and if relevant genetically.

The laboratory must ensure that there is consistency between the list of strains proposed (target and non target) and the expected phenotypic and/or genetic character of these strains. **The laboratory shall ensure the diversity of strains tested** (serotype and food origin). Any single *Salmonella* serotype shall be tested no more than twice.

INTERPRETATION OF CHAPTER SECTION 5.1.2.4 (INOCULATION)

Tests shall be performed **with the alternative method only**.

For positive strains (target microorganisms):

The inoculum level shall be between 10 and 100 times greater than the minimum relative detection level of the alternative method.
The Technical Committee recommends that volumes of roughly similar size to those used in current practices for the method be inoculated.
The complete protocol of the alternative method shall be used, including pre-enrichment step if existing. When false negative or doubtful results are obtained, the strain shall be tested again with both methods (alternative and reference).

Specific requirements of the technical committee: The confirmation method must also be implemented if an original method is used.

For negative strains (non-target microorganisms):

The final enrichment medium of the alternative method must be inoculated and incubated. If this medium is a selective broth this could be replaced by an appropriate non selective broth medium.

Specific requirements of the technical committee: The inoculum level must be in the order of the highest contamination level found in the food categories tested, and must be at least 105 cell/ml.

When the alternative method gives positive or doubtful results with non-target microorganisms, the test shall be repeated using the complete protocol, with the alternative method and the reference method.

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

- Morphology of non-targeted colonies
- Morphology of targeted colonies, if not typical : micro-colony, size, shape, color, halo,…
- Presence of less than 5 colonies (state number)

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

EXPRESSION OF THE RESULTS (SECTION 5.1.3.3)

Table 4 - Presentation of the results for the selectivity: the results concerning the reference method must be marked "if necessary", as the reference method is only used during additional testing for positive or doubtful results, and not systematically.

The expert laboratory keeps count of the target and non-target micro-organism suspensions used in the tests to ensure the relative detection level is reached.

When doubtful or non expected results are obtained, the test shall be performed again and in parallel with the reference method.

Other published data that meets the requirements of the EN ISO 16140 standard may also be used by the expert laboratory to provide further information on this criteria.

Praticability of the alternative method

Others technical committee specific requirements, not asked by the EN ISO 16140 standard.

A study of "**practicability**" shall be done. It concerns the alternative method only. Its aim is to test or obtain information on several criteria concerning the practical/adaptability aspect of the method.
For each of these criteria, the method of communicating the criteria is defined with the user and the control mode of the criteria. Indeed, some criteria require communication on the packaging or instructions whereas others require communication on the NF VALIDATION certificate.

The data resulting from this study shall be incorporated in:

- the preliminary study report for the criteria 1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 13,
- the interlaboratory study report for the criteria 7 and 10.

The list of the 13 criteria are as follows:

	Criteria to check	Communication on the criteria	Method for checking the criteria
1	Conditioning mode of the elements for the method	packaging or instructions	verification by the expert laboratory
2	Volume of reagents	packaging or instructions	verification by the expert laboratory
3	Storage conditions of the elements (+ time limit for unopened products)	packaging or instructions	verification by the expert laboratory that the conditions exist
4	Methods of use after first use (particularly existence of limit dates)	packaging or instructions	verification by the expert laboratory that the methods exist
5	Specific equipment or premises required	instructions	verification by the expert laboratory of the truthfulness of the written elements
6	Reagents ready to use or reconstitute (where a procedure exists)	packaging or instructions	verification by the expert laboratory of the truthfulness of the written elements
7	Time to train an operative not familiar with the method	report	measured by the expert laboratory (possibility of using the implementation times of the collaborative laboratories) and distributed in one of the following 3 categories: under 1 day, between 1 day and one week, longer than one week.
8	Real handling time/ Flexibility of the technique with respect to the number of samples to analyse, their bacterial load, etc.	report	handling time measured in comparison with the reference method: less than, equal to or greater than the handling time of the reference method
9	Time to obtain the results	report and attestation	establishment of 2 cycles describing each step in the method only in terms of time: - 1st cycle: negative samples - 2nd cycle: positive samples

	Criteria to check	Communication on the criteria	Method for checking the criteria
10	Type of operator qualification	report	specified by the expert laboratory with respect to the level required for the reference method: identical level or different from the one required for the reference method (the expert laboratory can use the data of the collaborative laboratories)
11	Common steps with the reference method	report	verification by the expert laboratory
12	If there is one, traceability of the analysis results	instructions	verification by the expert laboratory
13	Maintenance by the laboratory	report	time and frequency

QUALITATIVE METHODS :

INTERLABORATORY STUDY

(section 5.2 of EN ISO 16140 standard)

The aim of the interlaboratory study is to determine the variability of the results obtained in a number of laboratories using identical samples.

NOTE: compilation of the interlaboratory study results

For qualitative methods, two interlaboratory studies carried out on the same product can be compiled only after the go-ahead from the Technical Committee, and only if the following conditions are met:

- there are 10 different laboratories
- the analysis protocols are exactly the same from one study to the next
- all the planned studies in the project have been executed
- the set of results obtained by each collaborative laboratory are complete

There must be enough laboratories to ensure **usable results are presented from at least ten laboratories**.

A food product will be **artificially** contaminated with the target micro-organism. Reference materials may be used.

It is recommended to select a food type that is relevant with regards to the targeted microorganism. The level of contamination flora must be at least 10^3 CFU per mL or g, unless otherwise informed by the Technical Committee.

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

Specific requirements of the technical committee: When the interlaboratory study is conducted on a sample of milk, the laboratory must ensure that the level of the natural microflora is at least 1 X 10^3 CFU/ml or reaches this level by adding raw milk (in reasonable proportions).

Note: Where the interlaboratory validation study has already been completed with samples of milk containing levels below 10^3 CFU/ml, the study need not be repeated.

Each sample shall be individually contaminated to at least 3 contamination levels:

- level 0
- level slightly higher than the relative detection level
- level 10 times the previous level

e.g., 0; 3; 30 cells / 25g

At least 8 samples per level shall be prepared, making 24 samples in all for each laboratory.

Laboratories shall analyse each sample by the **alternative** method **and** by the **reference** method.

If the first stage of culture is different for each method, the number of samples should be doubled. A total of 240 results per method must be obtained.

The interpretation of the results shall be realized according to EN ISO 16140 standard (see tables §5.2.2.1 and calculation of percentages of relative specificity, sensitivity and accuracy).

Specific requirements of the technical committee:

For the **sensitivity** percentage, an additional calculation shall be performed by **combining levels L1 and L2**. The statistic interpretation calculations described in appendix L of the standard shall be made in every case. If the value of the odds ratio is greater than 1 (meaning concordance is less probable than discordance), the exact statistical test described in section L.4.2 must be used.

If the alternative method has several protocols, the interlaboratory study will **not be conducted systematically for each of the protocols**. The technical committee will propose the protocol(s) to be tested, according to the corresponding matrices. Indeed, the standard EN ISO 16140 can lock the variability of the method by means of the accuracy study on the naturally contaminated samples.

Interpretation of appendix H – Guidelines relating to the organisation and management of interlaboratory studies:

Concerning the preparation of food samples (H.1), the technical committee estimates that the homogeneity and stability studies are unnecessary for the qualitative methods. The laboratory must check that the mixture is sufficiently stable over several days in transport and preservation conditions.

Concerning the transport of the samples (H.2), the technical committee knows by experience that the posting and distribution test recommended in the standard is difficult to perform by the expert laboratory. Moreover, the technical committee considers that the organising laboratories must give priority to refrigerating rather than freezing the samples. Indeed, freezing may lead to a risk of losing pathogenic bacteria for samples of a low contamination rate. Hence, the technical committee 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 committee) are applied: *the temperature of the samples during transport must be less than or equal to 8°C and, on arrival at the laboratory, between 0°C and 8°C.*

Concerning the organisation of the interlaboratory study (H.3), in the 2nd paragraph (confirmation of the sample quality), the enumeration of the total bacterial count must be performed on a specific additional sample prepared by the expert laboratory.

Using branded media from different batches causes further variability.

QUANTITATIVE METHODS :

COMPARATIVE STUDY OF THE METHODS

(section 6.2 of EN ISO 16140 standard)

The purpose of this study is to compare the performances of the alternative method against to those of the reference method, testing the following parameters :

- Linearity and relative accuracy
- Relative sensitivity

And to determine the following parameters for the alternative method :

- Detection and quantification limits
- Specificity and selectivity
- Praticability

General remark: the "quantitative methods" section of the EN ISO 16140 standard was proposed by a biostatistician. It includes many statistical formulae and was designed as a complete tool for laboratories wanting to make the calculations themselves. It is possible to do away with some formulae in the standard by using commercially available calculation software. The expert laboratory must nevertheless have the expertise in statistics to perform the calculations and interpretations successfully.

Linearity and relative accuracy (section 6.2.1)

The result obtained after conversion of the signal to the number of cells shall be validated. The conversion procedures are not taken account of in this validation: they depend on the appliance used. The final graph is what must be linear, not the signal.

MEASUREMENT PROTOCOL (SECTION 6.2.1.2)

Specific requirements of the technical committee:

The number of samples planned for the standard is low with respect to the requirements of AFNOR VALIDATION in force before the application of the standard EN ISO 16140. That is why we recommend the following modalities, based on the **separation of the two protocols (linearity and accuracy)**:

For the two protocols (linearity and accuracy), among the **five** categories requested, there must be at least **4 human food categories**. The origin of the samples must be the most varied possible so as to reduce errors related to food specialities.

If the method studied includes a **protocol specific to a type or category of foo**d, this type or category must be tested.

If the validation scope includes one single food type within a food category, this particular food type is to be considered as a full category.

If the validation of the alternative method is requested for **the animal feeding stuffs**, the samples of animal feed must be considered to be a category and consequently must be tested.

If the validation of the alternative method is also requested for the **environmental samples**, they can be considered as a category and three types of environmental products must be tested from among the following:

- surface swabs (these can be made using sponges). *Note: some thought is needed on samples taken by contact box, which are outside the scope of the AFNOR VALIDATION mark*
- dust, sweepings, residues (on production lines, for example, cutters, vacuum cleaners),
- siphon brushes, drains,
- various types of water (washing, rinsing, etc.)

The use of a neutralizing buffer as well as environmental sample collection protocol must be described by the expert lab in the study report.

During presentation of the draft study, the Technical Committee will rule on the matrices and categories to be used depending on the micro-organism being sought.

LINEARITY:

Select **5 categories** of food so as to take **one matrix per category** (linearity is defined as "Ability of the method (...) with a given matrix").

Take **one strain per matrix**. Artificially contaminated samples must be used.

For each matrix, **5 levels** will be obtained by successive dilutions of the micro organism suspension (that is placed in the matrix - see section 6.2.1.2). If it is a liquid product, the sample shall be diluted. Carry out **2 repetitions at least per level** from the parent suspension (interpretation of appendix M).
Generally, it is advisable not to use a level corresponding to the counting of small numbers (in the sense of the standard ISO 7218).

The analysis will be performed by the **alternative method** and also by the **reference method**, to enable the relative sensitivity to be calculated. If reference materials are not used, the implementation of the reference method on the initial suspension enables an initial determination value to be obtained.

In all, at least 100 analyses shall be performed (5 categories x 5 levels x 2 repetitions (minimum) x 2 methods).

ACCURACY:

Select **5 categories** of food. For each category, **10 positive samples** – naturally contaminated if possible – must have statistically exploitable results. The samples for which the results are not statistically exploited must also figure in the report.

The foods to analyse are subdivided into **categories**, themselves subdivided into **types** (see appendix B). In each category, there must be three types of food, where a single type contains several food **matrices**. For example: in the "meats" category, the "*heat-processed*" type can be found, and in this type the matrices "*cooked meals, blood sausages, pâté, etc.*"

The samples must be the most varied as possible within a single category. Except in the case of impossibility, the products must be chosen from among **3 different types**.

Two repetitions will be performed per sample and the analysis will be realised by **both method**s.

In all, 200 analyses will be performed (5 categories x 10 samples x 2 repetitions x 2 methods).

CALCULATIONS (SECTION 6.2.1.3)

Specific requirements of the technical committee:

The results of the linearity can be used on the basis of 1 level per matrix and provided that the strain was stressed.

Beside the calculations required by the standard EN ISO 16140, calculate the **repeatability** for the two methods and the **bias** between the two methods, according to the calculation method used for the interlaboratory study (see section 6.3.5 and section 6.3.6 of the standard). These results will provide **additional information** for the **accuracy** criteria.

Detection and quantification limits (section 6.2.2)

MEASUREMENT PROTOCOL AND SAMPLES (SECTION 6.2.2.3)

The main document in the standard concerns the instrumental methods and does not give rise to any special interpretation problems. The notes concern counting methods.

Specific requirements of the technical committee:

Take **3 levels** and **replicating six times** per level from a suspension of microorganisms, which would provide a minimum level of information. The determination will be performed **with the alternative method only.**

Given the low levels sought, the Technical Committee recommends verifying contamination in the suspensions used for inoculating the samples, counting the values from at least 10 boxes to this end.

Relative sensitivity (section 6.2.3)

The protocol is the same as the linearity protocol (see interpretation of section 6.2.1.2).
For calculations, refer to standard EN ISO 16140.

Specificity and selectivity (section 6.2.4)

It is indeed inclusivity/exclusivity, as for the qualitative methods.

Concerning the **total bacteria**, the study of selectivity is unnecessary as there are no "non-target" strains.

As far as any other evaluation is concerned, adopt the measuring protocol shown in section 6.2.4.2.

The selectivity study need not be performed if published data already exists meeting EN ISO 16140 requirements.

Others characteristics of the alternative method (section 6.2.5)

In this section, the standard requests documenting other characteristics of the alternative method (stability, reliability, robustness, etc.) that lie **outside the scope of third party validation**.

Specific requirements of the technical committee:

A study of "**practicability**" will be done. It concerns the alternative method only. Its aim is to test or obtain information on several criteria concerning the practical/adaptability aspect of the method.

For each of these criteria, the method of communicating the criteria is defined with the user and the control mode of the criteria. Indeed, some criteria require communication on the packaging or instructions whereas others require communication on the NF VALIDATION certificate.

The data resulting from this study shall be incorporated in:

- the preliminary study report for the criteria 1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 13,
- the interlaboratory study report for the criteria 7 and 10.

The list of the 13 criteria are as follows:

	Criteria to check	Communication on the criteria	Method for checking the criteria
1	Conditioning mode of the elements for the method	packaging or instructions	verification by the expert laboratory
2	Volume of reagents	packaging or instructions	verification by the expert laboratory
3	Storage conditions of the elements (+ time limit for unopened products)	packaging or instructions	verification by the expert laboratory that the conditions exist
4	Methods of use after first use (particularly existence of limit dates)	packaging or instructions	verification by the expert laboratory that the methods exist
5	Specific equipment or premises required	instructions	verification by the expert laboratory of the truthfulness of the written elements

	Criteria to check	Communication on the criteria	Method for checking the criteria
6	Reagents ready to use or reconstitute (where a procedure exists)	packaging or instructions	verification by the expert laboratory of the truthfulness of the written elements
7	Time to train an operative not familiar with the method	report	measured by the expert laboratory (possibility of using the implementation times of the collaborative laboratories) and distributed in one of the following 3 categories: under 1 day, between 1 day and one week, longer than one week.
8	Real handling time/ Flexibility of the technique with respect to the number of samples to analyse, their bacterial load, etc.	report	handling time measured in comparison with the reference method: less than, equal to or greater than the handling time of the reference method
9	Time to obtain the results	report and attestation	establishment of 2 cycles describing each step in the method only in terms of time: - 1st cycle: negative samples - 2nd cycle: positive samples
10	Type of operator qualification	report	specified by the expert laboratory with respect to the level required for the reference method: identical level or different from the one required for the reference method (the expert laboratory can use the data of the collaborative laboratories)
11	Common steps with the reference method	report	verification by the expert laboratory
12	If there is one, traceability of the analysis results	instructions	verification by the expert laboratory
13	Maintenance by the laboratory	report	time and frequency

QUANTITATIVE METHODS :

INTERLABORATORY STUDY

(section 6.3 of EN ISO 16140/A1 : 2011 standard)

The aim of the interlaboratory study is to determine the variability of the results obtained in a number of laboratories using identical samples.

There must be enough laboratories to ensure **usable results are presented from at least ten laboratories**.

A food product will be **artificially** contaminated with the target micro-organism. Reference materials may be used.

It is recommended to select a food type that is relevant with regards to the targeted microorganism. The level of contamination flora must be at least 10^3 CFU per mL or g, unless otherwise informed by the Technical Committee.

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

Specific requirements of the technical committee: **Specific requirements of the technical committee**: When the interlaboratory study is conducted on a sample of milk, the laboratory must ensure that the level of the natural microflora is at least 1 X 10^3 CFU/ml or reaches this level by adding raw milk (in reasonable proportions).

Note: Where the interlaboratory validation study has already been completed with samples of milk containing levels below 10^3 CFU/ml, the study need not be repeated.

Each sample shall be individually contaminated to at least 4 contamination levels:

- level 0
- 3 levels covering use fo range (inferior / intermediate / superior)

At least **2 samples per level** shall be prepared, making 8 samples in all for each laboratory.

Laboratories shall analyse each sample by the **alternative** method **and** by the **reference** method.

If the first stage of culture is different for each method, the number of samples should be doubled.

A total of 128 results per method must be obtained (54 results per method).

The interpretation of calculation shall be made according to EN ISO 16140/A1 : 2011 standard.

If the alternative method has several protocols, the interlaboratory study will not be conducted systematically for each of the protocols. The technical committee will decide the protocol(s) to be tested, according to the corresponding matrices. Indeed, the standard EN ISO 16140 can lock the variability of the method by means of the accuracy study on the naturally contaminated samples.

INTERPRETATION OF APPENDIX H – GUIDELINES RELATING TO THE ORGANISATION AND MANAGEMENT OF INTERLABORATORY STUDIES:

Concerning the transport of the samples (H.2), the technical committee knows by experience that the posting and distribution test recommended in the standard is difficult to perform by the expert laboratory. Moreover, the technical committee considers that the organising laboratories must give priority to refrigerating rather than freezing the samples. Indeed, freezing may lead to a risk of losing pathogenic bacteria for samples of a low contamination rate. Hence, the technical committee 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 committee) are applied: the temperature of the samples during transport must be less than or equal to 8°C and, on arrival at the laboratory, between 0°C and 8°C.

Concerning the organisation of the interlaboratory study (H.3), in the 2nd paragraph (confirmation of the sample quality), the enumeration of the total bacterial count must be performed on a specific additional sample prepared by the expert laboratory.

Using branded media from different batches causes further variability.

STUDY PROCEDURE INSTRUCTIONS THE EXPERT LABORATORY SHALL FOLLOW

The manufacturer chooses the expert laboratory from the list of the qualified laboratories. It must inform the manufacturer at each stage of study execution and also in the event any deliberate deviation from the initially set protocol.

The expert laboratory must be qualified by AFNOR Certification (ACE), after the Technical Committee's findings. Qualification procedures for expert laboratories are shown in appendix 7 of the certification rules.

General notes

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

- ACE will only put on the next meeting agenda the validation files (draft study or study report) for which the complete documents are published before the deadline. Consequently only completed studies –the results of which are known when drafting the meeting agenda – shall be part of the next meeting agenda. Any study non completed when drafting the agenda will not be presented at the next meeting. Its presentation shall be delayed to another meeting.

1 Presentation of the preliminary draft study

The expert laboratory must set up a draft preliminary study and send it to ACE, before the deadline decided by ACE (3 to 4 weeks before the meeting date)

The expert laboratory and the manufacturer/requester are summoned by ACE to the Technical Committee meeting. The lab must present the draft preliminary study it has set up, using a visual medium.

During this first stage, the Technical Committee 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 preliminary draft study.

Two **reviewers** are appointed: they are selected within the technical committee and will study those dossiers for which they are responsible in more depth.

After the meeting, ACE communicates the Technical Committee's decision to the manufacturer/requester, expert laboratory, and recording secretaries by letter.

If necessary, all modifications relating to the draft preliminary study must be taken into account by the expert laboratory, who sends ACE a modified project description if the Technical Committee so requests.

2 Preliminary study and presentation of results

Important: A maximum period of **one year between each stage** of the dossier's technical processing procedure is permitted. Presentation of preliminary study results must take place no more than 1 year after presentation of the draft preliminary study.

The expert laboratory must inform ACE by the agreed date (usually 4 weeks before the date of the Technical Committee meeting) whether it is ready to present the preliminary study results.

The preliminary study report must be drawn up per the outline available from ACE.

The expert laboratory must send ACE the **preliminary study report** (along with any addenda) along with the documents in its **appendix** (draft technical instructions, etc.) before the deadline set by ACE (usually 3 to 4 weeks before the meeting) so the latter can circulate this file to the Technical Committee members.

In order to gain a week, the expert laboratory can directly circulate the file to the Technical Committee members provided two conditions are met:

- it is sent out **2 weeks before** the meeting date
- it asks ACE for an up-to-date list of Technical Committee members, paying particular attention to exclusion of any of the manufacturer's representatives

ACE summons the expert laboratory and the requesting manufacturer to the Technical Committee meeting. The expert laboratory must present the preliminary study report it has drawn up, using visual media.

During this 2nd stage, the Technical Committee gives its findings on the results obtained during the preliminary study.
To do so, a discussion takes place at the end of the presentation without the manufacturer/requester but including the expert laboratory. Then **a vote is taken** in the absence of both the manufacturer/requester and the expert laboratory, taking account of all results of the preliminary study.

This vote determines the Technical Committee's decision and whether the results of the preliminary study are accepted.
The voting outcome is communicated to the manufacturer and the expert laboratory at the meeting.

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

If the **full results of the preliminary study** (including complements 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 the meeting. The Technical Committee gives its opinion on the draft interlaboratory study. The list of collaborative laboratories can be included in the draft or held back until before the start of the study.

The expert laboratory must take account of any modifications relating to the interlaboratory study and subsequently send ACE its modified draft if the Technical Committee so requests.

After the meeting, ACE communicates all the decisions made at the meeting to the manufacturer/requester, the expert laboratory and the rewievers by letter.

3 Interlaboratory study and presentation of results

Important: Presentation of interlaboratory study results must take place no more than **1 year** after presentation of the draft interlaboratory study.

The expert laboratory must inform ACE by the agreed date (usually **4 weeks before** the date of the Technical Committee meeting) whether it is ready to present the preliminary study results.

The interlaboratory study report must be drawn up per the outline available from ACE.

The expert laboratory must send ACE the **interlaboratory study report** (along with any addenda) along with the documents in its **appendix** (draft technical instructions, etc.) before the deadline set by ACE (usually 3 to 4 weeks before the meeting) so the latter can circulate this file to the Technical Committee members.

In order to gain a week, the expert laboratory can directly circulate the file to the Technical Committee members provided two conditions are met:

- it is sent out **2 weeks before** the meeting date
- it asks ACE for an up-to-date list of Technical Committee members, paying particular attention to exclusion of any of the manufacturer's representatives

ACE summons the expert laboratory and the requesting manufacturer to the Technical Committee meeting.

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

During this 3rd stage, the Technical Committee 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 but including the expert laboratory. Then a vote is taken in the absence of both the manufacturer/requester and the expert laboratory, taking account of all results of the validation study (preliminary and interlaboratory).

This vote gives the final decision of the Technical Committee and takes account of all results presented (preliminary 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.

4 Preparation of the certificate of validation

ACE prepares a draft certificate and submits this to the expert laboratory for its opinion (with a copy to the manufacturer) before consulting the Technical Committee in writing.

Once this certificate is approved by the Technical Committee (including any manufacturers absent from presentation of the studies), it is signed by the General Manager of ACE.

The manufacturer receives the original copy of this certificate. The certificate is available to the public on the www.afnor-validation.com web site.

5 Summary study reports

Following the decision to validate, renew, or extend validation of a method, the expert laboratory must draw up a summary document for both the preliminary and interlaboratory studies.

This document covers the important items in these studies. Its purpose is to allow circulation to anyone who so requests. A document outline is available from ACE. All published summary reports are available to the public on the www.afnor-validation.org site.

The manufacturer must validate the contents with regard to the confidentiality of the items therein.

The expert laboratory sends ACE this document no later **than 2 months** after the Technical Committee has passed its favourable voted.

6 Duration of validity

NF VALIDATION certification lasts 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 is modified during the certification period, the decision remains valid until the original expiry date.

7 Extension/modification

If a complementary study is to be conducted, two rewievers are appointed at the Technical Committee meeting that follows the manufacturer's renewal request.

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

8 Renewal

The procedure for renewing validity of the study is defined in appendix 5 of the certification rules. If a complementary study is to be conducted, two recording secretaries are appointed at the Technical Committee meeting that follows the manufacturer's renewal request.

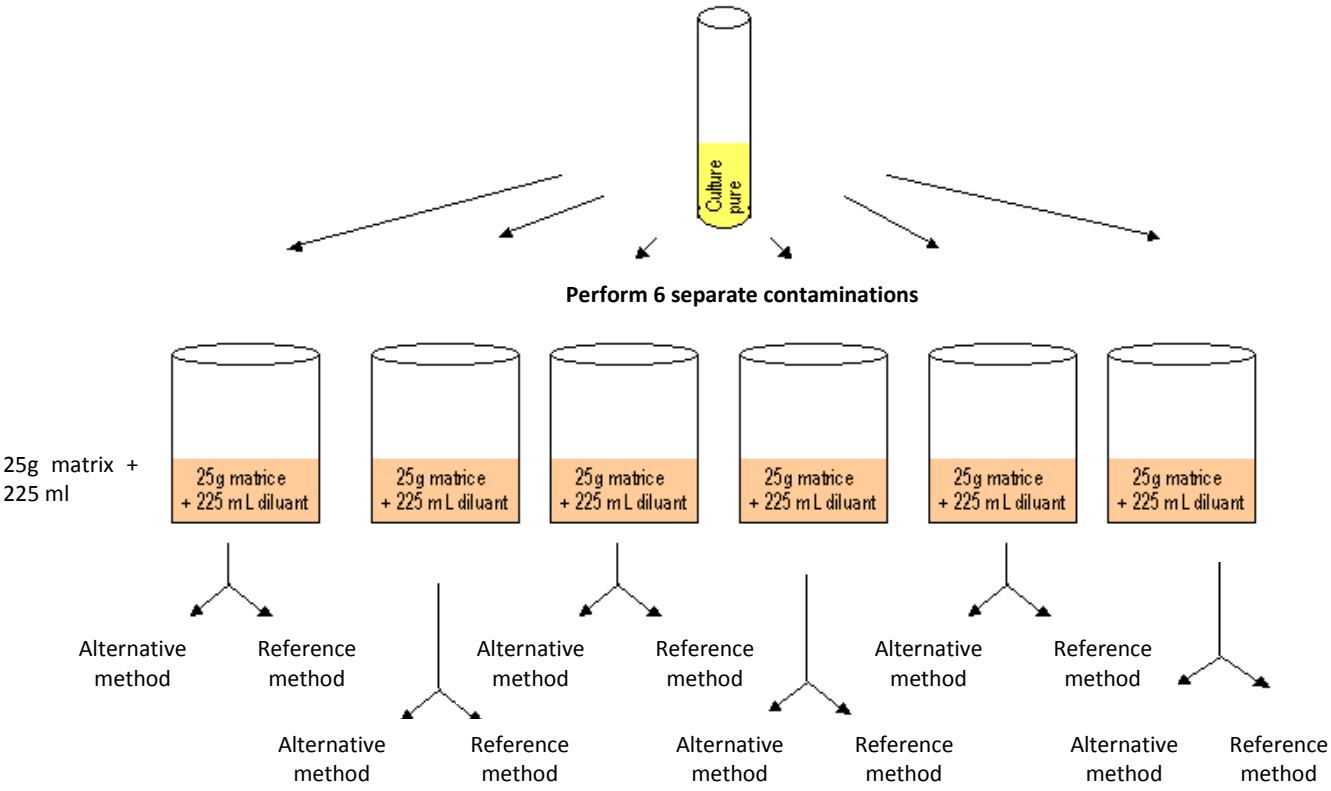
The summary report shall contain a recap of the main results obtained during the first validation study and a summary of the complementary studies conducted.

Appendix 1

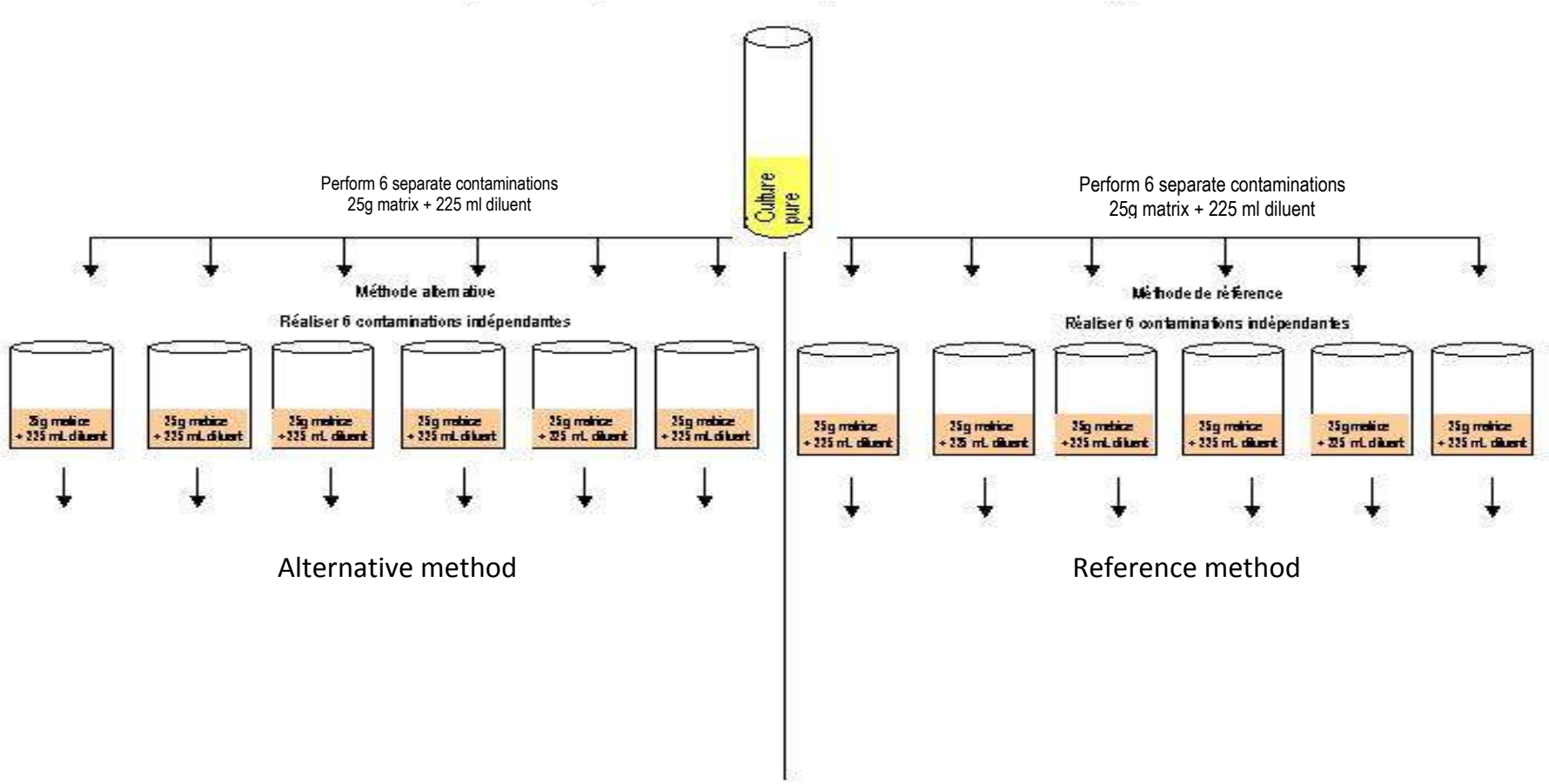
Qualitative methods - Relative detection level
Diagrams relating to the measurement protocol (section 5.1.2)

See the 2 following diagrams :

If the first steps are common to the alternative method and the reference method, for each combination perform (level/matrix/strain):



If there are no common steps between the alternative method and the reference method, for each combination perform (level/matrix/strain):



Appendix 2

Example of contamination and counting protocol for low rates

1 Calibration of parent suspensions of microorganisms

The calibration is performed using the formula: N = K x 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 UFC/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 D.O. 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 of 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 D.O. of the dilution;

f) Calculate N (number of UFC/ml) using the formula N = K x 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 the poissonian law.

Example: estimation of the confidence limit on the count of 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 limit on the suspension count will lay at most between:

m X 0.434 and m X 0.575 bacteria/ml

Example: estimation of the confidence limit on the count of 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 homogenous manner, no more than on dish out of the 10 must lay outside of the confidence limit given by the poissonian 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 limit for the suspension count will lay at the most between:

n X 0.0868 and n X 0.115 bacteria/ml

Theoretical level targeted (bacteria/25 ml)	Targeted level (bacteria/25 ml)	Concentration of the inoculum solution	Volume of inoculum (ml) per sample of 25 g	Estimation of the lower limit of the contamination per 25 g of sample	Estimation of the upper limit of the contamination per 25 g of sample
10 to 100	50	125 b / ml	0.4	m X 0.173	m X 0.23
5 to 50	25	125 b / ml	0.2	m X 0.0868	m X 0.115
2 to 20	10	25 b / ml	0.4	n X 0.035	n X 0.046
1 to 10	5	25 b / ml	0.2	n X 0.0173	n X 0.023

b = bacteria

Example: estimation of the confidence limit 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

Observed number on which the estimation is based	Lower limit factor	Upper limit factor		Observed number on which the estimation is based	Lower limit factor	Upper limit factor
1	0.0253	5.57		35	0.697	1.39
2	0.121	3.61		40	0.714	1.36
3	0.206	2.92		45	0.729	1.34
4	0.272	2.56		50	0.742	1.32
5	0.324	2.33				
6	0.367	2.18		60	0.770	1.30
7	0.401	2.06		70	0.785	1.27
8	0.431	1.97		80	0.798	1.25
9	0.458	1.90		90	0.809	1.24
10	0.480	1.84		100	0.818	1.22
11	0.499	1.79		120	0.833	1.200
12	0.517	1.75		140	0.844	1.184
13	0.532	1.71		160	0.854	1.171
14	0.546	1.68		180	0.862	1.160
15	0.560	1.65		200	0.868	1.151
16	0.572	1.62		250	0.882	1.134
17	0.583	1.60		300	0.892	1.121
18	0.593	1.58		350	0.899	1.112
19	0.602	1.56		400	0.906	1.104
20	0.611	1.54		450	0.911	1.098
				500	0.915	1.093
21	0.619	1.53				
22	0.627	1.51		600	0.922	1.084
23	0.634	1.50				
24	0.641	1.49		700	0.928	1.078
25	0.647	1.48				
				800	0.932	1.072
26	0.653	1.47				
27	0.659	1.46		900	0.936	1.068
28	0.665	1.45				
29	0.670	1.44		1000	0.939	1.064
30	0.675	1.43				

Appendix 3

Salmonella selectivity study - Lists of mandatory strains

Salmonella and non-*Salmonella* strains an expert laboratory shall mandatorily test in selectivity study for the validation of a *Salmonella* detection method. The lists shall be completed to respect the specific requirements of this current document (see section 5.1.3 “Inclusivity and exclusivity of the alternative method”).

1 Inclusivity

***Salmonella* strains**

« O » GROUP	SPECIE	SUB-SPECIE	SEROVAR	FORMULA
2 (A)	**S. enterica**	*enterica (I)*	**Paratyphi A**	1,2,12 : a : 1,5
4 (B)	**S. enterica**	*enterica (I)*	Paratyphi B	1,4,[5],12 : b : 1,2
	S. enterica	*enterica (I)*	Typhimurium	1,4,[5],12 : i : 1,2
	S. enterica	*enterica (I)*	Bredeney	1,4,12,27 : l,v : 1,7
	S. enterica	*enterica (I)*	Heidelberg	1,4,[5],12 : r : 1,2
	S. enterica	*enterica (I)*	Indiana	1,4,12 : z : 1,7
	S. enterica	*enterica (I)*	Saintpaul	1,4,[5],12 : e,h : 1,2
	S. enterica	*enterica (I)*	Derby	1,4,[5],12 : f,g : [1,2]
6,7 (C)	**S. enterica**	*enterica (I)*	Paratyphi C	6,7,[Vi] : c : 1,5
	S. enterica	*enterica (I)*	Livingstone	6,7,14 : d : l,w
	S. enterica	*enterica (I)*	Mbandaka	6,7,14 : z10 : e,n,z15
	S. enterica	*enterica (I)*	Virchow	6,7,14 : r : 1,2
	S. enterica	*enterica (I)*	Infantis	6,7,14 : r : 1,5
	S. enterica	*enterica (I)*	Rissen	6,7,14 : f,g : -
	S. enterica	*enterica (I)*	Montevideo	6,7,14 : g,m,[p],s : [1,2,7]
8 (C)	**S. enterica**	*enterica (I)*	Manhattan	6,8 : d : 1,5
	S. enterica	*enterica (I)*	Hadar	6,8 : z10 : e,n,x
	S. enterica	*enterica (I)*	Blockley	6,8 : k : 1,5
	S. enterica	*enterica (I)*	Kottbus	6,8 : e,h : 1,5
9 (D)	**S. enterica**	*enterica (I)*	Typhi	9,12,[Vi] : d : -
	S. enterica	*enterica (I)*	Napoli	1,9,12 : l,z13: e,n,x
	S. enterica	*enterica (I)*	Enteritidis	1,9,12 : [f],g,m,[p] : [1,7]
	S. enterica	*enterica (I)*	Dublin	1,9,12,[Vi] : g,p : -
	S. enterica	*enterica (I)*	Gallinarum	1,9,12 : - : -
3,10 (E)	**S. enterica**	*enterica (I)*	London	3,10[15] : l,v : 1,6
	S. enterica	*enterica (I)*	Anatum	3,10[15][15,34] : e,h : 1,6
	S. enterica	*enterica (I)*	Regent	3,10 : f,g,[s] : [1,6]
1,3,19 (E)	**S. enterica**	*enterica (I)*	Senftenberg	1,3,19 : g,[s],t : -
13 (G)	**S. enterica**	*enterica (I)*	Kedougou	1,13,23 : i : l,w
	S. enterica	*enterica (I)*	Havana	1,13,23 : f,g,[s] : -
18 (K)	**S. enterica**	*enterica (I)*	Cerro	6,14,18 : z4,z23 : [1,5]
48 (Y)	**S. enterica**	*arizonae (IIIa)*	S.III a	48 : z4,z23 : -
51	**S. enterica**	*arizonae (IIIa)*	S.III a	51 : z4,z23 : -
38	**S. enterica**	*diarizonae (IIIb)*	S.III b	38 : l,v : z53
61	**S. enterica**	*diarizonae (IIIb)*	S.III b	61 : k : 1,5,7
Variants of *Salmonella* Typhimurium	**S. enterica**	*enterica (I)*	S.I	1,4,[5],12 : i : -
	S. enterica	*enterica (I)*	S.I	1,4,[5],12 : - : 1,2
	S. enterica	*enterica (I)*	S.I	1,4,[5],12 : - : -

2 Exclusivity

Non-*Salmonella* strains

GENUS	SPECIE
Citrobacter *	*freundii, diversus, youngae, koseri, braaki,*
Escherichia	*coli, hermanii*
Proteus	*mirabilis, vulgaris*
Klebsiella	*pneumoniae, oxytoca*
Enterobacter	*cloacae, sakazakii, agglomerans (ou Pantoea agglomerans)*
Serratia	*marcescens*
Hafnia	*alvei*
Shigella	*flexneri*

*Choose 3 species among the five that are proposed.