NordVal Protocol 1B

Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures

This Protocol is equivalent to ISO/DIS 16140-6 2017-12-15

Contents

1. Scope

This Protocol describes validation of confirmation procedures of methods for detection of bacteria and fungi. The confirmation procedure advances a suspected (presumptive) result to a confirmed positive result. Validation of typing of pure strains (e.g. serotyping of *Salmonella*) are also included.

The validation protocol comprises two phases:

- a method comparison study of the alternative confirmation method against the reference confirmation procedure carried out in the organizing laboratory (Clause 3);
- an interlaboratory study (Clause 4).

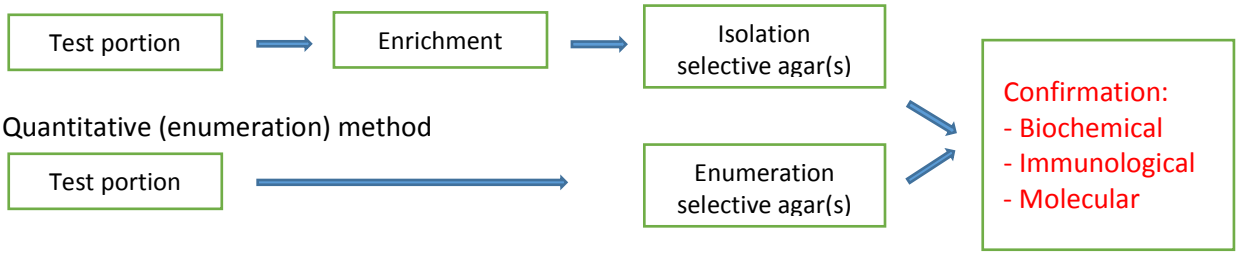
During the validation study, the performance of the alternative confirmation method will be compared to the performance of the reference confirmation procedure.
If successfully validated, the alternative confirmation method can only be used if strains are recovered on an agar that was used and shown to be acceptable within the validation study.

Validated alternative confirmation methods can replace (partly or completely) the confirmation procedure described in the reference method and/or in an alternative method validated according NordVal Protocol 1 (ISO 16140-2) providing that one of the isolation agars specified in the validation study of the alternative confirmation method is used.
This document also describes validation of alternative typing methods, where the reference method for example can be a serological method (e.g. serotyping of *Salmonella*) or a molecular method (e.g. typing of Shiga toxin-producing *E. coli*).

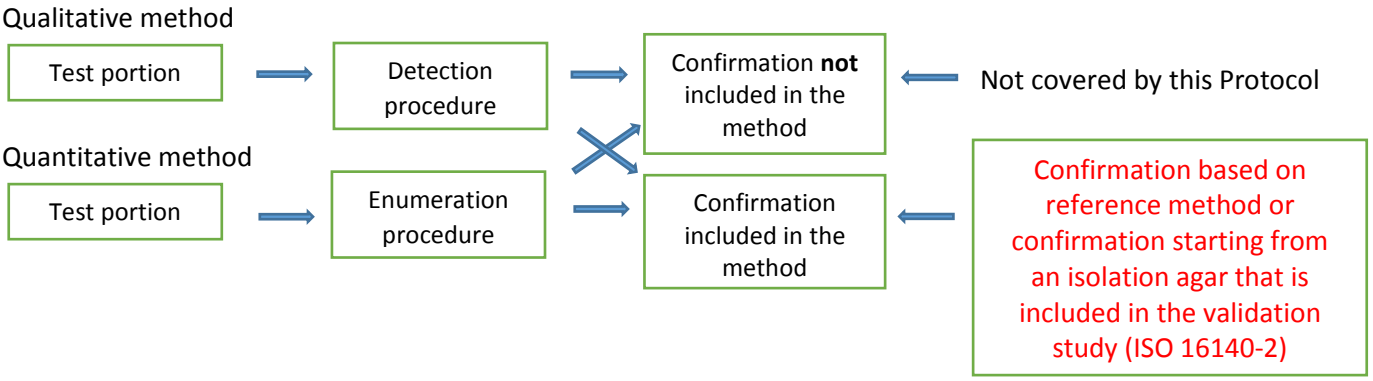
The flowcharts below shows where validation of an alternative confirmation method can be applied and which is described in this Protocol (see text in **red**).

Reference method

Qualitative (presence/absent) method



Alternative method validated according to NordVal Protocol 1 (ISO 16140-2)



It is possible to include data already obtained in inclusivity or exclusivity studies if relevant.

The Protocol describes validation of methods used for confirmation to the:

1. Family level (non-*Salmonella*);
2. Genus level (non-*Salmonella*);
3. Species level (non-*Salmonella*);
4. Microbial (sub)type level (non-*Salmonella*);
5. *Salmonella* genus or species level;
6. *Salmonella* serovar level.

2. Definitions

Acceptability limit, AL - maximum positive or negative acceptable difference between the reference value (or if not known, the accepted reference value) of a sample and an individual result obtained when applying the operating procedure of an analytical method

Confirmation procedure - number of defined tests that are performed on a strain, the combined results of which are used to definitively confirm the identity of that strain

Confirmation test - single test which is carried out to verify a presumptive result strain.

Non-target strain - strain, defined according to the scope of the reference method that would not reasonably be expected to be confirmed by the alternative method

Reference confirmation or typing procedure - combination of the confirmation or typing tests that are claimed to be replaced by the alternative confirmation or typing method
Note 1 to entry: The number of confirmation tests depends on the reference method for the specific microorganisms. The number of confirmation tests can also be one.

Microbial (sub)type - a group of closely related microorganisms (within a species) distinguished by their shared specific characteristics as determined by e.g. serological testing (serotype) or molecular testing (genotype)

Target strain - strain, defined according to the scope of the reference method, that is expected to be confirmed by the alternative method

Typing - the process of determining a particular microbial (sub)type

3. Method comparison study

The method comparison study consists of inclusivity and exclusivity study and shall be carried out in one laboratory.

A range of strains shall be used. Criteria for selecting test strains are given in Annex A. Suspect and interfering colonies should be included.

The pure strains have to be well characterized and shall follow the requirements as specified in ISO 7218. This identification information of each strain will be used to (additionally) confirm the result in cases of discrepancies between the results of the reference confirmation procedure and the alternative confirmation method.

The selected strains should take into account the measurement principle of the alternative method (e.g. culture based, immunological, molecular-based). Different measurement principles may require the use of panels of different test strains. It is important to include suspect and interfering colonies that may grow on the media used for the reference method. The rationale on the choice of the strains and their characteristics has to be included in the study report.

Each strain should be characterised biochemically and/or serologically and/or genetically in sufficient detail for its identity to be known. Strains should preferably have been isolated from foods, feed, the food-processing environment, or from primary production: depending on the scope of the validation. However, clinical, environmental and culture collection strains can also be used. The original source of all strains should be known and they shall be held in a local (e.g. expert laboratory), national or international culture collection to enable them to be used in future testing if required. See ISO 11133[3] for local maintenance of stock cultures.

Reference method results generated by a reference laboratory can be used if the laboratory performing the validation study is not able to perform the confirmation/typing of rare strains according to the reference method. For example, it is allowed to use serotyping results of a *Salmonella* reference laboratory in cases of rare *Salmonella* serovars.

3.1 Inclusivity study

Pure cultures of all target strains have to be tested with the reference confirmation procedure and with the alternative confirmation method. It is not necessary to repeat the reference confirmation procedure along with the alternative confirmation method if the required data for the reference procedure is available.

The number of strains to be tested under the various options, and the acceptability limits (ALs) are summarised in Table1.

Table 1: Inclusivity study - number of strains to be tested and the Acceptability Limits (AL)

Confirmation of	**Number of strains**	**Acceptance level ** ND - PD**	**Acceptance level ** ND+PD**
Non-*Salmonella*			
Family level * Ex: *Enterobacteriacea*	200 different target strains	2	4
Genus level * Ex: *Listeria* spp	150 different target strains	1	3
Species level * Ex: *L. monocytogenes*	100 different target strains per species	2	2
Microbial (sub)type level * Ex: *E. coli* O157, *E. coli* O111, *E. coli* O26, *E. coli* O103, and *E. coli* O145. (20 different strains of each of these 5 serogroups)	25 different target strains per microbial (sub)type If > 4 (sub)types: 100 strains & 5 strains per microbial (sub)type	1 [per microbial (sub)type]	1 [per microbial (sub)type]
Salmonella			
Salmonella Genus or species level	150 different target strains, See Note 1	1	3
Salmonella Serovar level EX: *Salmonella* Enteritidis & *Salmonella* Typhimurium: 25 different strains of *Salmonella* Enteritidis + 25 different strains of *Salmonella* Typhimurium	25 different target strains per serovar If > 10 serovars: 250 strains & 5 strains per serovar	1 [per serovar]	1 [per servoar]

* Not applicable for *Salmonella*

** For NP and PD, see section 3.3.

Note 1: For confirmation method of

- *Salmonella* spp.,: target strains shall include at least 2 strains each of *S. bongori, S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diazrizonae*, *S. enterica* subsp. *houtenae*, *S. enterica* subsp. *indica*, supplemented with strains of *S. enterica* subsp. *enterica* covering common serovars.
- *S. enterica*: target strains shall include at least 2 strains each of *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diazrizonae*, *S. enterica* subsp. *houtenae*, *S. enterica*

subsp. *indica*, supplemented with strains of *S. enterica* subsp. *enterica* covering common serovars.

- *S. enterica* subsp. *enterica*: target strains shall include different strains (and serovars) of *S. enterica* subsp. *enterica* only.

3.2 Exclusivity study

Pure cultures of all non-target strains have to be tested with the reference confirmation procedure and with the alternative confirmation method. It is not necessary to repeat the reference confirmation procedure along with the alternative confirmation method if the required data for the reference procedure is available.

Table 2: Exclusivity study - number of strains to be tested and the Acceptability Limits (AL)

Confirmation of	**Number of strains**	**Acceptance level * ND+PD**
Non-*Salmonella*		
Family level (non-*Salmonella*) Ex: *Enterobacteriacea*	100 different non-target strains	2
Genus level (non-*Salmonella*) Ex: *Listeria* spp	100 different non-target strains	2
Species level (non-*Salmonella*) Ex: *L. monocytogenes*	- 50 different strains from non-target genus - 50 different strains from non-target species within target genus	2
Microbial (sub)type level (non-*Salmonella*) Ex: *E. coli* O157, *E. coli* O111, *E. coli* O26, *E. coli* O103, and *E. coli* O145. (20 different strains of each of these 5 serogroups)	- 25 different strains from non-target genus - 25 different strains from non-target microbial (sub)type within target species - add up to a minimum of 100 strains in total	2

Confirmation of	**Number of strains**	**Acceptance level * ND+PD**
Salmonella		
Salmonella Genus or species level	100 different non-target strains See Note 2	2
Salmonella Serovar level EX: *Salmonella* Enteritidis & *Salmonella* Typhimurium: 25 different strains of non-*Salmonella* Enteritidis + 25 different strains of non-*Salmonella* Typhimurium	- 25 different strains from non-target genus - 75 different strains from non-target serovar within target subspecies	2

* For ND og PD, see section 3.3.

Note 2: For confirmation method of
S. enterica: non-target strains should include at least 2 strains of *S. bongori.*
S. enterica subsp. *enterica*, non-target strains include at least 2 strains each of *S. bongori, S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diazrizonae*, *S. enterica* subsp. *houtenae*, and *S. enterica* subsp. *indica*.

Preferably, select non-target strains that are able to grow on the selective agar(s) as used in the reference method.

3.3 Expression and interpretation of results

Tabulate the results for inclusivity and exclusivity in separate tables as illustrated in Table 3 and include the interpretation according to Table 4 and Table 5 respectively.

The results of the alternative confirmation method are first compared to the results of the reference confirmation procedure. Only in case of discrepancies between the reference method results and the identity of the strain the results are also compared to the identification information of the relevant strain. This will then become the final interpretation according to Table 4 or Table 5, including a remark on these findings. Any discrepancy in results needs to be investigated by the organizing laboratory, using relevant identification or characterisation methods (e.g. DNA sequencing) and to be included in the study report, preferably tabulated and with a rationale.

Table 3: Presentation of the results for inclusivity/exclusivity

Target strain	**Characteristics of the strain**	**Final result (+ / -) ***		**Interpretation of results (Table 4 for inclusivity Table 5 for exclusivity)**	**Remarks**
		Reference confirmation procedure	**Alternative confirmation method**		
1					
2					
3					
Etc.					
* + the train was confirmed to be the target - the strain was not confirmed to be the target					

Table 4: Comparison and interpretation of results between the reference and alternative methods for the inclusivity study

Result of the (reference or alternative) method per strain			***First interpretation*** **Alternative confirmation method compared to reference confirmation procedure***	***Final interpretation (if relevant)*** **Alternative confirmation method compared to identity of the strain ***
Reference confirmation procedure	**Alternative confirmation method**	**Identity of the strain**		
+	+	+	PA	Not relevant
+	-	+	ND	ND
-	+	+	PD	PA
-	-	+	NA	Not relevant
*PA: Positive agreement; ND: Negative deviation; PD: Positive deviation; NA: Negative agreement				

Table 5 — Comparison and interpretation of results between the reference and alternative methods for the exclusivity study

Result of the (reference or alternative) method per strain			***First interpretation*** **Alternative confirmation method compared to reference confirmation procedure****	***Final interpretation (if relevant)*** **Alternative confirmation method compared to identity of the strain ****
Reference confirmation procedure	**Alternative confirmation method**	**Identity of the (non-target) strain***		
-	-	-	NA	Not relevant
-	+	-	PD	PD
+	+	-	PA	Not relevant
+	-	-	ND	NA
* The identity of non-target strains when compared to the identity of target strains **PA: Positive agreement; ND: Negative deviation; PD: Positive deviation; NA: Negative agreement				

Summarise the results of the inclusivity and the exclusivity study as in Table 6 by taking the total numbers of PA, ND, NA, and PD found. Use the first evaluation results, and if applicable also the final evaluation results.

Table 6: Summary of the results in the inclusivity and the exclusivity study

	N	**PA**	**ND**	**NA**	**PD**	**ND-PD**	**ND+PD**
Inclusivity							
Exclusivity						not applicable	

For the inclusivity, use the calculated ND-PD and ND+PD to see if the outcome is no more than the given acceptability limits (ALs) in Table 1.
For the exclusivity, use the calculated ND+PD to see if the sum is no more than the given acceptability limits (ALs) in Table 2.

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

4. Interlaboratory study

The aim of the interlaboratory study is to determine the variability of the results obtained by different collaborators using identical strains (reproducibility conditions). Whenever possible the study conditions should reflect the normal variation between laboratories.

The interlaboratory study shall produce 10 valid data sets from at least 10 collaborators. The collaborators shall come from a minimum of 5 different organizations, but preferably 10 organizations, excluding the organizing laboratory. Technicians, involved in the preparation of the strains used in the interlaboratory study, shall not take part in the testing of those strains within the interlaboratory study.

NOTE: Laboratories in different locations but belonging to one company or institute are accepted as different organizations.

A total of 24 strains (16 target + 8 non-target) have to be tested per collaborator with the alternative confirmation method. Optionally, strains may also be tested by the participating laboratories with the reference confirmation procedure. The alternative confirmation method data will be compared to the reference confirmation procedure data available at the organizing laboratory.

For exclusivity, in the case of confirmation to the species level: a total of 8 different non-target strains; 4 strains from non-target genus and 4 strains from non-target species within the target genus.
Example: The alternative method claims to confirm *L. monocytogenes*.

- Inclusivity part: 16 different strains of *L. monocytogenes*.
- Exclusivity part: *Bacillus cereus*, *Enterococcus faecalis*, *Lactobacillus helveticus*, *Staphylococcus aureus*, *L. ivanovii, L. innocua, L. welshimeri,* and *L. seeligeri*.

For exclusivity, in the case of confirmation to the microbial (sub)type level (non-*Salmonella*): a total of 8 different non-target strains; 4 strains from non-target species and 4 strains from non-target microbial (sub)types within the target species.
Example: The alternative method claims to confirm *E. coli* O157, *E. coli* O111, *E. coli* O26, *E. coli* O103, and *E. coli* O145.

- Inclusivity part: 3 different strains each of *E. coli* O157, *E. coli* O111, *E. coli* O26, *E. coli* O103, and *E. coli O145*, plus 1 additional strain from the microbial (sub)types under study, making the total number of strains to 16.
- Exclusivity part: *Citrobacter freundii, Escherichia vulneris, Hafnia alvei, Salmonella enterica, E. coli* O6, *E. coli* O55, *E. coli* O78, and *E. coli* O128.

For exclusivity, in the case of confirmation to the *Salmonella* serovar level: a total of 8 different non-target strains; 4 strains from non-target genus and 4 strains from non-target serovars within the target subspecies.
Example: The alternative method claims to confirm *Salmonella* Enteritidis and *Salmonella* Typhimurium.

- Inclusivity part: 8 different strains of *Salmonella* Enteritidis and 8 different strains of *Salmonella* Typhimurium.
- Exclusivity part: *Citrobacter freundii, Enterobacter cloacae, Escherichia coli, Proteus mirabilis*, *Salmonella* Agama, *Salmonella* Berta, *Salmonella* Hadar, and *Salmonella* Virchow.

4.1 Procedure

The organizing laboratory:

- prepares strains, and ships them on agar transport swabs, agar tubes, or non-selective agar plates depending on the characteristics of the strains to be sent;
- provides the participants with adequate information on safe handling of the strains upon arrival and storage, on testing protocol(s) and on reporting results;
- indicates if plates and/or strains shall be retained for a certain period of time to be able to confirm results obtained by a collaborator.

The analysis of the strains may start from the isolation of the strains on non-selective agar and the testing of the strains has to be performed by the alternative confirmation method. If the alternative confirmation method claims to be able to directly confirm from selective agars, at least one of these selective agars shall be included in the interlaboratory study as the starting point of analysis.

The analysis of strains shall be performed by each collaborator within a stipulated timeframe, and the data should be submitted to the organizing laboratory.

At least 240 results should be generated for use in the interpretation.

Disregard data from collaborators:

- that received strains/test kits, etc. which were damaged during transportation;
- using media formulations that are not in accordance with the method;
- if the reported technical results suggest that the laboratory has deviated from either the testing protocol(s) or the critical operating conditions.

4.2 Expression of results

Tabulate the results for the interlaboratory study as in Table 7.

Table 7: Presentation of the results for the interlaboratory study by the alternative confirmation method

Collaborators	**Number of correctly confirmed strains in the inclusivity test (PA)**	**Number of correctly non-confirmed strains in the exclusivity test (NA)**
Collaborator 1	/16	/8
Collaborator 2	/16	/8
Etc.	/16	/8
Collaborator 10	/16	/8
Total	/160	/80

Any result discrepancies shall be included in the study report, accompanied by an explanation.

4.3 Interpretation and evaluation

Summarise the results of the interlaboratory study as in Table 6 (in total numbers of PA, ND, NA, and PD found), based on the interpretations according to Table 4 (inclusivity) and Table 5 (exclusivity).

The Acceptance Level (AL) is not met when the observed value is higher than the AL. The ALs are specified in Table 8.

Table 8 Number of strains to be tested in the inclusivity and exclusivity and the acceptability limits for the interlaboratory study

Number of collaborators	**Inclusivity**			**Exclusivity**	
	Number of target strains	**Acceptance level ND-PD**	**Acceptance level DN+PD**	**Number of non-targets strains**	**Acceptance level ND+PD**
10	160	2	4	80	2
11	176	2	4	88	2
12	192	2	5	96	2
13	208	3	5	104	3
14	224	3	6	112	3
15	240	3	6	120	3
16	256	3	6	128	3
17	272	3	7	136	3
18	288	4	7	144	4
19	304	4	8	152	4
20	320	4	8	160	4

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

Annex A — Selection of strains

In the selection of test strains the majority shall originate from foods, feed, the processing environment or the primary production, and cover the recognised range of the target analyte with respect to the following; diversity in identification characteristics e.g. biochemical, serovar, phage type, geographical distribution, incidence, and any other claims made by the producers of the alternative method. In addition, (food-borne related) clinical, environmental and culture collection strains can also be used.

Target group categories

- Undefined group, for example total count, coliform, yeast, lactic acid bacteria
- Family, for example *Enterobacteriaceae*;
- Genus, for example *Salmonella*, *Pseudomonas*, *Listeria*;
- Species, for example *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli*;
- Microbial (sub)type, for example *E. coli* O157, *Salmonella* Enteritidis.

Inclusivity study - Target groups selection

- For undefined groups, the strains used shall be selected from those capable of typical growth in the reference method.
- For families: use strains from a range of genera in that family and if possible include a representative member of all genera in the family.
- For genera: use a range of species from that genus and test as many species as possible in the genus.
- For species: use a range of strains from that species. For the selection of strains, other more detailed ways for subtyping need to be considered. For example, *Salmonella* and *Listeria* are serotyped and phage typed and/or typed with other (genetic) typing methods. In defining the positive strains to be used, organizing laboratories should use available up to date information to ensure that strains are relevant to the target (food) categories, at the time of testing.
- For microbial (sub)type: use a range of origins (like different foods, different feed, etc.) of that microbial (sub)type.

Exclusivity study - Non-target groups selection

The non-target groups (that is those expected to be negative and being used for cross reactivity tests) should be specified according to the target group.

- When the target group is a family: non-target strains shall include families.
  When the target group is a genus: non-target strains shall include other genera considered to be similar to the target genus.
- When the target group is a species: non-target strains shall include other species within the target genus.
  When the target group is a microbial (sub)type: non-target strains shall include other microbial (sub)types.