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NordVal International Certificate

Issued for:	Microbiologique Salmonella IEH Test Kit
NordVal No:	056
First approval date:	17 October 2024
Valid until:	17 October 2026

Manufactured and supplied by:

Microbiologique, inc.
8315 Lake City Way NE
Seattle, WA 98115

NordVal International has reviewed the method validation documentation. The validation was conducted by Labor Kneißler GmbH & Co according to ISO 16140-2. The reference method was ISO 6579-1:2017 Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1: Detection of *Salmonella* spp.

NordVal International concludes that it has been satisfactorily demonstrated that the data and interpretations comply with the EN ISO 16140-2:2016 requirements and demonstrate comparable performance of the alternative method Salmonella IEH Test Kit (Microbiologique, WA USA), to the ISO reference method for the detection of *Salmonella* spp. in raw milk and dairy products, raw and ready to eat meat products, eggs and egg products, fish and seafood, multicomponent foods, and environmental samples.

The production of the kits fulfills the requirements outlined in ISO 9001.

Date: 14. October 2024

Yours sincerely,

Hrólfur Sigurðsson

Chair of NordVal International

Eystein Oveland

NMKL Executive Director

PRINCIPLE OF THE METHOD

The IEH Test System uses cultural enrichment at 37±1°C for minimum 14±2 hours in non-selective media buffered peptone water followed by application of Polymerase Chain Reaction (PCR) technology. PCR involves a three-step process: DNA preparation, gene amplification, and detection. Bacterial DNA is released from organisms in enriched samples through a lysis procedure aided by heat. In this assay several multiplex PCRs, employing multiple Salmonella spp. specific amplicons, are used (ES6, S7; Salmonella IEH Test Kit Microbiologique Product Code: PB100701SAL). PCR assays are rapid, sensitive and highly specific methods, which are routinely used for screening procedures in food testing laboratories. The specificity of PCR assays arises from the set of two short (~21 bases) DNA oligonucleotides which bind in a complementary fashion to the single stranded target DNA. The DNA-Polymerase is used to replicate the complementary strand starting from the respective DNA oligonucleotide. By restricting the elongation time, only a part of the target DNA is replicated. By repeating these steps, the so-called amplicon, flanked by the two specific DNA oligonucleotides, is exponentially multiplied. The unique DNA sequences amplified in the PCR reactions are subsequently separated by size using agarose gel electrophoresis and visualized using an UV transilluminator.

Initially, the IEH Test System applies a PCR assay (designated ES6) to amplify unique sequences of DNA present in *Salmonella* spp. ES6 targets two *Salmonella* specific genes together with four *E. coli* (STEC) virulence genes.

Samples at this stage either yield a negative result and are not further analyzed, or an initial reactive (IR) result. For *Salmonella* IRs, the original PCR assay (ES6) and an additional PCR assay (S7) is applied on the enrichment sample with or without the use of immunomagnetic separation beads specific for *Salmonella*.

The presumptive molecular identification assay completes the screening test. Based on the results, a sample is either declared to be negative, and not further analyzed, or is declared to be molecularly confirmed.

FIELD OF APPLICATION

The Salmonella IEH Test Kit Microbiologique (Product Code: PB100701SAL) is designed for horizontal testing of food matrices and environmental samples. Accordingly, the comparison studies will be performed with 6 test categories summarized in **Table 1**.

METHOD COMPARISON STUDY

Selectivity study

Selectivity for *Salmonella* spp. was demonstrated by inclusivity testing for 100 *Salmonella* spp. strains and exclusivity for 32 non-target strains.

Sensitivity study

Table 1: List of test categories and types.

Category	Type
Raw milk and dairy products	raw creams
	hard and semi-hard cheese (e.g., Comte, Beaufort)
	soft cheese (e.g., Brie Munster)
Raw and ready-to-eat meat products	fresh meats (e.g., meat cuts, Carpaccio)
	minced meat, meat preparations
	Fermented or dried meat (e.g., Salami)
Eggs and egg products	eggs
	egg products (e.g., heat processed yolk, egg white, whole egg liquids) with additives (salt or sugar <2%)
	dry (egg powder)
Fish & seafoods	raw fish (unprocessed)
	cooked fisher products
	smoked or cured fish
Multicomponent food	hot meals
	cooked chilled foods, boiled rice or pasta
	bagged raw vegetable salads with dressing
Environmental samples	swabs
	washing water and process water

Table 2: Sensitivity characteristics of the alternative method.

Category	SE_{alt}	SE_{Ref}	RT	FPR	ND-PD	ND+PD
Raw milk and dairy products	93.8	100.0	96.7	0	2	2
Raw and ready-to-eat meat products	100.0	100.0	91.7	8.3	0	0
Eggs and egg products	90.6	100.0	95.0	0	3	3
Fish & Seafood	90.5	95.2	95.0	0	1	3
Multicomponent Food	90.5	90.5	96.7	0	-2	2
Combined Food Categories	92.2	97.7	96.7	3.0	4	10
Environmental swabs	100.0	100.0	100.0	0	0	0
Water samples	76.5	100.0	90.0	0	4	4

Abbreviations: SE, sensitivity (Alt, Alternative method; Ref, reference method); RT, relative trueness; FPR, False positive ratio; ND, Negative deviation; PD, Positive deviation.
The acceptability levels for a paired study and 5 categories are ND - PD <= 5 and ND + PD <= 14, for 2 categories ND – PD <= 4 and ND + PD <= 8 and for 1 category ND – PD <= 3 and ND + PD <= 6. These thresholds were met by the results of the alternative method.

Level of Detection (LOD_{50}) and Relative Level of Detection (RLOD)

The relative level of detection of the method was tested with artificially contaminated samples (according to 4.5) using the concentrations i.e. 0, 0.25, 0.75, 2.5 CFU per 25 g/or 100 ml (sample unit) to cover the different detection levels, no detection, partial recovery and full recovery.

To enable the estimation of the concentration of each inoculum, the different concentrations were achieved by serial dilutions. An estimate of the concentration of the contamination was made by plating 100 µl fractions of different dilution steps from the serial dilution onto Plate count Agar plates. This way the original concentration of *Salmonella* in the starting culture was determined and concentrations of dilutions were calculated by using the respective dilution factor.

For the 0 CFU/25 g concentration, five samples were prepared, for the intermediate concentration of contamination at least 20 samples and for the highest concentration (i.e. 2.5 CFU) five samples were evaluated.

Table 3 and **Table 4** show the level of detection values for the reference method and the alternative method, respectively.

Table 3. Level of detection (LOD) and confidence levels for the reference method.

Category	LOD50 Log cfu/test portion	Lower confidence level log cfu/test portion	Upper confidence level log cfu/test portion
Raw milk and dairy products	0.522	0.278	0.980
Raw and ready-to-eat meat products	0.574	0.309	1.064
Eggs and egg products	0.386	0.181	0.824
Fish & seafoods	0.790	0.384	1.628
Multicomponent food	0.440	0.233	0.831
Environmental swaps	0.336	0.178	0.634
Combined	0.498	0.380	0.653

Table 4. Level of detection (LOD) and confidence levels for the alternative method.

Category	LOD50 log cfu/test portion	Lower confidence level log cfu/test portion	Upper confidence level log cfu/test portion	1.5 x LOD Ref
Raw milk and dairy products	0.461	0.248	0.854	0.783
Raw and ready-to-eat meat products	0.655	0.350	1.226	0.861
Eggs and egg products	0.386	0.181	0.824	0.579
Fish & seafoods	0.945	0.453	1.967	0.579
Multicomponent food	0.499	0.260	0.956	1.185
Environmental swaps	0.316	0.168	0.594	0.504
Combined	0.523	0.398	0.687	0.783

Table 5 shows the calculated RLOD values (LOD alternative method/ LOD reference method) for all the categories tested.

Table 5. Relative limit of detection (RLOD)

Category	RLOD
Raw milk and dairy products	0.88
raw and ready-to-eat meat products	1.17
Egg and Egg products	1.00
Fish & seafoods	1.26
multicomponent food	1.22
environmental swaps	0.94
Combined	1.05

The acceptability level of the RLOD is 1.5. The results are satisfactory for all categories tested with respect to RLOD.

INTERLABORATORY STUDY

The study was carried out in pasteurized milk with 10 collaborators and 5 participating laboratories. The samples were artificially contaminated with *Salmonella* Typhimurium ATCC 1331 at 3 inoculation levels (see **Table 6**).

Table 6. Planned and real inoculation of the samples

Contamination Level	N Samples	Planned Inoculation CFU / 25ml	Actual Inoculation CFU / 25 ml
L_0	10	0	0
L_1	10	0.5	<1
L_2	10	5	10

Each collaborator received 30 samples and analyzed them with the alternative and the reference method. The samples were shipped at 4°C to the participants, who reported the temperature upon arrival and processed the samples immediately.

The results from the laboratories that participated in the ILS are shown for the reference method and alternative method in **Table 7** and **Table 8**, respectively.

Table 7. Number of positive results by the reference method

Laboratories	L_0	L_1	L_2
L01-1	0	10	10
L02-1	2	9	10
L03-1	1	10	10
L04-1	0	8	10
L05-1	0	8	10
L01-2	0	10	10
L02-2	0	10	10
L03-2	1	10	10
L04-2	0	10	10
L05-2	0	10	10
Total (positive samples)	4	95	100

Table 8. Number of positive results by the alternative method

Laboratories	L_0		L_1		L_2	
	Screening	Confirmed	Screening	Confirmed	Screening	Confirmed
L01-1	0	0	10	10	10	10
L02-1	0	0	9	9	10	10
L03-1	0	0	10	10	10	10
L04-1	4	0	9	8	10	10
L05-1	0	0	8	8	10	10
L01-2	0	0	10	10	10	10
L02-2	0	0	10	10	10	10
L03-2	0	0	10	10	10	10
L04-2	0	0	10	10	10	10
L05-2	3	2	10	10	10	10
Total (positive samples)	7	2	96	95	100	100

SPECIFICITY

The specificity of reference and alternative method were calculated based on the contamination level before and after confirmation (**Table 9**).

Table 9. Specificity.

L_0	SP_{Ref}	SP_{Alt} before confirmation	SP_{Alt} after confirmation
	96.0 %	93.0 %	98.0 %

SENSITIVITY

For the sensitivity calculation the results from the contamination levels L_1 and L_2 are summarized in **Tables 10-11** as follows.

Table 10. Results for level 1.

L_1		**Reference method**		
		positive	negative	Total
Alternative method	positive	95	0	95
	negative	0	5	5
	Total	95	5	100

Table 11. Results for level 2.

L_2		**Reference method**		
		positive	negative	Total
Alternative method	positive	100	0	100
	negative	0	0	0
	Total	100	0	100

With these summarized results the following sensitivity characteristics were calculated (**Table 12**).

Table 12. Sensitivity results.

	SE_{Ref}	**SE_{Alt}**	**RT**	**FPR**	**ND-PD**	**ND+PD**
L_1	100.0	100.0	100.0	1.0	0	0
L_2	100.0	100.0	100.0	0	0	0

CONCLUSION

The completed studies demonstrate that the alternative method fulfills the requirements of the NordVal International Protocol No. 1 / ISO 16140-2 and provides equivalent results as the reference method.