NMKL - NordVal International
c/o Institute of Marine Research
P.O. box 1870 Nordnes, N-5817 Bergen, Norway
www.nmkl.org

NordVal International Certificate

Issued for:	**iQ-Check Salmonella II kit**
NordVal No:	038
First approval date:	10 October 2009
Extension date:	31 January 2022
Valid until:	01 February 2024

iQ-Check Salmonella II kit

Manufactured by:
Bio-Rad Laboratories, Inc.
2000 Alfred Nobel Drive,
Hercules, California,
94547- USA

Supplied by:
Bio-Rad Laboratories,
3 Blvd Raymond Poincare,
92430 Marnes-la-Coquette,
France

fulfils the requirements of the NordVal Validation Protocol 1. The reference method was EN ISO 6579-1/Amd 1 2020 2020): Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* spp. - Part 1: detection of *Salmonella* spp. Amendment 1: Broader range of incubation temperatures, amendment to the status of Annex D, and correction of the composition of MSRV and SC.

NordVal International has studied the enclosures to the application and evaluated the results obtained in the validations conducted by the expert laboratories l'Institut Pasteur de Lille and ADRIA Développement, France, respectively. The validations were first carried out according to ISO 16140:2003 and thereafter additional studies and extensions according to ISO 16140-2:2016. NordVal International concludes that it has been satisfactorily demonstrated that results document no difference in the performances between the iQ-Check *Salmonella* II kit and the reference method.

The production of the iQ-Check Salmonella II kit is fulfilling the requirements given in ISO 13485:2016.

Date: 31.01.2022

Yours sincerely,

Hilde Skår Norli
Chair of NordVal International

Eystein Oveland
NMKL Secretary General

PRINCIPLE OF THE METHOD

The iQ-Check Salmonella II kit is based on gene amplification and detection by real-time PCR. Ready-to-use PCR reagents contain oligonucleotides (primers and probes) specific for *Salmonella* spp., as well as DNA polymerase and nucleotides. Detection and data analysis are optimized for use with a Bio-Rad real-time PCR instrument, such as the CFX96 or the CFX96 Deep Well systems.

iQ-Check *Salmonella* II describes now seven procedures depending on the categories or test portions tested

Procedure	Scope	Test portion	Enrichment	Extraction	Year of validation
1	Food and feed products Environmental samples	25 g or sampling device	Buffered peptone water (BPW) 20h ± 4h at 37°C ± 1°C d 1/10	Standard I: 1 ml (tube)	2007
2			BPW 22h ± 2h at 37°C ± 1°C d 1/10	Easy I: 100 µl (tube or Deep Well plate)	
3	Primary production samples	25 g or sampling device	BPW + supplement (RAPID'Salmonella capsule) 22 h ± 4 h at 41.5°C ± 1°C d 1/10 + 5 h ± 1 h in BPW (100 µL + 900 µl) at 37°C ± 1°C	Standard II: 1 ml (tube or Deep Well plate), Easy I: 100 µl, (tube or Deep Well plate)	2011
4	Infant formula and infant cereals with and without probiotics including ingredients	375 g	Pre-warmed BPW (37°C) + PIF supplement 22 h ± 4 h at 37°C ± 1°C d 1/4	Easy I: 100 µl with or without FDRS (tube or Deep Well plate)	2020
5	Raw dairy product	50 g	Pre-warmed BPW (41.5°C) + novobiocin d 1/10 22 h ±2 h at 41.5°C	Easy I: 100 µl (tube or Deep Well plate)	
6	Raw meats	25 g	Pre-warmed BPW (41.5°C) 8 - 16 h at 41.5°C ± 1°C - d 1/10	Easy II: 100 µl (tube or Deep Well plate)	
7		375 g	Pre-warmed BPW (41.5°C) 10 - 18 h at 41.5°C ± 1°C - d 1/4	Easy II: 100 µl (tube or Deep Well plate)	

The PCR can be performed using either the classical iQ-Check APF (Application Protocol File) which corresponds to a 1h50 min PCR run or the APF Fast (reduction of the number of cycles + time reduction of some steps) to reduce the PCR run time down to 1h10 min without impact on the performances.

FIELD OF APPLICATION

The method is applicable for the detection of *Salmonella* spp in a broad range of food, animal feed, production environmental samples and primary production samples.

HISTORY

Year	Summary of studies
2004	The first validation study was conducted at l'Institut Pasteur de Lille
2007	The method was extended with the Easy extraction protocol, new enrichment protocols (18h ± 2h for the Standard extraction protocol and 21h ± 1h for the Easy extraction protocol), and new reagents for lysis and PCR steps. The study was carried out at ADRIA Développement, France.
2008	The method was extended to for raw meats: enrichment step for 10 h ± 2 h at 37°C, and new extraction protocol (Easy II) with use of lysis beads.
2011	A modification of the extraction of DNA from meat products, using a new "Deep Well plate" and a new protocol of extraction for meat products, Easy Protocol II, 18h ± 2h were introduced. ADRIA Développement, France carried out the studies.
2017	The existing results were evaluated according to the acceptance criteria of ISO 16140-2:2016.
2021	A completely new method comparison study was carried out on food, feed, production environmental samples and on primary production samples. The method was extended to be certified for analysing raw meats (25 and 375 g – 8h and 10h enrichment), raw dairy products (50 g, using Novobiocin) and infant formula and infant cereals with or without probiotics including ingredients (using PIF supplement). The extension also included the use of the iQ-Check Free DNA Removal Solution for the Infant Formula matrix category. For all matrices, a shorter PCR (from 110 minutes down to 72 minutes) with the "Salmo Fast" APF (Assay Protocol File) was validated. ADRIA Développement, France carried out the studies in 2020.

METHOD COMPARISON STUDY

Sensitivity study

The sensitivity (SE) is the ability of the method to detect the analyte by either the reference or alternative method.

For the sensitivity study, only the results of the renewal study are included in this certificate.

Combining all the categories and protocols, the number of samples tested were:

- 607 samples providing, 250 positive and 357 negative results with the Standard extraction protocol,
- 864 samples with the Easy extraction protocol providing 380 positive and 484 negative results.

22.3% of the samples were naturally contaminated. For the artificially contaminated samples, about 50% of the samples were contaminated with levels of no more than 3 cfu/sample.

All samples were analysed in by both the alternative and the reference method.

The results for relative trueness (RT), the sensitivity (SE) and the false positive ratio (FPR) for the Standard Extraction protocol and Easy Extraction protocol are given in Table 1 and 2, respectively.

Table 1. Results Standard Protocol

Category	PA	NA	PD	ND	PP ND	PP NA	SEalt (%)	SEref (%)	RT (%)	FPR (%)
Ready to eat and ready to reheat products (25 g) Standard I	32	42	0	0	0	2	100.0	100.0	100.0	4.5
Meat products (25 g) Standard I	29	36	0	1	0	0	96.7	100.0	98.5	0.0
Dairy products (25 g) Standard I	28	39	2	2	0	2	93.8	93.8	94.5	4.9
Egg products (25 g) Standard I	30	41	1	1	0	1	96.9	96.9	97.3	2.4
Fishery and vegetables (25 g) Standard I	29	47	1	0	0	0	100.0	100.0	100.0	0.0
Animal feed (25 g) Standard I	31	42	0	0	0	0	100.0	100.0	100.0	0.0
Environmental samples Standard I	29	56	0	2	0	4	93.5	100.0	97.8	6.7
Primary prod. Samples Standard **II**	26	45	2	4	0	0	87.5	93.8	92.2	0.0
Total	234	348	6	10	0	9	**96.0**	**97.6**	**97.4**	**2.5**

PA = number of obtained results that are positive with both the alternative and the reference method.
NA = number of obtained results that are negative with both the alternative and the reference method.
ND = number of obtained results that are negative with the alternative method and positive with the reference method.
PD = number of obtained results that are positive with the alternative method and negative with the reference method.
PPND = number of positive results for both methods before confirmation but the alternative method is negative after confirmation.
PPNA = number of negative results with the reference method found positive with the alternative method but found to be negative after confirmation.
RT = relative trueness method.
SE = the sensitivity; the ability of the method to detect the analyte.
FPR = the false positive rate.

Table 2. Results Easy Extraction protocol

Category	PA	NA	PD	ND	PP ND	PP NA	SEalt (%)	SEref (%)	RT (%)	FPR (%)
Ready to eat and ready to reheat products (25 g) Easy I	31	41	0	1	0	3	96.9	100.0	98.7	6.8
Meat products (25 g) Easy I	29	36	0	1	0	0	96.7	100.0	98.5	0.0
Dairy products (25 g) Easy I	27	38	1	3	0	3	90.3	96.8	94.5	9.5
Egg products (25 g) Easy I	31	41	1	0	0	1	100.0	96.9	98.6	2.4
Fishery and vegetables (25 g) Easy I	27	43	1	2	0	4	93.3	96.7	96.1	6.4
Animal feed (25 g) Easy I	30	39	1	1	0	2	96.9	96.9	97.3	4.9
Environmental samples Easy I	30	48	0	1	0	12	96.8	100.0	98.9	13.3
Raw meat products (25 g) Easy **II**	25	30	8	3	0	0	91.7	77.8	83.3	0.0
Raw meat products (375 g) Easy **II**	8	30	11	11	1	0	61.3	64.5	62.3	3.3
Raw dairy products (50 g) Easy I	14	37	11	6	0	0	80.6	64.5	75.0	0.0
Infant formula and infant cereals with and without probiotics and ingredients (375 g) Easy I	21	30	7	4	0	0	87.5	78	82.2	0.0
Primary prod. Samples Easy I	26	45	2	5	0	0	84.4	93.8	90.9	0.0
Total	298	457	43	38	1	27	**89.7**	**88.7**	**90.5**	**5.8**

See abbreviations below Table 1.

Discussion of the results

For large sample sizes, the sensitivity is low for both the reference and the alternative method, especially for raw meat products of 375g sample where the sensitivity for both methods is just above 60%. The protocol of the alternative method shows 6 positive deviations (PD) and 10

negative deviations (ND) for the overall categories when using the Standard extraction protocols, 43 positive deviations and 39 negative deviations when using the Easy extraction protocols. The results from the (ND+PPND)-PD calculations meet the acceptability limits (AL) whatever the categories, and as well for the 11 tested categories.

Level of Detection (LOD) and Relative Level of Detection (RLOD)

LOD: the level for which the probability of detection is p. Here the p = 0.50, i.e. LOD50 is the level of detection for which 50 % of tests give a positive result.

RLOD is the LOD50 of the alternative method divided by the LOD50 of the reference method.

For the 2021 study, the LOD50 calculations each matrix for the standard and easy extraction protocols are given in Table 3 and 4. The calculations are carried out according to Wilrich & Wilrich[1].

Table 3. LOD50 and RLOD for Standard extraction protocol

Category	(Strain / matrix) pair	Test portion	Level of detection at 50% (CFU / sample size)		RLOD
			Reference method	Alternative method	
1	Deli salad - S. Typhimurium Ad1603	25 g	1,2 [0,7-2,0]	1,2 [0,7-2,0]	1.0
2	Ground beef - S. Infantis 14	25 g	0,9 [0,5-1,8]	0,9 [0,5-1,8]	1.0
3	Raw milk - S. Mbandaka Ad2296	25 g	1,3 [0,7-2,3]	1,4 [0,7-2,6]	1.2
4	Liquid egg - S. Enteritidis 2532	25 g	0,7 [0,4-1,5]	0,7 [0,4-1,5]	1.0
5	Perch fillet - S. Saintpaul F31	25 g	0,8 [0,4-1,6]	0,8 [0,4-1,6]	1.2
6	Balls for dog - S. Agona AOOV038	25 g	0,6 [0,3-1,1]	0,6 [0,3-1,1]	0.6
7	Process water - S. Derby AOOE084	25 g	0,6 [0,3-1,3]	0,6 [0,3-1,3]	1.0
8	Bootsocks - S. Kentucky Ad1756	Sample device	0,8 [0,5-1,5]	0,9 [0,5-1,5]	1.0
Combined			**0,9 [0,7-1,1]**	**0,9 [0,7-1,1]**	**1.0**

Table 4. LOD50 and RLOD for Easy extraction protocol

Category	(Strain / matrix) pair	Test portion	Level of detection at 50% (CFU / sample size)		RLOD
			Reference method	Alternative method	
1	Deli salad - S. Typhimurium Ad1603	25 g	1,2 [0,7-2,0]	1,2 [0,7-2,0]	1.0
2	Ground beef - S. Infantis 14	25 g	0,9 [0,5-1,8]	0,9 [0,5-1,8]	1.0
3	Raw milk - S. Mbandaka Ad2296	25 g	1,3 [0,7-2,3]	1,6 [0,9-3,0]	1.5
4	Liquid egg - S. Enteritidis 2532	25 g	0,7 [0,4-1,5]	0,7 [0,4-1,5]	1.0
5	Perch fillet - S. Saintpaul F31	25 g	0,8 [0,4-1,6]	0,9 [0,5-1,7]	1.2
6	Balls for dog - S. Agona AOOV038	25 g	0,6 [0,3-1,1]	0,4 [0,2-0,7]	0.6

[1] Wilrich, C., and P.-Th. Wilrich: Estimation of the POD function and the LOD of a qualitative microbiological measurement method. AOAC International **92** (2009) 1763 - 1772.

Category	(Strain / matrix) pair	Test portion	Level of detection at 50% (CFU / sample size)		RLOD
			Reference method	Alternative method	
7	Process water - S. Derby AOOE084	25 g	0,6 [0,3-1,3]	0,6 [0,3-1,3]	1.0
8	Bootsocks - S. Kentucky Ad1756	Sample device	0,8 [0,5-1,5]	0,9 [0,5-1,5]	1.0
9	Veal meat - S. Enteritidis Ad926	25 g	0,6 [0,4-1,1]	0,6 [0,4-1,1]	1.0
10	Ground beef - S. Newport Ad2730	375 g	0,7 [0,4-1,4]	1,0 [0,5-1,9]	1.4
11	Raw milk - S. Mbandaka Ad2296	50 g	0,4 [0,3-0,7]	0,3 [0,2-0,6]	0.8
12	Infant formula with probiotics - S. Cerro Ad2727	375 g	0,7 [0,4-1,2]	0,3 [0,1-0,5]	0.4
Combined			**0,8 [0,7-0,9]**	**0,7 [0,6-0,9]**	**0.9**

Discussion of the results

The RLOD values (using the confirmed alternative method results) meet the acceptability limit of 1.5 for paired studies or 2.5 for unpaired studies, for all matrix/strain pairs tested. The LOD50 varies: - for the Standard extraction protocol, from 0.6 to 1.3 CFU/sample size for the reference method and from 0.6 to 1.4 CFU/ sample size for the alternative method, - for the Easy extraction protocol, from 0.4 to 1.3 CFU/sample size for the reference method and from 0.3 to 1.6 CFU/sample size.

Inclusivity /exclusivity

The studies carried out in 2007 and 2008, respectively, for the alternative method showed that:

- 156 strains of Salmonella were detected out of the 156 tested, regardless of the lysis protocol used.
- The study of 30 non-Salmonella strains resulted in no cross-reactions regardless of the lysis protocol used.

In addition, the study carried out in 2004 for the alternative method showed that:

- 51 strains of Salmonella were detected out of the 51 tested, regardless of the protocol used.
- The study of 31 non-Salmonella strains resulted in no cross reactions, regardless of the protocol used.

For renewal in 2022, the validations carried out in 2020/21 additional inclusivity study was carried out due to the extension (see History):

- The 100 tested target strains were detected by the iQ-Check *Salmonella* II method (positive PCR results).
- For *Salmonella* Abortusovis Ad2320, *Salmonella* Gallinarum biovar Pullorum Ad300 and *Salmonella* Luciana CIP105626, 48 h incubation time of the plates were necessary to obtain typical colonies growth.
- For *Salmonella* Wayne Ad502, positive PCR result was observed with a late Ct value (43,57), no typical colony was observed on RAPID'*Salmonella* plates after direct streaking or after subculture in RVS broth. Typical colonies were observed after subculture in RVS or MKTTn broths and streaking onto XLD plates. The strain was tested again in parallel with the alternative method and the reference method. The same result was observed for PCR. For the reference method, micro-colonies were

observed only on RAPID'*Salmonella* plates from MKTTn broth.

- *Salmonella* Strasbourg CIP 5632 gave atypical blue colonies on RAPID'*Salmonella*.

INTERLABORATORY STUDY

The interlaboratory study was conducted in 2008 using Easy Protocol I.

In total, 19 collaborators participated. Results from 8 laboratories were excluded due to intralaboratory contamination of the samples, and one laboratory received the samples too late and hence was unable to perform the tests.

The analyses were performed on samples of pasteurized milk, artificially contaminated with a strain of *Salmonella typhimurium* at the following three contamination levels:

- L0 = 0 cfu/25 ml
- L1 = [7.1; 9.4] cfu/25 ml
- L2 = [31.4; 41.6] cfu/25 ml

The laboratories analysed 8 replicates for each level using both the alternative and the reference method. The results from the collaborators are given in Table 5.

Table 5. Positive results before and after confirmation by the alternative method and the reference method

Cont. level	**L0**			**L1**			**L2**		
Collaborators	PCR	Conf. result	Ref. method	PCR	Conf. result	Ref. method	PCR	Conf. result	Ref. method
A	2	2	2	8	8	8	8	8	8
D	1	0	0	8	8	8	8	8	8
G	0	0	0	8	8	8	8	8	8
H	0	0	0	8	8	8	8	8	8
J	0	0	0	8	8	8	8	8	8
K	3	0	0	8	8	8	8	8	8
N	3	2	2	8	8	8	8	8	8
O	0	0	0	8	8	8	8	8	8
R	0	0	0	8	8	8	8	8	8
S	1	0	0	8	8	8	8	8	8
TOTAL	**P_0=10**	**CP_0=4**	**P_{0R}=4**	**P_1=80**	**CP_1=80**	**P_{1R}=80**	**P_2=80**	**CP_2=80**	**P_{2R}=80**

The percentage specificities (SP) of the reference method and of the alternative method, using the data after confirmation, based on the results of level L0 are calculated in Table 6.

Table 6. Specificity of the methods

Specificity for the reference method	$SP_{ref} = \left(1 - \left(\frac{P_{0R}}{N_-}\right)\right) x\ 100\ \% =$	100 %
Specificity for the alternative method	$SP_{alt} = \left(1 - \left(\frac{CP_0}{N_-}\right)\right) x\ 100\ \% =$	100 %

N = total number of all L_0 tests.
P_{0R}= total number of false-positive results obtained with the reference method.
CP_0= total number of false-positive results obtained with the alternative method.

Calculation of the sensitivity for the alternative method (SE_{alt}), the sensitivity for the reference method (SE_{ref}), the relative trueness (RT) and the false positive ratio for the alternative method (FPR) are provided in Table 7.

Table 7. Results of the interlaboratory study (sensitivity, relative trueness and false positive rate)

Level	PA	NA	PD	ND	FP	N	SE_{alt} (%)	SE_{ref} (%)	RT (%)	FPR (%)
L1	80	0	0	0	0	80	100.0	100.0	100.0	0
L2	80	0	0	0	0	80	100.0	100.0	100.0	0

There are no deviations in the results for level 1 and 2 and hence the acceptance limit is met for both levels.

CONCLUSION

According to the comparison and the interlaboratory study equivalent performance have found between the iQ-check *Salmonella* II test and the reference method, ISO 6579-1:2017/Amd1 2020, for the detection of *Salmonella* spp. in a broad range of food, animal feed, production environmental samples and primary production samples.