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NordVal International Certificate

Issued for:	*Salmonella* detection method by real-time PCR
NordVal No:	041
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Salmonella detection method by real-time PCR

by Eurofins Steins, Ladelundsvej 85, 6600 Vejen, Denmark, produced by Eurofins Gene Scan GmbH, fulfils the requirements of the NordVal Validation Protocol 1.

The Salmonella detection method using DNA extraction by boiling or King Fisher is applicable for raw meat and swabs from cattle and pig carcasses. The Salmonella detection method using DNA extraction by King Fisher is also applicable for poultry faeces sock swabs.

The performance of this PCR method has been compared to the following reference methods:

- √ NMKL 71, 5th Ed., 1999: *Salmonella* spp.. Detection in foods.
- √ NMKL 187, 2007: *Salmonella* spp.. Detection in foods, faeces and materials from primary animal production using MSRV.

The results document no statistical difference in the performances between the methods.

Date: 8 March 2018

Yours sincerely

Hilde Skår Norli
Chair of NordVal International

Nina Skall Nielsen
NMKL Secretary General

PRINCIPLES OF THE METHOD

The method describes a shortened pre-enrichment in buffered peptone water followed by DNA extraction and subsequent real-time PCR analysis.

Pre-enrichment

The samples are homogenised by hand and pre-enriched at 37 °C.

- Raw meat for 12 ± 2 hours
- Swabs from carcases for 14 ± 1.5 hours

DNA extraction

Either:

DNA extraction using boiling:

Re-suspend the sample pellet in 200 µL of Tris-HCL EDTA (TE) buffer and heat the tubes at 95-98 °C for 10 min. Use 9 µL of the extracted DNA as template in the real-time PCR.

or

Automated DNA extraction (KingFisher + MagneSil KF Genomic System) or equivalent

Re-suspend the sample pellet in 200 µL of lysis buffer and prepare the plates for the automated DNA extraction. Use 9 µL of the extracted DNA as template in the real-time PCR.

PCR Analysis

The PCR analysis can be performed on an MX real-time PCR thermal cycler (Stratagene) or on "fast cycling PCR system"; StepOne/StepOnePlus system from Applied Biosystems or the ARIA MX system from Agilent Technologies.

FIELD OF APPLICATION

The method is validated on raw meat and swabs from beef and pork carcases. This Salmonella real-time PCR method can be used without further confirmation, i.e. if it is not required according to legislation.

HISTORY

Comparison studies and collaborative studies have been organised by the National Food Institute of the Technical University of Denmark in 2007, 2010 and 2015, respectively.

In 2007, the KingFisher and real-time PCR method was compared against the reference methods; NMKL 71 and NMKL 187 (NordVal certificate 031).

In 2010, the boiling and real-time PCR method was compared against the reference method, NMKL 187, and against the KingFisher and real-time PCR-method.

In 2012, the NordVal certificate 031 and the NordVal certificate 041 were combined into one. The previous NordVal certificate 031 was tested only by the use of the King Fisher DNA extraction method.

In 2014/2015, the thermocycler used in *Salmonella* 12 hour method (Agilent MX3000 series) first approved in 2010 was compared in two studies with two new "fast cycling PCR systems"; StepOne/StepOnePlus from Applied Biosystems and ARIA MX from Agilent Technol-

ogies, respectively. The comparison between Agilent MX3500 and StepOnePlus was carried out at Danish Crown's laboratory in Herning, Denmark in November 2014. The comparison between Agilent MX3500 and ARIA MX was carried out by Tican's laboratory in Thisted, Denmark, April 2015.

In 2017, Eurofins applied for renewal of the certificate. In the 2018 renewal additional information about the results obtained in previous studies have been included in order to meet the requirements including the acceptance criteria of the new validation protocol ISO 16140-2.

COMPARISON STUDY

Accuracy, sensitivity, specificity

The following results were obtained in the studies conducted in 2007:

Table 1: The KingFisher/real-time PCR method compared against NMKL 71

Sample type	No. of samples						% Value			Kappa
	N	PA	NA	FN	TP	FP	AC	SE	SP	
Minced meat (raw pork and veal)	60	30	30	0	0	0	100	100	100	1.00
Poultry neck-skins	60	27	31	0	2	0	100	107	100	0.97
Pig carcass swabs	120	21	98	1	0	0	99	95	100	0.97
Total	240	78	159	1	2	0	100	103	100	0.97

N: Number of samples, PA: Positive Agreement, NA: Negative Agreement, TP: True Positive, FN: False Negative, FP: False Positive, AC: Relative Accuracy, SE: Relative
Sensitivity, SP: Relative Specificity, N = PA +NA + FN + TP + FP.
Meat samples were artificially contaminated and swab samples naturally contaminated.

According to ISO 16140-2, for 3 categories in an unpaired study, the maximum number for FN-(FP+TP) = 5. For this study the FN-(FP+TP) = -1, and hence the results are considered acceptable.

The detection level of the two methods was 1–10 CFU/25 g sample (corresponding to a relative detection level of 100%) in all cases except for the swabs inoculated with *S*.Enteritidis, where it was 10–100 CFU/25 g for the NMKL method (relative detection level > 100%).

Study of 2010:

60 samples of each of the sample types meat (minced meat and veal) and carcass swabs (pig carcases) were included in the study. 30 of the samples were left uninoculated, 15 artificially inoculated with 1-10 CFU *Salmonella Typhimurium* and 15 with 10-100 CFU *Salmonella Typhimurium.*

The following results were obtained in the comparison studies:

Table 2: The boiling/real-time PCR method compared against NMKL 187

Sample type	No. of samples						% Value			Kappa
	N	PA	NA	FN	TP	FP	AC	SE	SP	
Carcass swabs	62	29	31	0	2	0	100	107	100	1.00
Minced meat (raw pork and veal)	60	29	29	0	1	1	98.3	103	96.7	0.97
Total	122	58	60	0	3	1	99.2	105	98.3	0.98

N: Number of samples, PA: Positive Agreement, NA: Negative Agreement,
TP: True Positive, FN: False Negative, FP: False Positive, AC: Relative Accuracy,

SE: Relative Sensitivity, SP: Relative Specificity, N = PA +NA + FN + TP + FP.

Table 3: Comparison between the boiling/real-time PCR method and King-Fisher/the real-time PCR method (KingFisher PCR considered as the reference method)

Sample type	No. of samples						% Value			Kappa
	N	PA	NA	FN	TP	FP	AC	SE	SP	
Carcass swabs	62	29	31	0	2	0	100	103	100	1.00
Minced meat (raw pork and veal)	60	30	30	0	0	0	100	100	100	1.00
Total	122	59	60	0	2	1	100	103	100	1.00

N: Number of samples, PA: Positive Agreement, NA: Negative Agreement,
TP: True Positive, FN: False Negative, FP: False Positive, AC: Relative Accuracy,
SE: Relative Sensitivity, SP: Relative Specificity, N = PA +NA + FN + TP + FP.

According to ISO 16140-2, the acceptance criteria for 2 categories in an unpaired study is FN-(FP+TP) = 4. Here the value is 0-(1+2)=-3, and hence it fulfils the criteria.

There was a very good agreement between the reference method, the KingFisher/real-time PCR method and the boiling/real-time PCR method. No significant statistical deviations were obtained, the kappa shown very good agreement (Kappa > 0.80).

Inclusivity and exclusivity

The selectivity, inclusivity and exclusivity, was tested with satisfactory results for the DNA extraction using KingFisher extraction and real-time PCR (Food Appl. Environ, Microbiol. 70, 7046-7052). The performances of these characteristics are considered to be valid for the method modification described in this NordVal validation.

COLLABORATIVE STUDY

In the collaborative study conducted in 2010, 12 laboratories analysed in duplicates 3 samples of minced meat and carcass swab samples. One laboratory obtained positive results for all of the four negative controls. This indicates cross contamination, and hence the results obtained by this laboratory are omitted in the overall results.

Table 4: Results of the collaborative validation of the alternative method (the boiling and real-time PCR)

Sample type	No. of samples						% Value			Kappa
	N	PA	NA	FN	TP	FP	AC	SE	SP	
Carcass swabs							95.8	100	87.5	0.90
L_0 = neg sample	24	-	21	-	-	3				
L_1 = 1-10 cells/ 25 g	24	24	-	-	-	-				
L_2 = 10-100 cells/25 g	24	24	-	-	-	-				
Minced meat							95.8	100	87.5	0.90
L_0 = neg sample	24	-	21	-	-	3				
L_1 = 1-10 cells/ 25 g	24	24	-	-	-	-				
L_2 = 10-100 cells/25 g	24	24	-	-	-	-				

According to ISO 16140-2 the maximum acceptable numbers of deviations for 12 laboratories are: FN-FP-TP = 4 and FN+FP+TP = 5. In this study the deviating number is 3, and hence the method meets the acceptance criteria. And is considered fit for purpose.

Study of 2014/2015:

Comparison between Agilent MX3500 and StepOnePlus and between Agilent MX3500 and ADRA MX PCR platform:

In the comparison between Agilent MX3500 and StepOnePlus, a total of 150 samples were compared in duplicate (33 positive and 117 negative), and in the comparison between Agilent MX3500 and ARIA MX PCR platform, 135 samples were compared in duplicate (37 positive and 98 negative). From each of the thawed samples, 2 x 1 mL was transferred, centrifuged and the pellet resuspended in 200 µL TE- Buffer (pH 8.0) and heated at 98°C for 10 minutes. Each DNA preparation was tested in both PCR systems by dispensing 9 µL to a microtiter plate, containing 16 µL MasterMix. The following time/temperature settings were used:

Table 5: Time/temperature settings

MXPro System (total time: 1^{52})	StepOnePlus system (total time: 0^{34})	ARIA MX PCR system (total time: 0^{45})
• 2 min at 50°C • 3 min at 95°C • 40 cycles of o 30 sec at 95°C o 60 sec at 65°C o 30 sec at 72°C	• 30 sec at 50°C • 20 sec at 95°C • 40 cycles of o 1 sec at 95°C o 20 sec at 65°C	• 2 min at 50°C • 20 sec at 95°C • 40 cycles of o 1 sec at 95°C o 20 sec at 65°C

For both systems, the fluorescence was measured in the HEX (IAC) and in the FAM *(Salmonella*) channel at "end-point" for each cycle.

Table 6 The results for comparison of Agilent MX3500 and StepOnePlus

	No. of negative	No. of positive	Dubious results
MXPro 3005p	116	33	1 (Ct=36)
OneStepPlus	117	33	0

Table 7 The results for comparison of Agilent MX3500 and ARIA MX PCR system

	No. of negative	No. of positive	Dubious results
MXPro 3005p	98	37	0
ARIA MX	97	37	1

The two comparison tests showed that all three PCR systems can be applied.

CONCLUSION

The results of the studies are satisfactory. There is no statistical difference between the alternative method using boiling and real-time PCR, the method using King Fisher DNA extraction and real-time PCR method and the reference method.

Further, it has been shown that the Agilent MX3000 series PCR platform may be replaced by the StepOne/StepOnePlus system from Applied Biosystems or the ARIA MX system from Agilent Technologies without affecting the method performance.