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NordVal Certificate

Issued for:	RAPID'*Salmonella* method, short protocol RAPID'*Salmonella* method, double enrichment protocol
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RAPID'*Salmonella* method, short protocol
RAPID'*Salmonella* method, double enrichment protocol

Manufactured and supplied by:
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fulfils the requirements of the NordVal validation protocol. The reference method was EN ISO 6579:2002: Microbiology of food and animal feeding stuffs -- Horizontal method for the detection of *Salmonella* spp.

After having reviewed the method descriptions and evaluated the results obtained in the validations, NordVal International concludes that it has been satisfactorily demonstrated that the RAPID'*Salmonella* method - short protocol and the RAPID'*Salmonella* method double enrichment protocol provide equivalent result to the reference method.

The validations performed before 2016 were carried out according to the ISO 16140:2003. The validations for the matrix extension of 2016 were carried out according to ISO 16140-2:2016. All existing data have now been evaluated according to ISO 16140-2:2016./ NordVal International Protocol 2016.

Date: 18 November 2016

Yours sincerely

Hilde Skår Norli
Chair of NordVal International

Nina Skall Nielsen
NMKL Secretary General

PRINCIPLE OF THE METHOD

RAPID'*Salmonella* is a chromogenic agar medium, the principle of which relies on demonstration of two enzymatic activities. The RAPID'*Salmonella* test methods approved by NordVal International are:

- **RAPID'*Salmonella* mehod - Short protocol**:
  - selective enrichment of 25 g sample (375 g for milk powders including infant formula) in Buffered Peptone Water and RAPID'*Salmonella* supplement at 41.5°C ± 1°C for 18h ± 2h (20 – 24h for milk powders including infant formula)
  - plating out on RAPID'*Salmonella*
  - selective isolation incubation at 37°C ± 1°C for 24h ± 2h
- **RAPID'*Salmonella* method – Double enrichment protocol**:
  - pre-enrichment in Buffered Peptone Water at 37°C ± 1°C for 18h ± 2h
  - selective enrichment in RVS for at 41,5°C ± 1°C for 24h ± 2h
  - plating out on RAPID'*Salmonella*
  - selective isolation by incubation at 37°C ± 1°C for 24h ± 2h.

Salmonella spp present appear as typical magenta colonies.

Confirmation of presumptive colonies can be performed by Oxoid *Salmonella* Latex test or conventional tests described in reference methods.

FIELD OF APPLICATION

The RAPID'*Salmonella* method - short protocol is applicable to a broad range of foods, including infant formula with and without probiotics, animal feed and environmental samples.

The RAPID'*Salmonella* method - double enrichment protocol are applicable to a broad range of foods and animal feeds. For the double enrichment protocol the agreement between the alternative method and the reference method is not satisfactory for meat, seafood and ovo products, due to a high number of false positives with the alternative method, and hence confirmation is necessary.

HISTORY

In 2005, the comparison study of the double enrichment protocol was carried out.

In 2009, the validation study of the short protocol and the *Salmonella* LATEX confirmation test were carried out.

In 2010, the scope of RAPID'*Salmonella* method – short protocol was extended to include environmental samples.

In 2011, the Short Protocol was modified in the sample preparation by addition of a capsule supplement as a concentrated solution to the enrichment broth". A red colouring agent was added to capsules RAPID' *Salmonella* QSP 2.5 mL, and the step of dissolving capsules with a concentrated NaOH solution was omitted.

In 2012, a new extension study for two confirmation tests, *Salmonella* Confirm Latex test and OXOID *Salmonella* Latex test, respectively, were carried out for confirmation of presumptive positive results obtained on RAPID'*Salmonella* agar" and on TCS agar.

In 2016, the method was extended to include milk powders including infant formula with and without probiotics and related dehydrated dairy ingredients. Further, the existing results were evaluated according to the new validation protocol, ISO 16140-2:2016.

COMPARISON STUDY

Accuracy, sensitivity, specificity

According to the ISO 16140-2:2016, for all selected categories at least 3 different types shall be included. The validations, which are performed by ADRIA Developpement, France, fulfil the required design in both the number of types and number of samples analysed.

The following categories have been studied with the following types for both protocols:

- Meat products: raw meats from pork and beef, poultry, cooked and raw deli cold cuts,
- Dairy products: unpasteurised cheese, unpasteurised milk, powdered milk, ice cream,
- Vegetables and seafood products: Shellfish, raw fish, smoked fish, frozen vegetables, prepared foods,
- Ovoproducts: liquid eggs, mayonnaise, powered eggs, egg-based preparations,
- Animal feed products: livestock feed, pet foods, meat meal

For the short protocol, the additional categories have been tested:

- Environmental samples: process water, surface sampling, siphon water, dust
- Milk powders: infant formula with probiotics, milk powders and infant formula without probiotics, milk powder ingredients

A summary of the results are given in the tables below for the RAPID'*Salmonella* method with Short and double enrichment protocol, respectively.

RAPID'*Salmonella* method – Short protocol

Table 1: Results after Screening

Matrices	PA	NA	ND	PD	Sum	Relative AC (%)	Relative SE (%)	Relative SP (%)	Kappa
Meat products	25	33	5	4	67	87	83	89	0.73
Dairy products	25	34	3	3	65	91	89	92	0.81
Vegetables and seafood products	25	31	4	2	62	90	86	94	0.80
Ovo products	29	30	0	3	62	95	100	91	0.90
Animal feed products	26	37	2	3	68	93	93	93	0.85
Environmental	37	41	5	2	85	92	88	95	0.84
Milk powders incl. infant formula with and without probiotics, and related dehydrated dairy ingredients	30	35	2	0	67	97	94	100	0.94
Total	207	241	21	17	486	92	91	93	0.84

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 (possible false negative)

PD = number of obtained results that are positive with the alternative method and negative with the reference method (possible false positive)

Relative AC = The relative accuracy; the degree of correspondence between the response obtained by

the alternative method and the reference method.

Relative SE = The relative sensitivity; the ability of the alternative method to detect the analyte compared to the reference method

Relative SP = The relative specificity is the ability of the alternative method not to detect the target microorganism when it is not detected by the reference method.

Kappa = The degree of agreement between the alternative method and the reference method, kappa of 0.80 or higher is considered to be very good agreement.

Table 2: Results after confirmation

Matrices	PA	NA	FN	TP	FP	Sum	Relative AC (%)	Relative SE (%)	Relative SP (%)	Kappa
Meat products	25	33	5	4	0	67	87	97	100	0.85
Dairy products	25	34	3	3	0	65	91	100	100	0.91
Vegetables and seafood products	25	31	4	2	0	62	90	93	100	0.87
Ovo products	29	30	0	3	0	62	95	110	100	1.0
Animal feed products	26	37	2	3	1	69	93	104	97	0.91
Environment	37	41	5	2	0	85	92	93	100	0.88
Milk powders incl. infant formula with and without probiotics, and related dehydrated dairy ingredients	30	35	2	0	0	67	97	94	100	0.94
Total	207	241	21	17	1	486	96	98	100	0.91

PA = positive agreement, NA = negative agreement, FN = false negative, TP = true positive, FP = false positive. Kappa = The degree of agreement between the alternative method and the “true result”, kappa of 0.80 or higher is considered to be very good agreement.

Agreement between the alternative method and the reference method

In the validation, the degree of agreement between the alternative method and the reference method is satisfactory when the statistical entity kappa is no less than 0.80. For meat products, before confirmation, kappa is 0.73. This low number is due to too many deviating results between the two methods. As the confirmation shows that the positive deviations are true positives, kappa is recalculated to 0.85. After confirmation, all sample groups show a very good agreement between results obtained by the alternative method and the “true results”.

Acceptability limit for the sensitivity study

For each category in a paired study having three matrix types (FN - (FP+TP)) should be no more than 3, and (FN+FP+TP) should be no more than 6. For the seven categories, the (FN - (FP+TP)) should be no more than 6 and the (FN+FP+TP) should be no more than 18. The results are summarised in Table 3.

Table 3: Acceptability limit of the sensitivity study

	(FN -(FP+TP)	(FN +FP+TP)
Meat products	5 – (0+4) = 1	5+0+4 = 9
Dairy products	3 – (0+3) = 0	3+0+3 = 6
Vegetables and seafood products	4 – (0+2) = 2	4+0+2 = 6
Ovo products	0 – (0+3) = -3	0+0+3 = 3
Animal feed products	2 – (1+3) = -2	2+1+3 = 6
Environment	5 – (0+2) = 3	5+0+2 = 7
Milk powders incl. infant formula with and without probiotics, and related dehydrated dairy ingredients	2 – (0+0) = 2	2+0+0 = 2
Total	21-(1+17) = 3	21+1+17 = 39

For all samples, the differences in the negative and positive deviations are less than or equal to 3, and hence satisfactory. For meat products and environment samples, the sums of the deviations are above the acceptability limit of 6. There are some false negative with the alternative method (21 of 486 samples, 4%), however the number of false negative with the reference method (17 of 486 samples, 3%) is in the same order of magnitude. This explains the high number of the sum of deviations, despite a good agreement between the methods.

RAPID'*Salmonella* method – Double enrichment protocol

Table 4: Results after Screening

Matrices	**PA**	**NA**	**ND**	**PD**	**Sum**	**Relative AC (%)**	**Relative SE (%)**	**Relative SP (%)**	**Kappa**
Meat products	56	64	3	22	145	83	95	74	0.66
Dairy products	28	28	0	6	62	90	100	82	0.81
Vegetables and seafood products	31	22	1	15	69	77	97	60	0.55
Ovo products	28	26	1	11	66	82	97	70	0.64
Animal feed products	28	33	2	3	66	92	93	92	0.85
Total	171	173	7	57	408	84	96	75	0.69

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 (possible false negative)

PD = number of obtained results that are positive with the alternative method and negative with the reference method (possible false positive)

Relative AC = The relative accuracy; the degree of correspondence between the response obtained by the alternative method and the reference method.

Relative SE = The relative sensitivity; the ability of the alternative method to detect the analyte compared to the reference method

Relative SP = The relative specificity is the ability of the alternative method not to detect the target

microorganism when it is not detected by the reference method.

Kappa = The degree of agreement between the alternative method and the reference method, kappa of 0.80 or higher is considered to be very good agreement.

Table 5: Results after Confirmation

Matrices	PA	NA	FN	TP	FP	Sum	Relative AC (%)	Relative SE (%)	Relative SP (%)	Kappa
Meat products	55	64	4	3	19	145	95	98	77	0.69
Dairy products	26	28	2	4	2	62	90	107	93	0.87
Vegetables and seafood products	31	22	1	0	15	69	99	97	60	0.55
Ovo products	26	26	3	3	8	66	91	100	77	0.67
Animal feed products	28	33	2	3	0	66	92	103	100	0.94
Total	166	173	12	13	44	408	94	101	80	0.73

PA = positive agreement, NA = negative agreement, FN = false negative, TP = true positive, FP = false positive. Kappa = The degree of agreement between the alternative method and the "true result", kappa of 0.80 or higher is considered to be very good agreement.

Agreement between the alternative method and the reference method

In the validation, the degree of agreement between the alternative method and the reference method is satisfactory when the statistical entity kappa is no less than 0.80. The agreement is satisfactory for dairy products and animal food products. For the other products the agreement is not satisfactory due to too high number of false positives.

Acceptability limit for the sensitivity study

For each category in a paired study having three matrix types (FN - (FP+TP)) should be no more than 3, and (FN+FP+TP) should be no more than 6. For the five categories, the (FN - (FP+TP)) should be no more than 5 and the (FN+FP+TP) should be no more than 14. The results are summarised in table 6.

Table 6: Acceptability limit of the sensitivity study

	(FN -(FP+TP)	(FN +FP+TP)
Meat products	4 – (19+2) = -17	4+19+3 = 26
Dairy products	2 – (2+4) = -4	2+2+4 = 8
Vegetables and seafood products	1 – (15+2) = -16	1+15+2 = 18
Ovo products	3 – (8+3) = -8	3+8+3 = 14
Animal feed products	2 – (0+3) = -1	2+0+3 = 6
Total	12-(44+13) = -43	12+44+13 = 69

According to the guidelines for the acceptability limit, the results are satisfactory for the dairy products and for animal products, however not for the other categories. The reason is the high number of false positives after the screening. Presumptive positive samples obtained with double enrichment protocol need therefore to be confirmed.

Confirmation with *Salmonella* LATEX test and the conventional test in the

After 24h incubation in RVS at 41.5°C (double enrichment protocol)

In 2009 an extension study was conducted using opaque agar and simplification of the confirmation test, using *Salmonella* LATEX test. 118 samples were analysed using the double enrichment protocol.

Table 7: Samples analysed

Categories	Positives	Negatives	Total
Meat products	20	28	48
Dairy products	5	19	24
Ovo products	13	3	16
Animal feed products	4	26	30
Total	42	76	118

During this study, two confirmation protocols were evaluated: The *Salmonella* LATEX test and the conventional test in the reference method. The following results were obtained:

Table 8: Results after Screening

Matrices	**PA**	**NA**	**ND**	**PD**	**Sum**	**Relative AC (%)**	**Relative SE (%)**	**Relative SP (%)**	**Kappa**
All matrices	39	75	2	2	118	97	95	97	0.93

Table 9: Results after Confirmation

Matrices	**PA**	**NA**	**FN**	**TP**	**FP**	**Sum**	**Relative AC (%)**	**Relative SE (%)**	**Relative SP (%)**
All matrices	39	75	2	1	1	118	98	98	99

Conclusion:

The agreement (kappa) between the methods is satisfactory (kappa ≥0.80).

In 2012, an additional study on confirmation tests was carried out. 152 strains were tested on RAPID'*Salmonella* agar – short protocol. One strain (*Salmonella* Kentucky CIP 105623) did not grow on RAPID'*Salmonella* agar. The presumptive positive *Salmonella* Strains were confirmed by *Salmonella* Confirm Latex test and OXOID *Salmonella* Latex test. The results are given in Table 8.

Table 10: Results of the confirmation tests

	RAPID'*Salmonella*		**TCS**	
	OXOID *Salmonella* Latex Test	***Salmonella* Confirm Latex test**	**OXOID *Salmonella* Latex Test**	***Salmonella* Confirm Latex test**
Number of strains	151	151	14	43
Positive	138	109	14	5
Negative	6	41	0	30
Weak agglutination	1	1	0	8
Inconclusive agglutination	6	0	0	0

Of the 151 strains obtained presumptive positive at RAPID'*Salmonella*, 109 strains were confirmed by

"*Salmonella* Confirm Latex test", however, 41 strains (27%) were obtained as negative.

Of the 43 strains obtained presumptive positive at TCS, only 5 strains (12%) were confirmed by "*Salmonella* Confirm Latex test", the remaining 30 strains were negative. Strains that were obtained as negatives were *Salmonella bongori*, *Salmonella arizonae*, *Salmonella diarizonae*, *Salmonella houtenae*, *Salmonella indicia*, *Salmonella enterica.*

This shows that the "*Salmonella* Confirm Latex test" has its limits, and that the "Oxoid *Salmonella* Latex test" provides more reliable results.

Relative level of detection, RLOD

Tests were carried out in 2005, for each category one relevant food type was used and artificially inoculated at 4 different levels.

The contamination levels are as follows:
- Level 1: 0 CFU/g or/ml
- Level 2: The level required to obtain from 0 to 50% positives.
- Level 3: The level required to obtain from 50 to 75% positives.
- Level 4: The level required to obtain100% positives.

Six replicates of each condition were conducted.

Table 11 – Results on relative detection levels - Short Protocol

Matched pairs (matrix / strain)	**LOD Alternative method**	**LOD reference method**	**RLOD**
Minced beef / *Salmonella* Infantis	0.4 [0.1 : 1.4] CFU/25 g	0.3 [0.1: 1.1] CFU/25 g	1.3
Unpasteurised milk / *Salmonella* Derby	0.4 [0.2 : 0.9] CFU/25 mL	0.5 [0.2: 1.3] CFU/25 mL	0.8
Haddock fillet / *Salmonella* Saintpaul	0.4 [0.2 : 1.1] CFU/25 g	0.6 [0.2: 1.5] CFU/25 g	0.7
Raw eggs / *Salmonella* Enteritidis	0.4 [0.1 : 1.2] CFU/25 g	0.3 [0.1: 1.0] CFU/25 g	1.3
Dog nuggets / *Salmonella* agona	0.6 [0.2 : 1.6] CFU/25 g	0.3 [0.1: 1.1] CFU/25 g	2.0
Poultry feces/*Salmonella* agona	1.7 [0.9 : 3.3] CFU/25 g	0.5 [0.3 : 1.0] CFU/25 g	3.4
Infant formula with probiotics	0.7 [0.4 : 1.0] CFU/375 g	0.6 [0.4 : 0.9] CFU/375 g	1.2

The acceptability limit (AL) for the RLOD is set at 1.5, meaning that the alternative method may not be higher than 1.5 times the LOD of the reference method. For dog nuggets and poultry feces, the AL is exceeded. For these products the alternative method has somewhat higher level of detection.

Table 12 – Results on relative detection levels – Double Protocol

Matched pairs (matrix / strain)	**LOD Alternative method**	**LOD reference method**	**RLOD**
Minced beef / *Salmonella* Infantis,	0.7 [0.3 : 2.1] CFU/25 g	0.8 [0.3: 2.4] CFU/25 g	0.9
Unpasteurised milk / *Salmonella* Typhimurium	0.6 [0.1 : 2.6] CFU/25 mL	1.8 [0.6: 5.6] CFU/25 mL	0.3
Ling fillets / *Salmonella* Saintpaul	0.4 [0.1 : 1.1] CFU/25 g	0.4 [0.1: 2.1] CFU/25 g	1.0
Raw eggs / *Salmonella* Enteritidis	0.8 [0.2 : 3.4] CFU/25 g	0.5 [0.1: 2.2] CFU/25 g	1.6
Morsel in aspic / *Salmonella* Agona	0.5 [0.1 : 2.5] CFU/25 g	0.5 [0.1: 2.5] CFU/25 g	1.0

The RLOD is below 1.5, the acceptability limit, for all matched pairs.

Inclusivity /exclusivity

RAPID'*Salmonella* method – Double enrichment protocol

Inclusivity: In a study conducted in 2005, 51 strains of *Salmonella* were detected out of 52 tested. The non-identified strain is *Paratyphi* A ATCC 9150. Two other strains of *Salmonella Paratyphi* A (ATCC 11511 and CIP 5541) were tested and found positive. All target strains show an Omni-0 positive/ONPG negative profile with the exception of *Salmonella arizonae* (lactose –positive phenotype) presenting a positive ONPG test

Exclusivity: The study of 30 non-*Salmonella* strains revealed typical colonies on RAPID'*Salmonella* agar in the case of a single strain of *Enterobacter sakazakii*. However, this latter presents a negative Omni-0-test, non-characteristics of *Salmonella*.

Certain strains of *Eschericihia hermanii* isolated during the course of the study demonstrate magenta colonies. Consequently 12 strains of this species were tested: 8 yielded positive reaction to Omini-0 test, but present a positive ONPG test, non-characteristic of *Salmonella*.

RAPID'*Salmonella* method – Short protocol

Inclusivity: In a study conducted in 2009, 47 strains of Salmonella were detected out of 51 tested. Three strains of *Salmonella* (*Salmonella Paratyphi* A ATCC 9150, *Salmonella Paratyphi* B Ad 301 and *Salmonella Paratyphi* C ATCC 13428) showed difficulty to grow, as well as *Salmonella gallinarium* Ad 300. Five strains of *Salmonella* gave a negative latex test: *Salmonella arizonae* Ad 450, *Salmonella bongori* Ad 599, *Samonella Cerrro* Ad 689, *Salmonella Houtenae* Ad 596 and *Salmonella Veneziana* Adria 233.

In a study conducted in 2005, 151 strains were tested in the above mentioned study in 2012. 145 of the 151 strains were confirmed positive on RAPID'Salmonella method using short protocol. Nine of the samples were negative

Exclusivity**:** 42 non-*Salmonella* strains, of which 12 strains of *Escherichia hermanii,* were studied. 11 of the *Escherichia hermanii* strains tested, 1 strain of *Citrobacter diversus* Adria 140 and 1 strain of *Serratia marescens* Ad 447. All these strains gave a negative latex test.

INTERLABORATORY VALIDATION:

RAPID'*Salmonella* method – Double enrichment protocol

The collaborative study was conducted in 2005

Number of participating laboratories: 15

The analyses were performed on samples of half-cream pasteurized milk, artificially contaminated with a strain of *Salmonella typhimurium* at the following levels

- 0 cfu/25 ml
- 5 cfu/25 ml
- 25 cfu/25 ml

The laboratories analysed 8 replicates for each level using both the alternative method and the reference method. Results from five laboratories were excluded to abnormal results apparently resulting from internal contamination and /or discordance in identification test. The results are given in table 13.

Table 13: Results of the collaborative study after confirmation

Alternative method	Reference method		Total
	+	-	
+	PA = 160	PD = FP = 2	162
-	FN= ND = 0	NA = 78	78
	160	80	240
Sensitivity:	100%		
Specificity:	97.5%		
Relative accuracy:	99.2%		
Kappa:	≥0.80		

Acceptability limit for the sensitivity study

For each category in a paired study having three matrix types (FN - (FP+TP)) should be no more than 3, and (FN+FP+TP) should be no more than 6.

According to table 13, the acceptability limit is not exceeded as (FN - (FP+TP)) = 0-(2+0)=-2, and (FN+FP+TP)= 0+2+0=2.

The collaborative study showed no statistical difference between the results obtained by the two methods.

No collaborative study has been carried out on the RAPID'*Salmonella* method – Short protocol.

CONCLUSION:

The comparison study and the collaborative study showed that RAPID'*Salmonella* double enrichment protocol (24h) and short protocol (18h) performs with a satisfactory sensitivity. Some false positives might occur with the double enrichment protocol and hence confirmation is necessary.