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NordVal International Certificate

Issued for:	RAPID'*Salmonella* method, short protocol RAPID'*Salmonella* method, double enrichment protocol
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Valid until:	01 December 2024

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 and ISO 6579-1: 2017: 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 for the matrices tested. The short protocol is applicable to a broad range of foods, milk powders including infant formula (with and without probiotics) and related dehydrated dairy ingredients, feed products and environmental samples (excluding primary production samples). The double enrichment protocol is applicable for dairy products. Confirmation is needed for presumptive colonies.

The validations of the methods have been carried out by ADRIA Developpement, France according to the ISO 16140:2003 and ISO 16140-2:2016. All existing data have now been evaluated according to ISO 16140-2:2016 & NordVal International Protocol 1. The production of RAPID'*Salmonella* is carried out according to ISO 9001:2008 and EN ISO 13485:2012.

Date: 21 November 2022

Yours sincerely,

Hilde Skår Norli
Chair of NordVal International

Eystein Oveland
NMKL Executive Director

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* method - Short protocol**:
    - selective enrichment of 25 g sample in Buffered Peptone Water and RAPID'*Salmonella* supplement at 41.5°C ± 1°C for 16h to 22h
    - For infant formula (375 g), the enrichment is prepared in pre-heated Buffered Peptone Water (37°C) without supplement and incubated at 37°C ± 1°C for 24h ± 2h or with PIF supplement incubated at 37°C ± 1°C for 20h ± 2h
    - 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.

The enriched Buffered Peptone Water can be stored for 72h prior to plating out.
The RAPID'*Salmonella* plates can be stored for 72 h at 5°C ± 1°C before reading.
Confirmation of presumptive colonies can be performed one of the following ways:

- Using the conventional tests
- Using nucleic probes as described in ISO 7218 standard (eg. iQ-Check *Salmonella*, **3578123)** using isolated colonies (with or without purification step).
- By the evaluation of oxidase activity (oxidase test, **934260**), followed by omnivalent Omni-O test (A60) (**3560781**) using 1 to 3 isolated suspect colonies. If reaction is positive to the Omni-O test, proceed with an ONPG biochemical test (**3553822**). *Salmonella* are negative to oxidase test, positive to Omni-O test (A60) and negative to ONPG test, with the exception of lactose-positive *Salmonella* which are ONPG+.
- Performing a latex agglutination test:
    - *Salmonella* latex (**3556710**) test on an isolated colony. *Salmonella* of groups B to E and G are positive to the latex test, or performing a *Salmonella* Confirm Latex test, using an isolated colony (**3556711**). Oxoid *Salmonella* Latex test was also validated.
- Using MALDI Biotyper® Complete Solution from Bruker directly from an isolated colony or after a purification step.

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 excluding samples for primary production.

The RAPID'*Salmonella* method - double enrichment protocol are applicable to dairy products (excluding raw milk).

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.

In 2018, there has been a renewal study in order to meet the new requirements of ISO 16140-2 for the double step enrichment protocol for dairy products (excluding raw milk) and for the short protocol for a broad range of food products, feed and environmental samples except for samples from primary production. Further there has been an extension for storage of RAPID'*Salmonella* plates for 72 h at 5°C ± 1°C before reading and an extension for using MALDI Biotyper from Bruker for the confirmation of the typical colonies isolated on RAPID'*Salmonella* plates after purification step on a non-selective agar plate.

In 2020, the method is extended to include infant formula and infant cereals with or without probiotics including ingredients for 375 g sample size using a PIF supplement (catolaog #12013322) in the enrichment in addition to Buffered Pepton Water.

METHOD COMPARISON STUDY

Sensitivity study

The sensitivity (SE) is the ability of the method to detect the analyte by either the alternative or the reference method. Taking into account the results carried out since 2005, 629 samples have been tested for short protocol and 70 samples are tested for double step enrichment protocol. For all selected categories at least 3 different types are included.

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

- Meat products: raw meat products (raw, frozen, seasoned), poultry meat, raw delicatessen
- Dairy products: raw milk, raw milk cheese, heat treated products
- Vegetables and seafood products: seafood, raw, ready-to-re-heat, ready-to-cook
- Egg products: liquid eggs, egg powders, egg-based products
- Ready to eat and ready to reheat: Ready to eat, ready to reheat, marinated smoked
- Animal feed products: high moisture fished products, low moisture finished products, raw materials
- Environmental samples: water, dust and residues, surfaces
- Milk powders: infant formula with probiotics, milk powders and infant formula without probiotics, milk powder ingredients
- Infant formula and infant cereals with and without probiotics and ingredients (Maltodextrin, NFDM, whey)

For the double enrichment protocol, the following category has been tested:

- Dairy products (excluding raw milk): raw milk cheese, milk powder, pasteurised dairy products

A summary of the results is given in Table 1.

Table 1: Sensitivity (SE) of the methods, relative trueness (RT) and the false positive ratio (FPR)

Matrices	PA	NA	PD	ND	FP	Sum	SEalt (%)	SEref (%)	RT (%)	FPR (%)	Kappa
Short Protocol											
Ready to eat and ready to reheat	25	39	5	2	0	71	94	84	90	0	0.8
Meat products	19	45	5	8	0	77	75	84	83	0	0.6
Dairy products	28	44	3	3	0	78	91	91	92	0	0.8
Egg products	31	36	3	0	0	70	100	9	96	0	0.9
Seafood products and vegetables	23	33	4	6	0	66	82	88	85	0	0.7
Feed products	28	48	3	3	0	82	91	91	93	0	0.8
Environmental	42	62	5	6	3	118	86	91	89	5	0.8
Milk powders	30	34	0	2	1	67	94	100	97	3	1.0
Infant formula and infant cereals with and without probiotics and ingredients (375 g)	21	30	7	4	0	62	88	78	82	0	0.6
Total	247	371	35	34	4	691	89	89	89	0.6	0.8
Double Protocol											
Dairy products 6 h	27	39	1	3	0	70	90	91	91	1	0.9
Dairy products 22 h	30	38	2	0	0	70	90	91	91	1	0.9

PA = number of results that are positive with both the alternative and the reference method
NA = number of results that are negative with both the alternative and the reference method.
PD = number of results that are positive with the alternative method and negative with the reference method
ND = number of results that are negative with the alternative method and positive with the reference method
FP = number of presumptive positive results with the alternative method confirmed as negatives
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.

Acceptability limit for the sensitivity study

For the short protocol, the reference and alternative method do not have a joint initial enrichment step, except for milk powders, and is therefore considered as an unpaired study. For each category in an unpaired study ND + FP - PD should be no more than 3. For the double enrichment protocol and short protocol for milk powders, the reference and the alternative method have the same initial step and it is therefore considered as a paired study. For one category the ND + FP - PD should be no more than 3 and ND + FP + PD should be no more than 6. See Table 2.

Table 2: Acceptability limit (AL) of the sensitivity study

Protocol	Categories – Unpaired Study	ND + FP - PD	AL
Short	Ready to eat and ready to reheat	2 + 0 - 5 = -3	3
	Meat products	8 + 0 - 5 = 3	3
	Dairy products	3 + 0 - 3 = 0	3
	Egg products	0 + 0 - 3 = -3	3
	Seafood products and vegetables	6 + 0 - 4 = 2	3
	Feed products	3 + 0 - 3 = 0	3
	Environmental	6 + 2 - 5 = 3	3

	Infant formula and infant cereals	4 + 0 - 7 = -3	3		
Protocol	Categories – Paired Study	ND + FP - PD	AL	ND + FP +PD	AL
Short	Milk powders	2 + 1 - 0 = 3	3	2 + 1 + 0 = 3	6
Double Enrichment	Dairy products (ex. raw milk) 6 h	3 + 0 - 1 = 2	3	3 + 0 + 1 = 4	6
	Dairy products (ex. raw milk) 22 h	0 + 0 - 2 = -2	3	0 + 0 + 2 = 2	6

For all samples, the differences in the negative and positive deviations are less than or equal to the AL, and hence satisfactory.

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 0.8. For meat products, seafood and vegetables, and for infant formulae and infant cereals kappa is below 0.8, indicating a not very good agreement. However, as shown in Table 2, the negative deviations are offset with the positive deviations. The sensitivity of the alternative method is equivalent or better than the reference method.

Storage

Storage of Enrichment broth and RAPID' *Salmonella* plates for 72 h at 5°C ± 3°C before reading

A total of 478 enrichment broth samples were analysed before and after storage at 72 h 5°C ± 3°C. Three changes were noticed, and the calculated values for the ND+FP-PD and the ND+ FP+PD for the individual categories meet the Acceptability Limit (calculated values ≤ AL).

220 RAPID'*Salmonella* plates (samples representative of all the tested categories) were stored after incubation and were observed a second time for the presence of characteristic colonies. The calculated values for the ND+FP-PD and the ND+ FP+PD for the individual categories meet the Acceptability Limit (calculated values ≤ AL). No evolution was observed for the plate storage during the extension study in 2020 for infant formulae and infant cereals.

Level of detection (LOD) and relative level of detection (RLOD)

Results of detection level studies have been carried out in 2009, 2010, 2015 and 2018, and are summed up in the table below.

Table 3. LOD for alternative and reference method, and RLOD

Protocol	Matrix	Level cfu/test portion	Alternative method		Reference method		RLOD =LODalt/LODref
			Pos/Total	LODalt cfu/test portion	Pos/Total	LODref cfu/test portion	
Short	Meat	0	0/6	0.71	0/6	0.77	0.92
		0.4	2/6		3/6		
		0.9	3/6		3/6		
		1.7	5/6		4/6		
		4.3	6/6		6/6		
	Raw milk	0	0/6	0.50	0/6	0.90	0.56
		0.2	1/6		1/6		
		0.4	1/6		2/6		
		0.8	5/6		2/6		
		2.1	6/6		5/6		
	Fish	0	0/6	0.59	0/6	0.74	0.80
		0.3	1/6		1/6		

Protocol	Matrix	Level cfu/test portion	Alternative method		Reference method		RLOD =LODalt/LODref
			Pos/Total	LODalt cfu/test portion	Pos/Total	LODref cfu/test portion	
		0.7	3/6		3/6		
		1.3	6/6		4/6		
		3.4	6/6		6/6		
	Egg	blank	0/6	0.66	0/6	0.49	1.5
		0.4	2/6		2/6		
		0.7	5/6		3/6		
		1.4	3/6		6/6		
		3.6	6/6		6/6		
	Feed	blank	0/6	0.84	0/6	0.57	1.5
		0.3	1/6		2/6		
		0.6	1/6		3/6		
		1.1	4/6		4/6		
		2.9	6/6		6/6		
	Environment	-	0/6	0.98	0/6	0.80	1.2
		0.4	2/6		1/6		
		0.8	4/6		2/6		
		1.6	2/6		5/6		
		4.1	6/6		6/6		
	Infant formula milk powder with probiotics 375 g	blank	0/5	0.79	0/5	0.69	1.1
		1.4	14/20		15/20		
		5.0	5/5		5/5		
	Ready-to-eat and ready to reheat		0/5	0.58	0/5	0.47	1.2
		0.2	5/20		5/20		
		0.6	2/5		3/5		
	Infant formula and infant cereals	blank	0/5	0.28	0/5	0.74	0.37
		1.2	19/20		13/20		
		3.0	5/5		5/5		
Double enrichment	Pasteurized milk	blank	0/5	1.2	0/5	1.2	1.0
		0.9	11/20		11/20		
		2.5	2/5		2/5		

The acceptability limit for paired samples is 1.5 and for unpaired samples 2.5. The results are satisfactory for all matrices tested.

Inclusivity/ Exclusivity and Confirmation Protocols

Confirmation of presumptive colonies has been tested by using the MALDI Biotyper from Bruker, Oxoid *Salmonella* Latex test or conventional tests described in reference methods.

Inclusivity and exclusivity studies using different confirmation protocols have been tested in 2005, 2009, 2011, 2012, 2015 and 2017.

RAPID'Salmonella method – Double enrichment protocol

Study conducted in 2005:

Inclusivity: 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*.

RAPID'*Salmonella* method – Short protocol

Study conducted in 2009:

Inclusivity: 47 strains of *Salmonella* were detected on RAPID'*Salmonella,* confirmed with *Salmonella* latex test, 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.

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.

Study conducted in 2011: A study was carried out in order to verify that *Salmonella* spp strains with low esterase activity show characteristic colonies on RAPID'*Salmonella* agar. Five strains of *Salmonella* Dublin, two of *Salmonella* bongori and one strain of *Salmonella* Braenderup, *Salmonella* Enteritidis and *Salmonella* Typhimurium were tested. All strains were positive on RAPD'*Salmonella*. For two of the *Salmonella* bongori strains the latex test were negative.

Study conducted in 2012: 152 strains were tested on RAPID'*Salmonella* agar confirmed with OXOID *Salmonella* Latex test and *Salmonella* Confirm latex test. 145 gave a positive result with the OXOID latex test and 110 with the *Salmonella* Confirm Latex test. The strains which gave a negative latex or a weak positive result were also tested form the TCS plates, whereof all strains were positive.
Of the latex tests, the OXOID *Salmonella* latex test is the most reliable confirmation method.

Study conducted in 2017 using MALDI Biotyper® from Bruker for confirming *Salmonella* strains, 156 positives and 101 negative strains were tested.
The results showed that in most of the cases a direct transfer procedure allowed to confirm the strains from TSA and RAPID'*Salmonella* plates (100% and 99%) for inclusivity and (96% and 94%) for exclusivity. The MALDI Biotyper is a reliable method for confirmation of the colonies in the RAPID'*Salmonella* method.

INTERLABORATORY STUDY

The interlaboratory study was conducted in 2015 using RAPID'*Salmonella* method – Double enrichment protocol.
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 valid results from the remaining laboratories were all satisfactory as listed in the table below.

Table 4 *Sensitivity (SE) of the methods, relative trueness (RT) and the false positive ratio (FPR)*

Sensitivity of the alternative method, SE_{alt}	100%
Sensitivity of the reference method, SE_{ref}	100%
Relative trueness, RE	100%
False positive ration for the alternative method	-
Kappa	1.0

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

CONCLUSION

The comparison study and the interlaboratory study showed that RAPID'*Salmonella* performs equivalent to the reference method. Further, it has been demonstrated that the RAPID'*Salmonella* plates can be stored for 72 h at 5°C ± 1°C before reading. Confirmation of presumptive colonies can be performed by using the MALDI Biotyper from Bruker, *Salmonella* latex test, *Salmonella* Confirm latex test, Oxoid *Salmonella* Latex test or conventional confirmation tests described in reference methods.