

# Detection of *Salmonella* spp. in 375 g test portions of animal feed and pet food using a PCR kit and a chromogenic medium

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## Introduction

Salmonellosis is a zoonotic disease of a considerable magnitude. Animal feed is a recognized source of *Salmonella enterica* for farm livestock and might also act as an indirect cause of infection of people consuming food of animal origin<sup>1</sup>. Human salmonellosis occurs mainly because of handling or consuming contaminated food products, with a small percentage of cases being related to other, less well-defined exposures, such as contact with companion animals and natural pet treats<sup>2</sup>. Animal feed and pet food remain challenging matrices for the detection of *Salmonella* due to the high level of background flora.

## Purpose

The study evaluated the suitability of a new unique enrichment protocol to improve the detection of *Salmonella* in animal feed and pet food by both a PCR kit and a chromogenic medium specific for the detection of *Salmonella*.

## Methods

### Enrichment conditions, PCR and chromogenic media detection

Samples belonging to raw material, animal feed and pet food types were diluted 1:6 in prewarmed buffered peptone water supplemented with the RAPID'*Salmonella* supplement and incubated at 37 ± 1°C for 18 hr. For PCR detection, the Bio-Rad iQ-Check *Salmonella* II Kit was used (Easy I lysis protocol with or without iQ-Check Free DNA Removal Solution treatment). Two thermal cyclers were tested: CFX96 Touch Deep Well and CFX Opus Deepwell Real-Time PCR Systems. In case of inhibition, 1:10 or 1:50 dilution was made in water, or the iQ-Check Purification Reagent was used. For the detection on a chromogenic media, 10 µl of the enriched samples was streaked on a RAPID'*Salmonella* plate. Confirmation was carried out using RAPID'*Salmonella* media when the primary testing was performed using the iQ-Check *Salmonella* II kit. When the primary detection method was RAPID'*Salmonella*, isolated colonies were either tested with the iQ-Check *Salmonella* II kit or confirmed using conventional tests described in the ISO 6579-1 reference method after subculture in MKTTn and RVS broths. All samples were tested in parallel with the ISO 6579-1 reference method.

### Sensitivity study according to the ISO 16140-2 standard (2016)<sup>3</sup>

In this unpaired study, 62 samples (31 positive and 31 negative) were tested in parallel with the iQ-Check *Salmonella* II, the RAPID'*Salmonella* and the ISO 6579-1 reference methods<sup>4</sup>. For the positive samples, 4 were naturally contaminated, 4 were contaminated using the spiking protocol (<5 CFU/sample) and 23 were spiked using a seeding protocol with a majority of sample (17/23) with a inoculation level ≤3 CFU.

### Relative level of detection (RLOD) according to the ISO 16140-2 standard (2016)<sup>3</sup>

Thirty samples of dry kibble dog food were tested in parallel with the iQ-Check *Salmonella* II, the RAPID'*Salmonella* and the ISO 6579-1 reference methods<sup>4</sup>. Five samples were uninoculated, 20

were inoculated with a lyophilized *Salmonella* Derby strain at a low level of spike allowing a fractional recovery (0.4 CFU/sample), and 5 were inoculated with the same strain at a higher level (5.1 CFU/sample). Samples were stored for 2 weeks at ambient temperature before continuing testing.

## Results

### Sensitivity study

The results obtained with iQ-Check *Salmonella* II method in comparison with the ISO 6579-1 reference method are described in Table 1.

**Table 1. Results of the sensitivity study on pet food and animal feed for the iQ-Check-Salmonella II method. PA = positive agreement, NA = negative agreement, PD = positive deviation, ND = negative deviation, PPND = presumptive positive non-confirmed leading to a negative deviation, PPNA = presumptive positive non-confirmed leading to a negative agreement.**

Category	PA	NA*	PD	ND**	PPND	PPNA	Total
Pet food and animal feed (375 g) without FDRS - CFX96 Touch Deep Well	25	31	2	4	0	0	62
Pet food and animal feed (375 g) with FDRS - CFX96 Touch Deep Well	25	31	2	4	0	0	62
Pet food and animal feed (375 g) without FDRS - CFX Opus Deepwell	25	31	2	3	0	0	61
Pet food and animal feed (375 g) with FDRS - CFX Opus Deepwell	25	31	2	4	0	0	62
<b>Without FDRS and CFX96 Touch Deep Well</b>	<b>323</b>	<b>488</b>	<b>45</b>	<b>42</b>	<b>1</b>	<b>27</b>	<b>926</b>
<b>With FDRS and CFX96 Touch Deep Well</b>	<b>323</b>	<b>488</b>	<b>45</b>	<b>42</b>	<b>1</b>	<b>27</b>	<b>926</b>
<b>Without FDRS and CFX Opus Deepwell</b>	<b>323</b>	<b>488</b>	<b>45</b>	<b>42</b>	<b>1</b>	<b>27</b>	<b>926</b>
<b>With FDRS and CFX Opus Deepwell</b>	<b>323</b>	<b>488</b>	<b>45</b>	<b>42</b>	<b>1</b>	<b>27</b>	<b>926</b>

\* PPNA not included

\*\* PPND not included

Four negative deviations (ND) were found except on the CFX Opus Deepwell when the FDRS treatment was not used (only 3 ND). For 3 out of 4 samples, *Salmonella* could be isolated in the enriched broth. Two positive deviations were also found with a total of NP+ PPND - PD (1 or 2) being inferior to the acceptable limit (3 in this case). When PCR inhibition occurred, a dilution of the DNA extract in water (1:10 or 1:50) was sufficient to remove inhibition in 90% of the cases. These results fit with the ISO 16140-2 expectations. Equivalent results were obtained after enrichment broth storage at 5 ± 3 °C for 24 hr or 72 hr (data not shown).

The results obtained with the RAPID'*Salmonella* method in comparison with the ISO 6579-1 reference method are described in Table 2.

**Table 2. Results of the sensitivity study on pet food and animal feed for the RAPID'Salmonella method.**

Category	PA	NA	PD	ND	PPND	PPNA	Total
Pet food and animal feed (375 g)	26	30	2	3	0	1	62
<b>Without FDRS and CFX Opus Deepwell</b>	<b>300</b>	<b>440</b>	<b>38</b>	<b>40</b>	<b>2</b>	<b>3</b>	<b>823</b>
<b>With FDRS and CFX Opus Deepwell</b>	<b>303</b>	<b>439</b>	<b>39</b>	<b>37</b>	<b>2</b>	<b>3</b>	<b>823</b>

Three negative deviations (ND) were found. For 2 out of 3 samples, *Salmonella* could be isolated in the enriched broth. Two positive deviations were also found with a total of NP+ PPND - PD (1) being inferior

to the acceptable limit (3 in this case). These results fit with the ISO 16140-2 expectations. Equivalent results were obtained after enrichment broth storage at 5 ± 3°C for 24 hr or 72 hr (data not shown).

### Relative level of detection (RLOD)

The results obtained with iQ-Check *Salmonella* II method in comparison with the ISO 6579-1 reference method are described in Table 3.

**Table 3. Results of the RLOD study on pet food and animal feed for the iQ-Check Salmonella II method.**

Matrix/strain pair	Test portion	RLOD	RLODL	RLODU	b = ln (RLOD)	sd(b)	z-Test statistic	p-value	AL
Dry kibble dog food	375 g	1.000	0.434	2.304	0.000	0.417	0.000	1.000	2.5
S. Derby Ad1878									
<b>Combined</b>		<b>0.949</b>	<b>0.753</b>	<b>1.197</b>	<b>-0.052</b>	<b>0.116</b>	<b>0.450</b>	<b>1.347</b>	<b>/</b>

The RLOD found was equal to 1, which is inferior to the Acceptability Limit (2.5). According to the ISO16140-2 calculation, this result indicates that the RLOD for dry dog kibble is conform. It corresponds to the detection level at 50% (LOD<sub>50</sub>) of 0.4 ± 0.2 CFU/ sample for both methods.

The results obtained with the RAPID'*Salmonella* method in comparison with the ISO 6579-1 reference method are described in Table 4.

**Table 4. Results of the RLOD study on pet food and animal feed for the RAPID'Salmonella method.**

Matrix/strain pair	Test portion	RLOD	RLODL	RLODU	b = ln (RLOD)	sdb	z-Test statistic	p-value	AL
Dry kibble dog food	375 g	1.000	0.434	2.304	0.000	0.417	0.000	1.000	2.5
S. Derby Ad1878									
<b>Combined</b>		<b>0.949</b>	<b>0.751</b>	<b>1.239</b>	<b>-0.036</b>	<b>0.125</b>	<b>0.289</b>	<b>1.277</b>	<b>/</b>

The RLOD found is equal to 1 which is inferior to the Acceptability Limit (2.5). According to the ISO16140-2 calculation, this result indicates that the RLOD for dry dog kibble is conform. It corresponds to the detection level at 50% (LOD<sub>50</sub>) of 0.4 ± 0.2 CFU/ sample for both the RAPID'*Salmonella* method and the reference method.

## Significance

These data show that both the iQ-Check *Salmonella* II and the RAPID'*Salmonella* methods can be used on pet food and animal feed with performance statistically equivalent to the ISO 6579-1 method. The AFNOR validation of both alternative methods has been successfully extended for animal feed and pet food.

## References

- Löfström et al., Rapid and Specific Detection of *Salmonella* spp. in Animal Feed Samples by PCR after Culture Enrichment. Appl Environ Microbiol 2004 Jan; 70(1): 69–75
- Finley et al., Human health implications of *Salmonella*-contaminated natural pet treats and raw pet food. Clin Infect Dis 2006 Mar 1;42(5):686–91
- ISO 16140-2:2016-Microbiology of the food chain — Method validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method
- ISO 6579-1:2017 Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of *Salmonella* — Part 1: Detection of *Salmonella* spp.

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