# ISO 16140-2 Validation of the Hygiena® foodproof® *Salmonella* plus *Cronobacter* Detection LyoKit for Infant Formula & Production Environmental Samples – Interlaboratory Study Results

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## INTRODUCTION:

Worldwide, *Salmonella* and *Cronobacter* species are considered the most harmful microorganisms in powdered infant formula. These types of bacteria pose serious health risks to infants. *Salmonella* can cause gastrointestinal illness, fever, and in severe cases, systemic infections. In infants, *Salmonella* infection can be particularly dangerous due to their underdeveloped immune systems. *Cronobacter* is less common but more dangerous for newborns. It can cause severe infections such as meningitis, sepsis, and necrotizing enterocolitis, often with high fatality rates in neonates. Due to their ability to adapt to dryness, these two pathogens can survive in dry foods even throughout the desiccation or manufacturing process. Because powdered infant formula is intended for the most vulnerable population, regulatory agencies require screening for *Salmonella* and *Cronobacter* species throughout the entire production process and in the final product.

## PURPOSE:

To obtain AFNOR certification for the foodproof® *Salmonella* plus *Cronobacter* Detection LyoKit, a collaborative study was carried out as the second part of the validation study in comparison to ISO 6579-1:2017 and ISO 22964:2017 standards according to the requirements of DIN EN ISO 16140-2:2016 and the AFNOR technical rules.

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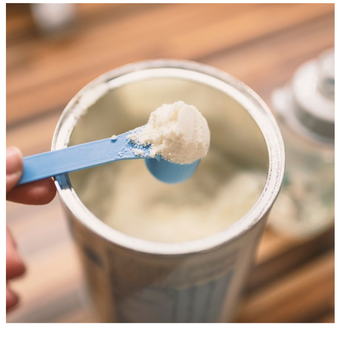
## METHOD:

For each test parameter of the PCR kit, one interlaboratory study was organized by ADRIA Développement. 16 sets of 24 blind-coded samples were prepared per collaborative study. Each set of test portions included 8 uninoculated, 8 low-level inoculated and 8 high-level inoculated samples. For *Salmonella* spp. detection, 25 g test portions and for *Cronobacter* spp. detection, 10 g test portions of powdered infant formula with probiotics were analyzed by 16 participants. Samples were enriched in buffered peptone water (1:10 dilution) with 10 mg/L vancomycin and incubated for 16 to 20 hours at 37 °C. Following incubation, DNA extraction was performed using the foodproof StarPrep® Three Kit or the BAX® Prep Gram-Negative Lysis Kit, followed by real-time PCR analysis using a BAX System Q7 or a Roche LightCycler 480 II instrument. Confirmation of PCR results was performed according to the respective ISO reference method (ISO 6579-1 or ISO 22964).



## INTERLABORATORY STUDY FOR *SALMONELLA* spp. DETECTION:

In preparation for the interlaboratory study for the detection of *Salmonella* spp., test portions of powdered infant formula containing probiotics were artificially contaminated with a lyophilized strain of *Salmonella* Anatum and co-inoculated with a *Cronobacter* spp. strain. The estimated inoculation levels for *Salmonella* were 1.0 CFU/test portion for the low-level inoculated samples and 3.3 CFU/test portion for the high-level inoculated samples. The study followed an unpaired study design, as different enrichment media were used for alternative and reference methods. For the candidate method, BPW supplemented with vancomycin was used, while for the ISO 6579-1:2017 reference method, samples were enriched in double-strength BPW. Eleven out of 16 participants provided valid data sets that fulfilled the requirements of the ISO 16140-2:2016 standard. Fractional positive results were only obtained for the low inoculation level (L1). The data generated for L1 was used for data interpretation according to ISO 16140-2 calculation rules for an unpaired study design.

**Table 1.** Data Generated During the Collaborative Study for *Salmonella* Detection

| Inoculation Level | PA | NA | PD | ND | $N_x$ | ND-PD | AL | Conclusion |
|---|---|---|---|---|---|---|---|---|
| L1 - low inoculation level - 1.0 CFU/test portion | 14 | 29 | 28 | 17 | 88 | -11 | 11.41 | PASSED |

**PA:** number of positive results obtained with both the alternative and the reference method; **NA:** number of negative results obtained with both the alternative and the reference method; **PD:** number of obtained results that are positive with the alternative method and negative with the reference method **ND:** number of obtained results that are negative with the alternative method and positive with the reference method; **$N_x$:** total number of results; **AL:** Acceptability Limit.

The difference between negative deviation (ND) and positive deviation (PD) is a value that is used to evaluate the performance of the candidate method compared to the reference method. According to ISO 16140-2, the observed value for (ND-PD) must not be higher than the acceptability limit (AL). The value obtained for ND-PD remains below the AL, fulfilling the requirements of the ISO 16140-2 standard.

## INTERLABORATORY STUDY FOR *CRONOBACTER* spp. DETECTION:

Test portions of powdered infant formula with probiotics were inoculated with a lyophilized *Cronobacter* sakazakii strain isolated from milk powder. The estimated inoculation levels were 0.5 CFU/test portion for the low-level inoculated samples and 1.3 CFU/test portion for the high-level inoculated samples. A paired study design was used since for both the candidate and ISO 22964:2017 reference method, BPW supplemented with vancomycin was used to enrich the samples. Ten out of 16 collaborators provided data sets that complied with the ISO 16140-2 standard requirements. The data generated for inoculation levels L1 and L2 was used as the basis for calculation since fractional recovery was observed for both inoculation levels.

**Table 2.** Data Generated During the Collaborative Study for *Cronobacter* Detection

| Inoculation Level | PA | NA | PD | ND | $N_x$ | ND-PD | ND+PD | AL (ND-PD) | AL (ND+PD) | Conclusion |
|---|---|---|---|---|---|---|---|---|---|---|
| L1 - low inoculation level 0.5 CFU/test portion | 36 | 43 | 0 | 1 | 80 | 1 | 1 | 3 | 4 | PASSED |
| L2 - high inoculation level 1.3 CFU/test portion | 36 | 44 | 0 | 0 | 80 | 0 | 0 | 3 | 4 | |
| **TOTAL** | **72** | **87** | **0** | **1** | **160** | **1** | **1** | **3** | **4** | |

**PA:** number of positive results obtained with both the alternative and the reference method; **NA:** number of negative results obtained with both the alternative and the reference method; **PD:** number of obtained results that are positive with the alternative method and negative with the reference method **ND:** number of obtained results that are negative with the alternative method and positive with the reference method; **$N_x$:** total number of results; **AL:** Acceptability Limit.

For a paired study design, the values obtained for (ND-PD) and (ND+PD) must remain below the acceptability limits (AL). The candidate method for *Cronobacter* spp. detection also fulfills the requirements of the ISO 16140-2 standard.

## SIGNIFICANCE:

With the foodproof *Salmonella* plus *Cronobacter* Detection LyoKit, Hygiena offers the first certified method on the market for the simultaneous detection of *Salmonella* spp. and *Cronobacter* spp. from a single enrichment culture. Based on the results of the entire validation study according to ISO standard 16140-2, the assay met all requirements compared to ISO reference methodologies to receive AFNOR certification (QUA 18/12-12/24 and QUA 18/13-12/24) for infant formula with and without probiotics and related ingredients for test portions up to 375 g and production environmental samples. This method offers the infant formula industry a rapid, reliable and easy-to-use PCR-based technology. By combining the detection of *Salmonella* spp. and *Cronobacter* spp. into one real-time PCR reaction, testing time and costs are significantly reduced. Providing different options for DNA extraction and different real-time PCR platforms gives the end user a high degree of flexibility in selecting a method that meets their individual requirements.