Study Report

Thermo Scientific SureTect Cronobacter species Assay (using the Thermo Scientific PikoReal Real-Time PCR instrument) NF VALIDATION ISO 16140: Method Comparison Study

Jonathan Cloke, Thermo Fisher Scientific, Wade Road, Basingstoke, Hampshire, RG24 8PW, UK

Method Comparison Study

A method comparison study was conducted by the AFNOR Certification Expert laboratory, ADRIA Développement, Quimper, France as part of the NF VALIDATION™ ISO 16140 for the Thermo Scientific™ SureTect™ Cronobacter species Assay. This report presents the results from both the expert and collaborative laboratory studies conducted to validate the performance of this SureTect assay method using a range of artificially contaminated powdered infant formula and production environment samples. Following the initial expert laboratory study, the final phase of the validation involved a collaborative study. This was conducted with a matrix of spiked dried infant formula which was analyzed by 15 independent laboratories across eight European countries.

Methodology

Expert laboratory study: A total of 128 samples (64 from powdered infant formula with and without probiotic supplementation and 64 production environment samples) were analyzed. Samples were artificially spiked using both seeding and spiking protocols as detailed in ISO 16140:2015. Powdered infant formula was seeded with different *Cronobacter* isolates at levels of 0.2-5.7 CFU/10g and stored for 1-2 weeks at ambient temperature. Additionally powdered infant formula was spiked by adding heat injured *Cronobacter* isolates directly to the prepared sample/enrichment broth before incubation. Production environment samples were spiked by adding a heat or pH stressed inoculum directly to samples or lyophilised cultures were prepared and added directly to dried environmental samples, such as factory dust samples.

Collaborative laboratory study: Ten gram samples of a powdered infant formula were spiked with *Cronobacter sakazakii* Ad 940, at low and high inoculum levels. Samples were then aliquoted to produce eight samples per spiking level for each of the participating collaborative laboratories. The actual spiking levels before contamination of the matrix were determined by the expert laboratory to be 1.0 (0.8-1.3) for the low level of contamination and 8.6 (7.0-10.7) CFU/10g for high levels of contamination. A further eight samples were prepared for each of the collaborative laboratories, which remained unspiked.

Upon receipt at each of the collaborative laboratories, half of the prepared samples were analyzed following the SureTect Assay method and the other half according to the ISO Technical Specification method detailed in ISO/TS 22964:2006. Statistically usable data was available from 11 of the 15 collaborative laboratories participating in the study.

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SureTect Assay Method: 10g samples of powdered infant formula were homogenized with 90ml of BPW (ISO) and incubated at 37 ±1°C for 16-20h. For production environment samples, 25g of solid samples or wipes were added to 225ml, swabs were added to 10ml and sponges were added to 100ml BPW (ISO) supplemented with 6mg/l vancomycin and incubated at 37°C for 18-22h. For PCR analysis, 10µl of SureTect Proteinase K reagent was added to each of the prefilled SureTect Lysis Tubes before adding 10µl of the enrichments to the Lysis Tubes, which were then heated at 37 ±1°C for 10 minutes, followed by 95 ±1°C for 5 minutes. The tubes were cooled by leaving in a rack at room temperature and 20µl aliquots of the lysates were transferred to SureTect PCR Tubes containing PCR tablets and then immediately transferred to the Thermo Scientific™ SureTect™ PikoReal™ Real-time PCR Instrument for processing. All samples, regardless of the PCR result were confirmed by plating 10µl of the BPW enrichments onto Chromogenic Cronobacter Isolation (CCI) Agar, which was incubated at 41.5 ±1°C for 22-26h and any presumptive positive blue-green colonies were confirmed using a biochemical micro-gallery kit.

ISO Reference Method: 10g of each sample were analysed using the method detailed in ISO/TS 22964:2006. Each sample was enriched in 90ml of BPW (ISO) and incubated at 37 ±1°C for 16-20h. Following incubation, 100µl of the enrichment was sub-cultured into 10ml modified Lauryl Sulphate Tryptone Broth (mLST) supplemented with 10mg/l vancomycin and incubated for 22-26 h at 44 ±0.5°C. 10µl of the mLST Broth enrichment was then streaked across the surface of a plate of Enterobacter sakazakii Isolation Agar, which was incubated at 44 ±1°C for 22-26 hours. Presumptive positive, blue-green colonies were streaked to Tryptone Soya Agar, which was incubated at 25 ±1°C for 44-48 h. Yellow colonies on TSA were confirmed by oxidase and biochemical identification tests as *Cronobacter* species.

Results

Expert Laboratory: The SureTect Cronobacter species PCR Assay method was shown to be a reliable alternative to the ISO/TS method for the detection of *Cronobacter* species from both powdered infant formula (with and without probiotics) and production environment samples during the expert laboratory phase of the AFNOR Certification ISO 16140 validation study.

Table 1: NF VALIDATION ISO 16140 Confirmed Results for the SureTect Cronobacter species Assay method: Powdered Infant Formula

	ISO/TS 22964:2006 positive results	ISO/TS 22964:2006 negative results
SureTect Assay method positive results	30	0
SureTect Assay method negative results	0	34

Table 2: NF VALIDATION ISO 16140 Confirmed Results for the SureTect Cronobacter species Assay method: Production Environment Samples

	ISO/TS 22964:2006 positive results	ISO/TS 22964:2006 negative results
SureTect Assay method positive results	23	5
SureTect Assay method negative results	3	33

There were no discordant results during the analysis of powdered infant formula. When testing production environment samples, there were five SureTect Assay method positive and ISO method negative discordant results. All five of these samples gave a positive result with the SureTect Assay method and were confirmed by culture, whereas the ISO reference method incorrectly gave a negative result. Additionally, there were three SureTect Assay method negative and ISO method positive discordant results for the environmental category. Further analysis of the enrichments processed for the SureTect Assay method failed to recover any *Cronobacter* from the samples. It is most likely that the spiking level in these samples was too low (at 0.2-2 CFU/sample) and no cells were spiked into these three samples.

According to the AFNOR Certification rules and ISO 16140:2015, statistical analysis of the expert laboratory study was undertaken to determine the sensitivity of the reference and alternative methods and the accuracy and false positive ratio during the study. The SureTect Assay and ISO reference methods demonstrated a sensitivity of 100% for all types of powdered infant formula. For production environmental samples, the sensitivity of the SureTect Assay method was demonstrated to be statistically better than the ISO reference method detailed in ISO/TS 22964:2006, as the sensitivity of the SureTect Assay method was shown to be 90.3%, compared to 83.9% for the ISO method.

Table 3: Statistical Analysis of the NF VALIDATION ISO 16140 Study

Category	Type	SureTect Method Sensitivity	ISO Reference Method Sensitivity	Study Accuracy	Study False Positive Ratio
Powdered infant formula	Unsupplemented without "probiotics"	100%	100%	100%	0.0%
	Supplemented with "probiotics"	100%	100%	100%	10.5%
	Total for category	100%	100%	100%	5.6%
Production environment samples	Process & cleaning water	100%	71.4%	88.2%	16.7%
	Dust and wastes	87.5%	87.5%	88.9%	0.0%
	Wipes, sponges & swabs	87.5%	87.5%	86.2%	20.0%
	Total for category	90.3%	83.9%	87.5%	11.1%
Total for all categories		95.1%	91.8%	94.1%	7.9%

Collaborative laboratory study

The collaborative study, performance according to the AFNOR Certification rules and the method detailed in the ISO Technical Specification, demonstrated that the SureTect Cronobacter species Assay method, reliably detected the *Cronobacter sakazakii* isolate from samples at both of the spiking levels evaluated. The acceptability limits during the study were determined to meet the requirements for alternative methods as detailed in ISO 16140:2015 (Tables 4 and 5), since the levels are both below the acceptability limits for a total of 11 laboratories with useable data.

Table 4: Comparison of the SureTect and ISO Method Results: Collaborative Study

	ISO/TS 22964:2006 positive results	ISO/TS 22964:2006 negative results
SureTect Assay method positive results	142	1
SureTect Assay method negative results	1	120

Table 5: Observed Acceptability Criteria for the SureTect Cronobacter species Assay

Observed Acceptability Criteria	ISO 16140:2015 Acceptability Limits
ND[1] – PD[2] = 1 – 1 = 0	4[3]
ND + PD = 1 + 1 = 2	4

[1]ND: Negative Deviations, [2]Positive Deviations, [3]Acceptabiltiy limits for 11 laboratories.

Conclusions

The results gained during this NF VALIDATION from AFNOR Certification validation study demonstrate that the SureTect Cronobacter species PCR Assay method has better performance than the reference method detailed in ISO/TS 22964:2006 for the detection of *Cronobacter* spp. from powdered infant formula (with and without probiotics) and production environmental samples. Where confirmation of a positive PCR result is required, this is easily performed by plating a 10µl loopful of the BPW enrichment onto Chromogenic Cronobacter Isolation (CCI) Agar and confirming any presumptive positive colonies with a biochemical micro-gallery kit. The ISO 16140 validation certificate and the official expert laboratory summary of this study are available from http://nf-validation.afnor.org/en/.

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Contact Information:
microbiology@thermofisher.com
USA +1 800 255 6730
International +44 (0) 1256 841144

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