STUDY REPORT

SureTect Cronobacter species PCR Assay Workflow NF VALIDATION ISO 16140 – Extension study: Method Comparison

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Summary

As part of the NF VALIDATION™ ISO 16140 extension study of the Thermo Scientific™ SureTect™ Cronobacter species PCR Assay workflow (alternative method), a method comparison study was conducted by ADRIA Développement, Quimper, France. The alternative method has previously been validated for 10 g powdered Infant Formula (PIF) samples and production environment samples with the Thermo Scientific™ SureTect™ PikoReal™ Real-Time PCR Instrument with Thermo Scientific™ SureTect™ version 1.2 software. The extension study was designed to validate the use of the alternative method with the SureTect PikoReal Instrument software for 300 g PIF, and the Applied Biosystems™ 7500 Fast Real-Time PCR System and Applied Biosystems™ Rapid Finder™ Express version 2.0 software for 10 g and 300 g PIF, and production environment samples.

This report presents the results from the expert laboratory study conducted to validate the performance of the alternative method using a range of artificially contaminated PIF and production environment samples.

Methodology

Expert laboratory study

A total of two-hundred and ten samples (64 10 g PIF, 77 300 g PIF, and 69 production environment samples) were analyzed during the expert laboratory study which was designed to validate the use of the alternative method using the SureTect PikoReal Instrument and the Applied Biosystems 7500 Fast System. A total of 89 samples were artificially contaminated by seeding and 65 samples by spiking protocols.

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Alternative method

- The 10 g PIF samples were homogenized with 90 ml of Thermo Scientific™ Buffered Peptone Water (BPW) (ISO) (Thermo Fisher Scientific) and enriched by incubating at 37±1 °C for 16-20 hours.

- The 300 g PIF samples were homogenized with 2700 ml of BPW (ISO) supplemented with 6 mg/ml vancomycin and enriched by incubating at 37±1 °C for 20-24 hours.

- For production environment samples, 25 g of process water samples were added to 225 ml, for surface sampling 1 wipe was added to 225 ml, 1 swab was added to 10 ml, and 1 sponge was added to 100 ml BPW (ISO) supplemented with 6mg/l vancomycin and enriched by incubating at 37±1 °C for 18-22 hours.

For PCR analysis, 10 µl of SureTect Proteinase K reagent were added to the required number of SureTect Lysis Tubes (supplied pre-filled with Lysis Reagent 1) before adding 10 µl 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 for around 2 minutes and 20 µl aliquots of the lysates were transferred to SureTect PCR Tubes containing SureTect *Cronobacter* spp. PCR tablets.

When performing PCR using the 7500 Fast System, a negative control sample was prepared by adding 10 µl sterile nuclease free water (or sterile medium) to a SureTect Lysis Tube, instead of the enriched sample.

The SureTect PCR Tubes were then immediately transferred to the SureTect PikoReal Instrument or the Applied Biosystems 7500 Fast System for processing.

All samples, regardless of the PCR result were confirmed by plating 10 µl of the BPW enrichments onto Thermo Scientific™ Chromogenic Cronobacter Isolation (CCI) Agar (Thermo Fisher Scientific), which was incubated at 41.5±1 °C for 22-26 hours. Any presumptive positive blue-green colonies were confirmed using Thermo Scientific™ Microbact™ GNB 24E (Thermo Fisher Scientific) biochemical micro-gallery kit.

ISO reference method

Ten grams of PIF and production environment samples were analysed using the method detailed in ISO/TS 22964:2006. Each sample was homogenized with 90 ml BPW (ISO) and enriched by incubating at 37±1 °C for 16-20 hours. Following incubation, 100 µl of the enrichment were sub-cultured into 10 ml modified Lauryl Sulphate Tryptone Broth (mLST) supplemented with 10 mg/l vancomycin and incubated for 22-26 hours at 44±0.5 °C. Ten microlitres of the mLST Broth enrichment were then streaked across the surface of a plate of Enterobacter sakazakii Isolation Agar (ESIA), which was incubated at 44±1 °C for 22-26 hours. Presumptive positive, blue-green colonies were streaked to Tryptone Soya Agar (TSA), which was incubated at 25±1 °C for 44-48 hours. Yellow colonies on TSA were confirmed by oxidase test and Microbact 24E biochemical micro-gallery kit.

Alternative method: Protocol used for PIF and production environment samples using the SureTect PikoReal Instrument

Day: 0

PIM samples:

- Add 10 g PIF samples to 90 ml BPW (ISO)
  Incubate at 37±1 °C for 16-20 hours
- Add 300 g PIF samples with 2700 ml BPW (ISO) + 6 mg/l vancomycin
  Incubate at 37±1 °C for 20-24 hours

Production environment samples:

- Add 1 swab to 10 ml BPW (ISO) + 6 mg/l vancomycin
  Incubate at 37±1 °C for 18-22 hours
- Add 1 sponge to 100 ml of BPW (ISO) + 6 mg/l vancomycin
  Incubate at 37±1 °C for 18-22 hours
- Add 25 g process water/ 1 wipe to 225 ml BPW (ISO) + 6 mg/l vancomycin
  Incubate at 37±1 °C for 18-22 hours

Day: 1

Remove ~1 ml of enrichment to a sterile sealable tube

First add 10 µl of SureTect Proteinase K to each required SureTect Lysis Tube

Second, add 10 µl enriched sample to the SureTect Lysis Tube

Incubate SureTect Lysis Tubes at 37±1 °C for 10 minutes followed by 95±1 °C for 5 minutes

Allow lysates to cool at room temperature for at least 2 minutes, then transfer 20 µl to SureTect PCR Tubes

Load SureTect PCR Tubes to the SureTect PikoReal Instrument and start PCR, review the results at end of run (approx. 80 minutes)

Report negative results

Confirm PCR positives by plating 10 µl of the BPW enrichments onto CCI Agar
Incubate at 41.5±1 °C for 22-26 hours

Confirm PCR positives by subculturing 100 µl of the BPW enrichments into 10 ml CSB
Incubate at 41.5±1 °C for 22-26 hours

Plate 10 µl of the CSB enrichment onto CCI Agar
Incubate at 41.5±1 °C for 22-26 hours

Day: 2 or 3

Any presumptive positive blue-green colonies were confirmed by an oxidase test and Microbact 24E biochemical micro-gallery kit

Alternative method: Protocol used for PIF and production environment samples using the Applied Biosystems 7500 Fast System

Day: 0

PIM samples:

- Add 10 g PIF samples to 90 ml BPW (ISO)
  Incubate at 37±1 °C for 16-20 hours
- Add 300 g PIF samples with 2700 ml BPW (ISO) + 6 mg/l vancomycin
  Incubate at 37±1 °C for 20-24 hours

Production environment samples:

- Add 1 swab to 10 ml BPW (ISO) + 6 mg/l vancomycin
  Incubate at 37±1 °C for 18-22 hours
- Add 1 sponge to 100 ml of BPW (ISO) + 6 mg/l vancomycin
  Incubate at 37±1 °C for 18-22 hours
- Add 25 g process water/1 wipe to 225 ml BPW (ISO) + 6 mg/l vancomycin
  Incubate at 37±1 °C for 18-22 hours

Day: 1

Remove ~1 ml of enrichment to a sterile sealable tube

First add 10 µl of SureTect Proteinase K to each required SureTect Lysis Tube

Second, add 10 µl enriched sample to the SureTect Lysis Tube. A negative control sample was prepared by adding 10 µl sterile nuclease free water to a SureTect Lysis Tube

Incubate SureTect Lysis Tubes at 37±1 °C for 10 minutes followed by 95±1 °C for 5 minutes

Allow lysates to cool at room temperature for at least 2 minutes, then transfer 20 µl to SureTect PCR Tubes

Load SureTect PCR Tubes to the Applied Biosystems 7500 Fast System and start PCR, review the results at end of run (approx. 80 minutes)

Report negative results

Confirm PCR positives by plating 10 µl of the BPW enrichments onto CCI Agar
Incubate at 41.5±1 °C for 22-26 hours

Confirm PCR positives by subculturing 100 µl of the BPW enrichments into 10 ml CSB
Incubate at 41.5±1 °C for 22-26 hours

Plate 10 µl of the CSB enrichment onto Chromogenic Cronobacter Isolation (CCI) Agar
Incubate at 41.5±1 °C for 22-26 hours

Day: 2 or 3

Any presumptive positive blue-green colonies were confirmed by an oxidase test and Microbact 24E biochemical micro-gallery kit

ISO reference method: Protocol used for PIF and production environment samples

Day: 0

Add 10 g of sample to 90 ml of BPW (ISO)

Incubate at 37 °C for 16–20 hours

Day: 1

Add 0.1 ml of the enrichment to 10 ml of mLST + 10 mg/l vancomycin

Incubate at 44±0.5 °C for 22-26 hours

Day: 3

Streak 10 µl of the mLST+ 10 mg/l vancomycin enrichment onto ESIA

Incubate at 44±1 °C for 22-26 hours

Day: 4-6

Presumptive positive, blue-green colonies were streaked onto TSA

Incubated at 25±1 °C for 44-48 hours

Yellow colonies on TSA were confirmed by oxidase test and
Microbact GNB 24E biochemical micro-gallery kits

Results

Expert Laboratory

The alternative method was shown to be a reliable alternative to the ISO reference method for the detection of *Cronobacter* spp. from PIF (10 and 300 g sample size), and production environment samples, using both the SureTect PikoReal Instrument and the Applied Biosystems 7500 Fast System during the expert laboratory phase of the AFNOR Certification ISO 16140 validation study.

Tables 1, 2 and 3 show the confirmed results for the ISO reference method and the alternative method using the SureTect PikoReal Instrument and Applied Biosystems 7500 Fast System for 10 g PIF, 300 g PIF and production samples respectively.

Table 1: NF VALIDATION ISO 16140 extension study confirmed results for the ISO reference method and the alternative method when testing PIF 10 g sample size

		ISO reference method positive results	ISO reference method negative results
SureTect PikoReal Instrument (initial validation)	Alternative method positive results	30	0
	Alternative method negative results	0	34
Applied Biosystems 7500 Fast System (extension study)	Alternative method positive results	30	0
	Alternative method negative results	0	34

Table 2: NF VALIDATION ISO 16140 extension study confirmed results for the ISO reference method and the alternative method when testing: PIF 300 g sample size

		ISO reference method positive results	ISO reference method negative results
SureTect PikoReal Instrument (initial validation)	Alternative method positive results	22	10
	Alternative method negative results	5	40
Applied Biosystems 7500 Fast System (extension study)	Alternative method positive results	22	10
	Alternative method negative results	5	40

Table 3: NF VALIDATION ISO 16140 extension study confirmed results for the ISO reference method and the alternative method when testing production samples

		ISO reference method positive results	ISO reference method negative results
SureTect PikoReal Instrument (initial validation)	Alternative method positive results	23	5
	Alternative method negative results	3	38
Applied Biosystems 7500 Fast System (extension study)	Alternative method positive results	23	5
	Alternative method negative results	3	38

During the expert laboratory study 15 positive deviations (PD) and 8 negative deviations (ND) were observed. All 15 PD samples were confirmed via the culture method, indicating the PCR result is correct. For the 8 ND samples, no *Cronobacter* spp. were recovered from the corresponding samples of the alternative method.

Due to the low spike level, it is likely that no cells were spiked into these samples, and as the study is unpaired, natural variation can occur. The statistical analysis of the relative accuracy of the alternative method is shown below in tables 4 and 5.

Table 4: Statistical analysis of the NF VALIDATION ISO 16140 extension study when using the SureTect PikoReal Instrument

Category	Type	Alternative Method Sensitivity	ISO Reference Method Sensitivity	Study Accuracy	Study False Positive Ratio
PIF (10 g) (initial validation)	Unsupplemented without probiotics	100%	100%	100%	0.0%
	Supplemented with probiotics	100%	100%	100%	11.8%
	Total for category	100%	100%	100%	5.9%
PIF (300 g) (initial validation)	Unsupplemented without probiotics	81.0%	71.4%	77.8%	4.3%
	Supplemented with probiotics	93.8%	75.0%	84.4%	14.3%
	Total for category	86.5%	73.0%	80.5%	2.6%
Production environment samples (initial validation)	Process & cleaning water	100%	71.4%	90.0%	8.3%
	Dust and wastes	87.5%	87.5%	90.0%	0.0%
	Wipes, sponges & swabs	87.5%	87.5%	86.2%	16.7%
	Total for category	90.3%	83.9%	88.4%	7.9%
Total for all categories		91.8%	84.7%	89.0%	2.7%

Table 5: Statistical analysis of the NF VALIDATION ISO 16140 extension study when using the Applied Biosystems 7500 Fast System

Category	Type	Alternative Method Sensitivity	ISO Reference Method Sensitivity	Study Accuracy	Study False Positive Ratio
PIF (10 g) (initial validation)	Unsupplemented without probiotics	100%	100%	100%	0.0%
	Supplemented with probiotics	100%	100%	100%	11.8%
	Total for category	100%	100%	100%	5.9%
PIF (300 g) (initial validation)	Unsupplemented without probiotics	81.0%	71.4%	77.8%	0.0%
	Supplemented with probiotics	93.8%	75.0%	84.4%	6.7%
	Total for category	86.5%	73.0%	80.5%	2.6%
Production environment samples (extension study)	Process & cleaning water	100%	71.4%	90.0%	0.0%
	Dust and wastes	87.5%	87.5%	90.0%	0.0%
	Wipes, sponges & swabs	87.5%	87.5%	86.2%	0.0%
	Total for category	90.3%	83.9%	88.4%	0.0%
Total for all categories		91.8%	84.7%	89.0%	2.7%

Conclusion

The results gained during this NF VALIDATION from AFNOR Certification validation study demonstrate that the SureTect Cronobacter species PCR Assay workflow showed equivalent or improved sensitivity performance to the reference method detailed in ISO/TS 22964:2006 for the detection of *Cronobacter* spp. from PIF (with and without probiotics) (10 g and 300 g sample size) and production environmental samples. Where confirmation of a positive PCR result is required, this is easily performed by plating 10 µl 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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