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Performance Summary

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Technology Platform:
3M™ Molecular Detection Assay 2
Originating Location: St. Paul, MN

3M™ Molecular Detection Assay 2 - *Cronobacter*

The 3M™ Molecular Detection Assay 2 – *Cronobacter* was developed for the rapid and specific detection of *Cronobacter* in powdered infant formula (PIF), raw materials and supplements utilized in PIF formulations and environmental samples after enrichment in Buffered Peptone Water (BPW) ISO. The assay uses loop-mediated isothermal amplification to rapidly amplify unique DNA nucleic acid sequences with high specificity and sensitivity, combined with bioluminescence to detect the amplification.

Performance of the 3M method was evaluated for inclusivity, exclusivity and performance compared to the ISO 22964 reference method with a wide range of samples. The limit of detection of the method and of the assay were also determined.

Inclusivity and Exclusivity Testing

- Inclusivity: The ability of the method to detect the target analyte from a wide range of *Cronobacter* strains. One hundred and six (106) *Cronobacter* isolates were tested. The strains were cultured in BPW ISO overnight at 37 $\pm$ 1 °C and diluted to ~10^5 -10^6 CFU/mL. Cultures were tested according to AOAC guidelines (Table 1 and Appendix Table A2).

- Exclusivity: The lack of interference from a relevant range of non-target strains. One hundred and two (102) different non-*Cronobacter* stains including closely related organisms were tested. The strains were cultured in BPW ISO and then diluted to levels of ~10^7 CFU/mL prior to testing with the 3M method (Table 1 and Appendix Table A3).

Table 1. Inclusivity and exclusivity results

3M Molecular Detection Assay 2 – *Cronobacter* **Results**	**Analysis**
106/106 *Cronobacter* strains were detected (results were "positive")	100% Inclusivity
102/102 non-*Cronobacter* strains were not detected (results were "negative")	100% Exclusivity

*See Appendix, Tables A2 and A3, for list of cultures tested.

Internal Methods Comparison Study

Studies were conducted to assess the performance of the 3M method compared to ISO 22964 as a reference method for the detection *Cronobacter spp.* Matrices that are commonly tested for the presence of *Cronobacter* were included. Five hundred and seventy four (574) samples were evaluated (See appendix, Table A1). Portions of 10-375 g were analyzed according to the 3M method product instructions. Matrices with a sample size of 10 g (n=319 samples) were evaluated as paired samples within the 3M method and the ISO method. Matrices with samples size larger than 10 g and for 10 g samples that required a 1:100 dilution (minerals) were evaluated as non-paired samples, with one portion analyzed by the 3M method and one analyzed by the ISO method (n=255 samples).

Results from the paired study, comparing 3M method performance with a sample size of 10 g, were analyzed according to ISO 16140-2 (Figure 1).

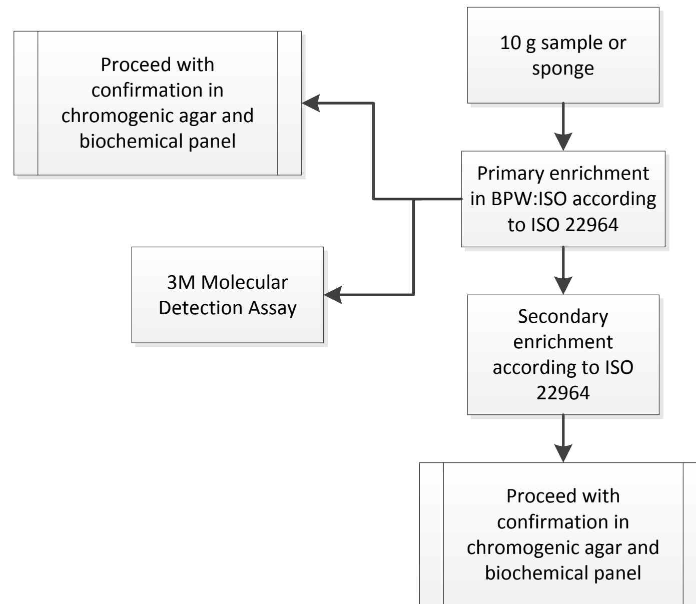

Figure 1. Paired study design to compare 3M Molecular Detection Assay 2 – *Cronobacter* with ISO 22964.

The difference between negative deviation (ND) and positive deviation (PD) was not significant differences between ISO 22964 and the 3M Molecular Detection Assay 2 - *Cronobacter* in all of the categories tested (Table 2). The ND+PD was significantly different for the environmental sampling category. *Cronobacter* was detected with the 3M method and further confirmed according to ISO 22964 (Table 2). In 12 out of the 190 environmental samples, confirmation was achieved only from the

BPW:ISO enrichment in agreement with the results obtained with the 3M Molecular Detection Assay 2 – *Cronobacter*, but not from the secondary enrichment (modified lauryl sulphate tryptose broth – mLST) described in the ISO 22964 method. The ND+PD was not significantly different for raw materials and cereals or for PIF sample. There was no observed interference as confirmed by valid results for all samples tested in parallel using the 3M™ Molecular Detection Matrix Control.

Table 2. Paired comparison between ISO 22964 and the 3M method with 10 g samples.

Category	Number of samples	Comparison of 3M Method to ISO method[a,b]
Powdered infant formula	66	Conforms with ISO standard
Raw materials and cereals	63	Conforms with ISO standard
Environmental samples	190	Does not conform with ISO standard (favors 3M method)[c]

[a]Statistical analysis was performed in accordance to ISO 16140-2:2015.

[b]Acceptance criterion for paired samples: No significant difference is established between methods when ND-PD $\leq$ 3 for individual categories and ND+PD $\leq$6 according to ISO 16140-2, where ND= number of samples with Negative Deviation results, and PD= number of samples with Positive Deviation results.

[c]*Cronobacter* was detected and confirmed from BPW:ISO enrichment but in 12 out of the 190 environmental samples it was not recovered from the mLST enrichment.

The unpaired study which involved the comparison of samples sizes larger than 10 g, including 30, 100, 300, 375 g and 10 g samples that required a 1:100 dilution were analyzed utilizing Chi Square (X^2) discordant analysis. There is not a significant difference between the ISO 22964 and the 3M Molecular Detection Assay 2 - *Cronobacter* in all the categories and sample sizes tested (Table 3). In addition, there was no observed interference as confirmed by valid results for all samples tested in parallel using the 3M Molecular Detection Matrix Control.

Table 3. Unpaired comparison between ISO 22964 and 3M method with samples larger than 10 g or at a dilution other than 1:10.

Category	Analysis[a] X^2 (ISO 22964)	Comparison of 3M Method to ISO method
PIF	0.0003	No statistical difference
Raw materials	0.0194	No statistical difference
Cereals	0.0087	No statistical difference
Environmental samples	NA	NA
All samples, n=255	*0.9020*	*No statistical difference*

NA, not applicable. Environmental samples were analyzed utilizing a paired method (Table 2)

[a]Acceptance criterion for paired samples: No significant difference is established between methods when X^2 less than 3.84.

Probability of Detection

The Probability of Detection (POD) is the proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. POD is concentration dependent may vary with different species, strains, samples and methods. POD measures can be calculated for the reference method results, the confirmed 3M results, the presumptive 3M results and the difference between any two POD values, called dPOD.

This part of the study was conducted by a third party accredited laboratory. Three hundred gram (300 g) samples were inoculated with a low level (0.2-2 CFU/sample) and with a high level (< 5 CFU/sample) of *Cronobacter*. Inoculated samples were stabilized for a period of two weeks. Samples were analyzed after 18 h of enrichment at 37±1 °C in BPW ISO. Results were confirmed according to ISO 22964. For the 3M method the lowest concentration detected was demonstrated to be 1-2 CFU/sample (Table 4) and the 3M method is within agreement with the ISO 22964 method when tested in samples inoculated with < 2 CFU/sample (Table 5).

Table 4. Third party laboratory data for the detection of *Cronobacter* in 300-g samples.

Powdered Infant Formula	**Presumptive positives[a] 3M method 300 g**	**Confirmed results[a] 3M method 300 g**	**Positives [a] ISO 22964 10 g**
Zero level samples (n=5)	0/5	0/15	0/5
High level samples (n=5)	5/5	5/5	5/5
Low level samples (n=20)	15/20	15/20	11/20

[a]Results represent the total number of positives/total number of analyzed samples

Table 5. Third party laboratory data for the detection of *Cronobacter* ISO 22964 and 3M method.

Powdered Infant Formula	**MPN/test portion (MPN range) for low (fractional) level**	**dPOD (95% CI) ISO 22964 / 3M method**	**p-value**	**Comparison of 3M Method (300 g) to ISO method (10 g)**
3M method Presumptive positives	1.0 (0.64, 1.77)	0.2 (-0.09, 0.45)	0.02	No statistical difference
3M method Confirmed positives		0 (-0.26, 0.26)	0.00	No statistical difference

The results on the table represent samples spiked with a low level of *Cronobacter*.
Difference in the probability of detection (dPOD)
Confidence interval (CI)
Acceptance criteria – dPOD CI must contain a 0.00 to indicate no statistical difference.

Assay Limit of Detection

The Assay Limit of Detection (LOD) can be determined by testing a range of known concentrations of a target organism. LOD may vary when considering sample matrix composition and strain variations. 3M internal validation studies to determine LOD were performed with five strains of *Cronobacter* (Table 6). The 3M Molecular Detection Assay 2 – *Cronobacter* was shown to have an LOD of approximately 10^3 CFU/mL in BPW ISO enrichment (Table 6).

Table 6. 3M Molecular Detection Assay 2 – *Cronobacter* assay limit of detection.

Microorganisms chosen for genetic diversity, isolation source, implication in outbreaks and type strain status	**LOD (CFU/mL)[a]**
C. sakazakii (ATCC BAA-894)	2.06×10^3
C. sakazakii (ATCC29544)	2.75×10^3
C. condimenti	4.75×10^3
C. dublinensis subsp. l*actaridi*	1.66×10^3
C. muytjensii	3.24×10^3
Average	2.89×10^3

[a]Results represent the average of results from three different manufacturing lots of the 3M Molecular Detection Assay 2 – *Cronobacter*.

References:

1. ISO 16140:2003 Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods.
2. ISO 16140-2:2015 Microbiology of the food chain – Method validation – Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method.
3. ISO 22964 Milk and milk products. Detection of *Enterobacter sakazakii.*
4. 3M Molecular Detection Assay 2 – *Cronobacter* Product Instructions.
5. AOAC International, 2012. Method Committee Guidelines for the Validation of Microbiological Methods for Food and Environmental Surfaces

3M Food Safety
3M Center
Building 275-5W-05
St. Paul, MN 55144-1000 USA

1-800-328-6553
www.3M.com/foodsafety

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Appendix

Table A1. Matrices tested included in the method comparison study.

Category	Matrices included	Description	
Raw materials N=138 samples	Vitamins and fatty acids	Multivitamin (cocktail) drops Multivitamin cocktail (powder) Multivitamin drops with docosahexanoic acid (DHA) Multivitamin containing iron Vitamin D3 Vitamin A Palmitate	Vitamin B5 Vitamin B2 Vitamin B12 Vitamin C Vitamin B12 Taurine
	Salts and minerals	Magnesium citrate Ferrous sulfate Zinc sulfate Potassium citrate Copper sulfate	Calcium carbonate Magnesium oxide Manganese sulfate monohydrate
	Oils	Coconut Sunflower	Coconut (labeled as organic) Sunflower (labeled as organic)
	Flours	Whole ground soy Arrowroot starch	
	Probiotics	Cereals with probiotics Yogurt culture starter Probiotic complex tablets Infant probiotic powder (various formulations)	Probiotics included: • *Bifidobacterium lactis, B. breve, B. bifidum, B. infantis, B. longum* • *Lactobacillus reuteri*, *L. fermentum, L. acidophilus, L. salvarius, L. helveticus, L. casei, L. bulgaricus*, *L. plantarum* and *L. rhamnosus* (including GG)
	Carbohydrates	Maltodextrin Lactose	Corn syrup solids Dextrose
	Protein	Whey protein isolate Dry milk powder	Nonfat dry milk powder Soy protein isolate
Cereals N=84 samples	Baby cereals	Brown rice flakes Whole grain rice flakes Brown Rice (labeled as organic) Rice (containing fruit) Cereal containing probiotics Rice with milk Rice with DHA and probiotics	Quinoa flakes Whole grain buckwheat flakes Buckwheat flakes Whole grain oatmeal Oat flakes Oatmeal (labeled as organic) Baby cereal (labeled as organic) Wheat with milk

Table A1 (continuation). Matrices tested included in the method comparison study.

Category	Matrices included	Description
Environmental N=190 samples	Sponges and dry samples	Surface samples collected using 3M™ Sponge–Stick with D/E broth Dry samples from vacuum or dust collection
Infant formula N=162 samples	Powdered infant formula	Formulations included: soy, dairy and corn syrup-based. Types included (according to manufacturer's label): organic, gentle/soothe, sensitive, supplemented with iron, hypoallergenic, with added rice starch, with probiotics, without probiotics, for neonates.

Table A2. Panel for inclusivity strains

Inclusive strains N=106	Number of strains included
Cronobacter condimenti	1
Cronobacter dublinensis	4
Cronobacter malonaticus	18
Cronobacter muytjensii	1
Cronobacter sakazakii	80
Cronobacter turicensis	1
Cronobacter universalis	1

Table A3. Panel for exclusivity strains

	Family/group	Genus/species/serotype	
Exclusives N=102	*Alcaligenaceae*	*Alcaligenes faecalis*	
	Bacillaceae	*Bacillus atrophaeus* *Bacillus cereus* *Bacillus circulans*	*Bacillus licheniformis* *Bacillus pumilus* *Bacillus thuringiensis*
	Bifidobacteriales	*Bifidobacterium breve*	
	Debarymycetaceae	*Candida albicans*	
	Enterobacteriaceae	*Buttiauxella noackiae* *Citrobacter brakii* *Citrobacter freundii* *Citrobacter koseri* *Citrobacter sedlakii* *Enterobacter aerogenes* (3) *Enterobacter amnigenus* *Enterobacter asburiae* *Enterobacter cancerogenus* *Enterobacter cloacae* (2) *Enterobacter gergoviae* *Enterobacter hormaechei* *Enterobacter kobei* *Enterococcus gallinarum* *Enterococcus faecalis* *Enterococcus faecium* (2) *Enterococcus raffinosus* *Escherichia coli* (11) *Escherichia coli* O157:H7 (2)	*Escherichia hermanii* *Escherichia vulneris* *Franconibacter helveticus* (2) *Franconibacter pulveris* (2) *Klebsiella oxytoca* (6) *Klebsiella pneumoniae* (4) *Leclercia adecarboxylata* *Salmonella enterica* sv. Arizonae *Salmonella enterica* sv. Bareilly *S. enterica* sv. Diarizona *S. enterica* sv. Enteritidis *S. enterica* sv. Infantis *S. enterica* sv. Newport *S. enterica* sv. Typhimurium *S. entrica* sv. Virchow *Serratia marcescens* *Shigella flexneri* *Shigella sonnei* *Siccibacter turicensis* (2)
	Erwiniaceae	*Pantoea agglomerans*	
	Hafniaceae	*Hafnia alvei*	
	Lactobacillaceae	*Lactobacillus acidophilus* (2) *Lactobacillus brevis* *Lactobacillus buchneri* *Lactobacillus casei* *Lactobacillus curvatus*	*Lactobacillus fermentum* *Lactobacillus lactis* subsp. *lactis* *Lactobacillus plantarum* *Pediococcus acidilactici*
	Micrococcaceae	*Kocuria rosea*	
	Moraxellaceae	*Acinetobacter calcoaceticus*	
	Morganellaceae	*Morganella morganii* subsp. *morganii* *Proteus mirabilis*	*Proteus vulgaris* *Providencia rettgeri*
	Pseudomonadaceae	*Pseudomonas aeruginosa* *Pseudomonas fluoresencens*	
	Saccharomycetaceae	*Saccharomyces cerevisiae*	
	Streptococcaceae	*Lactococcus lactis* *Streptococcus salivarius*	*Streptococcus thermophilus*
	Yersiniaceae	*Yersinia enterocolitica* *Yersinia kristensenii*	*Yersinia pseudotuberculosis*

(Number of strains tested, if more than one was included)