Cronobacter Sakazakii ISO 22964:2017 Testing of Milk Powders Using Commercially Available PCR

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Introduction

The detection of *Cronobacter sakazakii* in milk powder is important as a major health issue[1] as it can survive for long periods in dry conditions[2]. Faster detection using PCR is possible and currently need to be compatible with traditionally standardised ISO methods.

Viable cells are detected by PCR when dead cells and free DNA are diluted out, inhibited or destroyed. Biorad IQ–check DNA removal solution eliminates the detection of dead cells (based on endonuclease activity) or Biotecon Reagent D, (a light reactive aqueous reagent solution) is a dye designed to eliminate dead bacterial cell amplification, both avoid false-positive PCR results from dead cells.

With ISO 20838: Real-Time PCR can be self-confirming and no further confirmation necessary with faster in house control and release of product quicker based on PCR results.

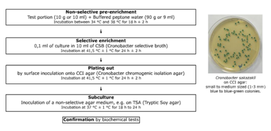
Objectives

The aim of this study was to test the ISO 22964:2017 method, along with PCR detection, to determine the sensitivity and interference from dead cells.

Materials and Methods

Sample preparation

To follow the ISO22964:2017 samples were prepared as follows. One loop from stock *Cronobacter sakazakii* ATCC 29004, ATCC 29544 was separately aseptically transferred to 5 ml of Buffer Peptone Water (BPW), incubated at 37 °C overnight to obtain a maximum cell concentration of ~log 9 cfu/ml and then diluted to -2, -1 and 1 log cfu/ml spiked to 10% milk powder (n=7 different milk powder sources) and 90% BPW, as in non selective enrichment step 1.



ISO 22964:2017 steps

After enrichment, samples were measured for cfu/ml by plate count using (Tryptic Soy Agar) TSA. All enriched samples were then confirment for enumeration to levels >log 3 cfu/ml. Step 2 enrichment samples were prepared with a second dilution, 0.1ml to 10ml Cronobacter Selective Broth. Samples were then diluted and tested by PCR methods (see below). Dead cells were also prepared at log 4 and 5 by autoclaving and tested by PCR.

PCR test 1

Samples were also tested using Biotecon Reagent D and DNA extraction with manual and automated methods using Biotecon Foodproof starprep DNA or Roche MagNAPure Compact Instrument respectively, using the Biotecon Diagnostic Cronobacter Detection LyoKit following manufactures instructions and run with the Roche lightcycler 96 system

Biotecon Reagent D.

Foodproof starprep DNA

Roche MagNAPure® Compact Instrument

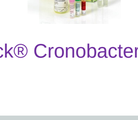
BIOTECON Diagnostic Cronobacter Detection LyoKit

Roche LightCycler® 96 System

PCR test 2

Samples were DNA extracted using iQ-Check® Free DNA Removal Solution and the Biorad Deepwell method prior to using the iQ-Check® Cronobacter spp. Kit following manufactures instructions and run with the Biorad PCR system.

iQ-Check® Free DNA Removal Solution



iQ-Check® Cronobacter spp. Kit

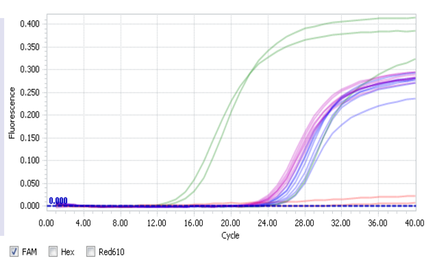
FCFX96 Touch Deep Well Real-Time PCR Detection System

Results

PCR test 1

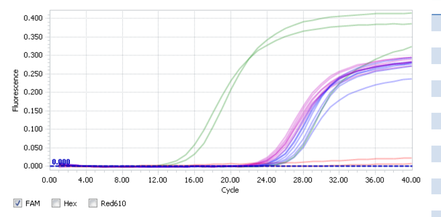
Following the ISO 22964:2017 enrichment method all samples resulted in positive PCR reactions. Spiked samples were prepared at 5,6,7 and 8 log cfu/ml for one powder, all detected, and at log 6 for all 7 powders

Log 5 for 8 different skim milk powders from different sources using foodproof® StarPrep One Kit of BIOTECON Diagnostic with Reagent D where one sample inhibited



	CT Strain 29004	CT Strain 29544
Powder 1	33.55	34.94
Powder 4	32.62	33.77
Powder 9	32.35	37.22
Powder 13	35.21	Inhibition
Powder 15	33.01	30.56
Powder 17	33.89	31.68
Powder 32	31.7	38.48
Average	**33.190**	**34.442**
Standard Deviation (SD)	**1.068**	**2.811**
%RSD (Relativity SD)	**3.2**	**8.2**
Positive control strain 29004	16.75	
Positive control strain 29544	16.08	
Positive control (kit)	28.65	

Log 6 for 8 different skim milk powders from different sources using foodproof® StarPrep One Kit of BIOTECON Diagnostic with Reagent D where all samples detected

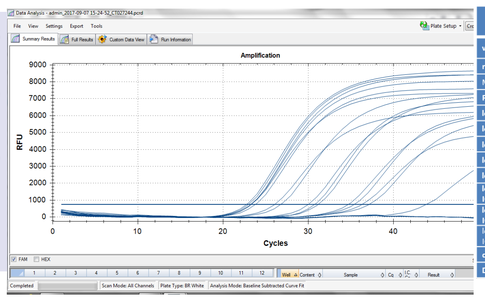


	CT Strain 29004	CT Strain 29544
Powder 1	26.91	26.15
Powder 4	27.3	25.9
Powder 9	27.63	25.14
Powder 13	26.5	25.34
Powder 15	26.58	25.63
Powder 17	26.18	25.64
Powder 32	27.92	25.52
Average	**27.003**	**25.617**
Standard Deviation (SD)	**0.590**	**0.311**
%RSD (Relativity SD)	**2.2**	**1.2**
Positive control strain 29004	16.6	
Positive control strain 29544	15.73	
Positive control (kit)	27.82	

Using the automated Roche MagNA Pure Compact System for DNA extraction, all 7 powders resulted in positive detection at 5 log cfu/ml.

PCR test 2

Enriched samples spiked at Log 4 to log 7 using Biorad IQ check DNA removal solution showing the elimination of dead cells at log 4 using the Biorad PCR



Sample	Cronobacter CT	Internal Control CT	Result
water blank	N/A	33.00	Negative
neg control	N/A	32.54	Valid Ctrl
Milk blank	N/A	32.51	Negative
Pos Ctrl	30.74	32.07	Valid Ctrl
log 7 spike in milk + IQ	23.79	31.07	Positive
log 7 spike in milk + IQ	23.00	30.44	Positive
log 7 spike in milk + IQ	23.31	31.66	Positive
log 7 spike in milk + IQ	22.75	31.37	Positive
log 7 spike in milk + IQ	23.25	31.24	Positive
log 6 spike with milk + IQ	26.76	31.58	Positive
log 5 spike with milk + IQ	31.17	32.19	Positive
log 4 spike with milk + IQ	35.38	32.57	Positive
dead cells log 5 + IQ	36.31	32.17	Positive
Dead cells log 4 + IQ	**N/A**	**32.52**	**Negative**

Dead cells

In both PCR tests when 4 log cfu/ml dead cells were added to the same matrix as used for live cell detection, no cells were detected by PCR. When 5 log cfu/ml dead cells were added, there was detection.

Conclusion

- PCR methods are suitable for use with the ISO 2264:2017 method.
- The automated DNA extraction Roche MagNA Pure Compact System was the more suitable for detection of *C. sakazakii* in skim milk powder samples using the Biotecon kit;
- There will be no interference from dead cells if the numbers are < 4 log cfu/ml.

References
1. Kalyantanda G, Shumyak L, Archibald LK. 2015. Cronobacter Species Contamination of Powdered Infant Formula and the Implications for Neonatal Health. Front Pediatr 3: 56.
2. Fei P, Jiang Y, Feng J, Forsythe SJ, Li R, Zhou Y, Man C. 2017. Antibiotic and Desiccation Resistance of Cronobacter sakazakii and C. malonaticus Isolates from Powdered Infant Formula and Processing Environments. Front Microbiol 8: 316.

Teagasc Agriculture and Food Development Authority

DPTC Dairy Processing Technology Centre