

Detection of *Cronobacter sakazakii* in Soy-Based Drinks Using the Innovate™ System and RapiScreen™ Dairy Kit

Introduction

Cronobacter sakazakii is a motile, gram-negative, non-spore-forming, rod-shaped coliform bacterium within the family *Enterobacteriaceae*, genus *Cronobacter*. It has been implicated in outbreaks of neonatal illness (premature infants), in isolated cases of severely immunocompromised individuals and in the elderly, but it rarely causes disease in healthy adults. *Cronobacter* can survive in dry foods, like powdered infant formula, powdered milk, herbal teas and starches, even throughout the desiccation process.

To minimize risk, it is vital to test final product for microorganism contamination. The Innovate™ Rapid Microbial Screening System is designed for the rapid detection of microorganisms in a range of products, including milk and infant formula. To detect very low levels of contaminants in these types of products, an enrichment step is required to ensure that there is sufficient ATP present for detection. Typically, a product is incubated in its own packaging to enrich the ATP from any contaminating microbial cells. Pre-established baselines obtained from uncontaminated product are used to determine positive results.

Objective

The goal of this study was to validate the Innovate System using the RapiScreen™ Dairy Kit for the detection of *Cronobacter sakazakii* in various soy-based protein drink products to demonstrate equivalence to traditional plate techniques.

Equipment, Supplies and Reagents

- Sterile inoculating loops
- Sterile pipettes and tips
- Incubators capable of $35 \pm 2^\circ\text{C}$
- Alcohol wipes
- RapiScreen Dairy Kit (includes reagents, polypropylene (PP) vials, microtiter plates)
- Potato Dextrose Agar (PDA)
- Tryptic Soy Agar (TSA) Plates
- Tryptic Soy Broth (TSB)
- pH meter and electrodes (i.e., Mettler-Toledo InLab® sensors)
- Syringes, 1 mL and 3 mL
- Dulbecco's Phosphate Buffered Saline - DPBS (1X)
- Shoe Goo, Clear Shoe Repair and Protective Coating
- Precision Glide Needles, 16 gauge 1 ½"
- ATP Positive Control
- Innovate System instrument

Test Organisms and Products

- Microorganisms
 - *Cronobacter sakazakii*, ATCC# 29544
- Milk product types tested
 - Soy-based complete protein drink, chocolate
 - Soy-based complete protein drink, vanilla
 - Soy-based UHT shake, chocolate
 - Soy-based UHT shake, vanilla



Methods

1. Cultures

Cronobacter sakazakii cultures were prepared by plating lyophilized organism onto TSA plates. Cultures were prepared in TSB and cultured for 24 hours at 35 ± 1 °C. A ten-fold serial dilution set was then made using DPBS, and plate counts were prepared on TSA plates to find a concentration of <100 CFU. The actual inoculum for the organism used during sample spiking was determined by plating it in duplicate onto TSA plates. The plates were incubated at 35 ± 2 °C and counted after 24 hours.

2. pH Assessments

Products assessed for pH were measured in triplicate to ensure the accuracy of measurements. The pH meter used for measurement of products was calibrated before use. This demonstrated that additional testing was not required to confirm that the RapiScreen Dairy Kit was capable of sufficiently neutralizing the products.

3. Background and Baseline RLU Determination

To determine ATP baseline levels, each product was initially incubated for 24 hours at 37 °C. Samples were mixed thoroughly and 20 mL of each product was transferred to a sterile container for testing. Both pH and background/baseline testing using the RapiScreen Dairy Kit were completed.

The background ATP level of each product was determined by running an assay using ATX buffer solution in place of reconstituted ATX reagent. The assay was then repeated using reconstituted ATX to allow for the depletion of the background ATP signal. These results are referred to as the Baseline RLU values. Baseline RLU values should be low and consistent, demonstrating that the background RLU signal has been fully depleted. A stable baseline RLU value allows for setting a threshold (positive/negative) cutoff value for identifying contaminated samples.

- Baseline Protocol: Dispense 60 µL ATX – 10 min shake – follow RapiScreen Dairy instructions for detection
- Background Protocol: Dispense 60 µL ATX buffer – 10 min shake – follow RapiScreen Dairy instructions for detection

Once baselines were established and cultures were prepared, each product type was inoculated in duplicate at <100 CFU per container. The microorganisms were spiked using a syringe through the top of the container and sealed with Shoe Goo glue. A non-inoculated container was incubated with each inoculated set as a negative control. Positive controls were set up by inoculating TSB or DPBS with 100 µL of the <100 CFU culture.

Samples were incubated at 35 ± 2 °C for up to seven days. On days 1 - 7, aliquots were taken from each container and tested on the Innovate System using the RapiScreen Dairy Kit. In parallel, each day, 10 µL of each product sample was inoculated onto TSA plates and incubated for 24 hours at 35 ± 2 °C for growth and morphology confirmation.

Results

In all drink types tested, the growth of low spike levels of *Cronobacter sakazakii* was detected after 24 hours of incubation using the Innovate System and the RapiScreen Dairy Kit (Table 1). RLU values ranged from 1,600 to over 81,000, demonstrating robust growth and ATP production, even when spiked at <100 CFUs. No data was taken after day 3 in any of the four inoculated products due to product leaking out, a result of excessive gas formation. All positive results on the Innovate System were confirmed by streaking products on TSA. The uninoculated controls had baseline values between 3 and 14 CFUs, typical for properly processed UHT products.

Table 1. Detection of *Cronobacter sakazakii* in Various Soy-based Drink Products

Product Background: N/A		Product Baseline: N/A		Threshold: N/A						
<i>C. sakazakii</i>	Target Inoculum	Actual Inoculum	Confirmation	Days: RLU Value (Avg)						
	Cell No. CFU	Cell No. CFU	Plate Result	1	2	3	4	5	6	7
Soy Based Chocolate (SBC)	<100	90	Growth	4,379	2,861	1,601	Leak*	Leak	Leak	Leak
Soy Based Vanilla (SBV)	<100	90	Growth	32,269	45,726	8,208	Leak	Leak	Leak	Leak
UHT Chocolate (UC)	<100	90	Growth	31,496	31,367	Leak	Leak	Leak	Leak	Leak
UHT Vanilla (UV)	<100	90	Growth	65,270	81,627	5,553	Leak	Leak	Leak	Leak
Blank Panel	Target Inoculum	Actual Inoculum	Confirmation Plate Result	1	2	3	4	5	6	7
<i>SBC Negative Control: 35 °C</i>	0	0	Negative	5	4	4	4	6	4	5
<i>SBV Negative Control: 35 °C</i>	0	0	Negative	13	11	10	12	12	14	14
<i>UC Negative Control: 35 °C</i>	0	0	Negative	5	5	3	5	6	4	4
<i>UV Negative Control: 35 °C</i>	0	0	Negative	12	10	9	8	12	11	11

*Leak – Gas production from the organism caused the product container to burst or leak.

Conclusions

As shown in the above table (Table 1), *C. sakazakii* was detected using the Innovate System and RapiScreen Dairy Kit after 24 h incubation in all spiked products. As for the traditional plating method, positive results were obtained for all samples tested, with the expected morphology.

Results for growth on day 4 and beyond were not completed on inoculated products due to the excessive growth of the organism in all the products. By day 3, all the product containers were bloated and by day 4, were leaking from excessive gas production, indicative of organism growth.

Summary

Aseptic processing of soy-based drink products may help reduce the risk of microbial contamination of the products. This is clearly shown in the low baseline values for ATP detection in uninoculated samples. In addition, *Cronobacter sakazakii* contamination at low levels (<100 CFU per container) could be detected at 24 hours using the Innovate System and RapiScreen Dairy Kit. In fact, very high RLU values were detected from *C. sakazakii* in Soy-Based Complete Protein Chocolate, Soy-Based Vanilla, UHT Vanilla and UHT Chocolate, thereby validating that the Innovate System would be a useful tool in detecting *C. sakazakii* in all these product types.

Based on these results, Hygiena® recommends using the Innovate System for the detection of low levels of *Cronobacter* species in UHT milk products.