



One Health Diagnostics™

Gabriela Tarlowska¹, Bernard Linke¹, Mat Lovemith¹, Colm Scully,² Johanna Ramirez²
1. Hygiena®, Guildford, United Kingdom
2. The Coca-Cola Company, 1 Coca Cola Plaza, Atlanta, GA

INTRODUCTION:

Non-Dairy Milk Alternative products are growing in popularity and have similar sterility challenges to established UHT Dairy products. ATP measurements have been successfully used to demonstrate commercial sterility of dairy products for many years, enabling rapid release of quality products. Non-Dairy Milk Alternative products need similar rapid methods to establish their commercial sterility.

The Innovate™ System is a microbial screening method offering rapid detection of bacteria, yeast, and molds which confirms the quality of food and beverage products in 48 hours. The Innovate System relies on the detection of microbial ATP via ATP bioluminescence measured in Relative Light Units (RLU) and can measure up to 96 samples per 30-minute run. RLU output from the product is considered positive when the result is at least three times higher than the baseline RLU of the product.

The Innovate System consists of an automated benchtop luminometer that utilizes RapiScreen™ reagents automatically injected into a microtiter plate by three injectors, providing fast and efficient microbial ATP detection. The mechanism of the reaction utilized in the Innovate System consists of non-microbial ATP removal by an ATX reagent containing ATP-ase and subsequent microbial ATP release with CellSolver, which disrupts the microbial cell membranes. A third reagent – Sensilux – contains the bioluminescent substrates that react with the released microbial ATP to generate an RLU signal.

Detection of seven microorganisms in Non-Dairy Milk Alternatives has been demonstrated using Hygiena’s Innovate System and compared to the traditional Miles Misra plating method.

PURPOSE:

This study demonstrates that the Innovate System and RapiScreen Dairy reagents are a suitable method to rapidly detect low levels of spoilage organisms, allowing rapid release of products - Almond Milk and Oat Milk.

REGISTERED TRADEMARKS:

RapiScreen™ and Innovate™ are registered trademarks of Hygiena.

REFERENCES:

1. Hygiena® (2022) Innovate™ Rapid Microbial Screening System. Accessed 2022.
Available from: <https://www.hygiena.com/food-safety-solutions/microbial-screening/innovate/>

Detection of Commercial Sterility in Non-Dairy Milk Alternative Products Using Hygiena’s Innovate™ System

Innovate™

Innovate™
AUTOSAMPLER III

METHODS:

Initial baseline RLU levels were established in sterile Almond Drink (6 RLU) and Oat Drink (6 RLU) products by taking 32 replicate ATP measurements. An RLU pass/fail level was established from these baselines and used for the analysis of spiked products.

Culture mixes: Non-*Bacillus* Cocktail – (*Streptococcus salivarius*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*), *Geobacillus stearothermophilus*, *Bacillus coagulans* and Heat Resistant Mold cocktail (*Talaromyces pinophilus*, *Byssoschlamys fulva*) were inoculated into product bottles through a sterile closure at 10-20 CFU per pack. Ten replicate bottles per each culture mix were used and incubated at 30 °C and 55 °C with five unspiked cartons of each product used as negative controls. At the following timepoints – 24, 48, 72, 96 and 120 hours - samples were drawn from each bottle and tested for ATP content using the Innovate System and plated on Potato Dextrose Agar (PDA) and Tryptic Soya Agar (TSA) using the Miles Misra plating technique. After 120 hours, samples were plated and moved to 30 °C +/-2 °C temperature, then further incubated and subjected to qualitative reference streaking after 240 hours to confirm the recovery of target organisms.

Organism	ATCC/NCTC	Supplier
<i>Bacillus coagulans</i>	ATCC 7050	Public Health England
<i>Byssoschlamys fulva</i>	NCTC 7156	Public Health England
<i>Talaromyces pinophilus</i>	ATCC 11797	Trafalgar Scientific
<i>Geobacillus stearothermophilus</i>	ATCC 12980	Public Health England
<i>Streptococcus salivarius</i> subsp. <i>thermophilus</i>	ATCC 19258	Public Health England
<i>Enterococcus faecalis</i>	ATCC 19433	Public Health England
<i>Pseudomonas aeruginosa</i>	ATCC 10145	Public Health England

Table 1. Microorganisms used for the study

CONCLUSIONS:

Two Non-Dairy Milk Alternative drinks – Almond Milk and Oat Milk - were submitted for assessment and inoculation studies using the Innovate System and RapiScreen™ Dairy reagent technology. The baseline and background studies showed a successful removal of background ATP and stable baseline RLU signal, which allowed positive/negative RLU thresholds to be established. Four different panels of microorganisms were submitted for assessment with a target spike of 10-20 CFU, including Heat Resistant Mold cocktail (*Byssoschlamys fulva* and *Talaromyces pinophilus*), non-*Bacillus* cocktail (*S. salivarius*, *E. faecalis* and *P. aeruginosa*), *G. stearothermophilus* and *B. coagulans*. The non-*Bacillus* cocktail and *B. coagulans* were spiked in the form of vegetative cells, the Heat Resistant Mold cocktail and *G. stearothermophilus* were spiked in the form of spores.

All four microorganism panels were successfully detected by the Innovate System in both Non-Dairy Milk Alternative products submitted for assessment. *G. stearothermophilus* was detected after 24 hours in both Almond Milk and Oat Milk, showing that both products provide a suitable environment for the germination of spores and growth as vegetative cells. RLU signal and colonies on Miles Misra plates started decreasing after the 24-hour timepoint with no detection in Oat milk at 72 hours or beyond. This phenomenon could be explained by quick nutrient utilization and subsequent sporulation of *G. stearothermophilus* in response to nutrient depletion in this product type. Low levels of heat-resistant spores were successfully detected by the Innovate System in both products at the 72-hour timepoint, with the Innovate RLU signal and detection on Miles Misra plates remaining high until the 120-hour timepoint. *B. coagulans* was successfully detected by the Innovate System and Miles Misra plates in both products at 72 hours, with the signal remaining high for the duration of the study. The non-*Bacillus* cocktail spiked into both products was detected after 48 hours, with the CFU and RLU signal remaining high for all remaining timepoints. The reference streaks conducted at the 240-hour timepoint confirmed the successful isolation of all target microorganisms.

Overall, the results indicate that Hygiens’s Innovate System and RapiScreen Dairy kit enable early detection of spoilage organisms in Non-Dairy Milk Alternative products using microbial ATP detection. The Innovate System reduces the time to result compared to standard plating methods, allowing for quicker product release.

RESULTS:

Organism	Sample type	Oat Milk									Almond Milk								
		Timepoints (hours)									Timepoints (hours)								
		24 h		48 h		72 h		96 h		120 h	24 h		48 h		72 h		96 h		120 h
		Innovate	Miles Misra	Innovate	Miles Misra	Innovate	Miles Misra	Innovate	Miles Misra	Miles Misra	Innovate	Miles Misra	Innovate	Miles Misra	Innovate	Miles Misra	Innovate	Miles Misra	Miles Misra
Heat Resistant Mold Cocktail (<i>T. pinophilus</i> and <i>B. fulva</i>)	Spiked cartons	0/10	0/10	3/10	4/10	8/10	8/10	10/10	10/10	10/10	0/10	0/10	0/10	0/10	3/10	3/10	10/10	10/10	10/10
	Controls	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	Average positive RLU / CFU/ml	N/A	N/A	306	8.1x10^2	2,265	2.5x10^2	15,920	8.6x10^3	4.1x10^3	N/A	N/A	N/A	N/A	742	9.3X10^3	11,372	9.3X10^2	4.1X10^3
G. stearothermophilus	Spiked cartons	10/10	10/10	4/10	3/10	0/10	0/10	1/10	0/10	0/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	5/10
	Controls	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	0/5	0/5	0/5	0/5	2/5	2/5	4/5	5/5	5/5
	Average positive RLU / CFU/ml	8,069	2.5x10^8	30	8.4x10^4	N/A	N/A	21	N/A	N/A	47,033	1.26x10^8	473	2.3x10^8	175	1.6x10^8	908	2.3x10^8	8.0x10^7
Non-Bacillus cocktail (<i>S. salivarius</i> , <i>E. faecalis</i> , <i>P. aeruginosa</i>)	Spiked cartons	4/10	7/10	9/10	9/10	9/10	9/10	9/10	9/10	9/10	1/10	2/10	7/10	9/10	10/10	10/10	10/10	10/10	10/10
	Controls	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	Average positive RLU / CFU/ml	35	2.2X10^8	134,427	2.5X10^8	102,412	2.5X10^8	101,977	2.5X10^8	2.5X10^8	220	2.5x10^1	41,256	1.75x10^8	65,727	2.5x10^8	2.5x10^8	2.5x10^8	2.5x10^8
B. coagulans	Spiked cartons	0/10	1/10	5/10	10/10	10/10	10/10	10/10	10/10	10/10	0/8	0/8	0/8	7/8	7/8	8/8	8/8	8/8	8/8
	Controls	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	Average positive RLU / CFU/ml	N/A	2.5x10^2	20	6.1x10^3	16,530	1.4x10^6	93,481	2.5x10^8	2.5x10^5	N/A	N/A	N/A	2.5x10^	391	2.2x10^4	73,039	2.5x10^8	1.3x10^7

Table 2. Summary of the time to detection of target organisms on the Innovate System and Miles Misra plates in Almond and Oat milk. The actual inoculum level for all the target organisms in both products was 7CFU. The ‘Spiked cartons’ and ‘Controls’ columns represent the number of cartons that turned positive on the Innovate System and Miles Misra plates. Positives on the Innovate System were samples that passed the detection threshold of 18RLU. The ‘Average positive RLU / CFU/20µl’ column represents the average RLU and CFU/20µl at each timepoint. The minimum time for detection for *G. stearothermophilus* was 24 hours and 48 hours for non-*Bacillus* cocktail in Almond milk and Oat milk. Heat resistant molds and *B. coagulans* were detected at 72 hours on the Innovate System and Miles Misra plates in both products. The 240 hours reference streak confirmed the recovery of all target organisms. The following colors were used to indicate the results: **detection**, **no detection**, **contamination**.