



One Health Diagnostics™

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Detection of Thermophilic Spore Formers in Plant-Based Drinks with the Hygiena Innovate™ System

INTRODUCTION:

Rapid detection of microorganisms based on adenosine triphosphate (ATP) measurement can be used for sterility control of a broad range of products. Spore formers pose a challenge for ATP-based detection systems because spores have low or no metabolic activity and, therefore, no measurable ATP. But thermophilic spore formers, in particular, which survive as spores in the sterilization process or as vegetative cells due to insufficient heat treatment, must be detectable.

In this study, we want to demonstrate that an optimal incubation temperature can delay the time between germination and resporulation, the period in which the vegetative phase enables the measurement of ATP.

To prove this, various plant-based beverages were inoculated with low concentrations of different spores from thermophilic bacteria. After incubating the product at different timepoints and temperatures, the samples were successfully tested for sterility using the Innovate Rapid Microbial Screening System using the RapiScreen™ Dairy Kit. This examination demonstrates the possibility of detecting spore formers using a bioluminescence-based ATP method.

PURPOSE:

The objective of this study was to demonstrate the possibility of detecting various thermophilic spore formers in plant-based beverages with a bioluminescent ATP system analyzed with the Innovate System and RapiScreen™ Dairy Kit from Hygiena® in comparison to the plate-count-based reference method.

REGISTERED TRADEMARKS / GLOBAL CERTIFICATIONS:

Hygiena® is a registered trademark of Hygiena.
Innovate™ and RapiScreen™ are trademarks of Hygiena.
RapiScreen™ Dairy Kit is AOAC RI certified, #092301.

METHOD:

In this study, spores from six thermophilic strains, *Geobacillus stearothermophilus*, *Anoxybacillus geothermalis*, *Aneurinibacillus flavithermus*, *Anoxybacillus kamchatkensis* and *Aneurinibacillus thermoaerophilus* were spiked directly into plant-based beverage products (Oat-, Almond- and Soy-Drinks).

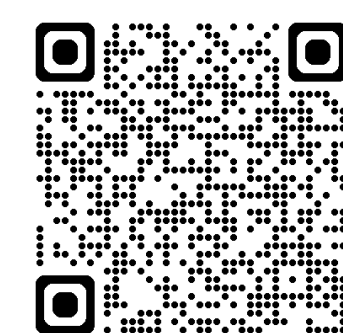
The 1 Liter samples were inoculated with a low-level spiking of 1-11 CFUs/Sample.

After incubation for one, two and three days at 48 °C and 55 °C, samples were analyzed using the standard RapiScreen Dairy Kit, and pH values were determined in parallel. The standard Dairy protocol was used for the measurement of Relative Light Units (RLUs) by the Innovate System.

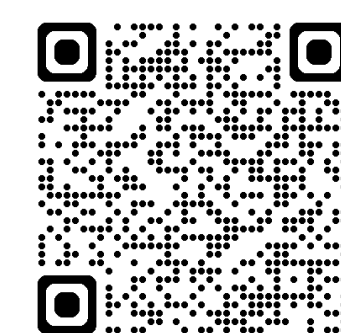
For comparison of the results, the samples were analyzed with the horizontal reference method based on ISO 4833-2:2013 and Miles-Misra.

Table 1: Enrichment and Measurement Protocol

Enrichment	Inoculum Concentration	1 - 11 CFU/Sample
	Incubation time	1 - 3 days
	Temperature:	48 °C and 55 °C
	Kit	Rapiscreen™ Dairy Kit
Measurement with Innovate System	Sample Volume	50 µL
	Protocol	standard dairy protocol
	Time	30 min
Confirmation	Method	According to ISO 4833-2:2013 Microbiology of the food chain - Horizontal method for the enumeration of microorganisms - Part 2: Colony count at 30°C by the surface plating technique
	Sample Volume	100 µL
	Plate Incubation Time	24 - 72 h



Innovate



RapiScreen™ Dairy Kit

RESULTS:

Table 2: Results of Almond Drink

	Inoculum CFUs/sample	Temperature (°C)	Matrix: Almond Drink, pH 7.85						
			Threshold RLU: 36						
			Day 1		Day 2		Day 3		
			RLUs	ISO [pos/neg]	RLUs	ISO [pos/neg]	pH-drop > 1.5	RLUs	ISO [pos/neg]
<i>Geobacillus stearothermophilus</i> 1	9	48 °C	3,326	pos	20,185	pos	✓	2,010	pos
			2,199	pos	18,164	pos	✓	304	pos
		55 °C	1,738	pos	85	pos	✓	20	pos
<i>Geobacillus stearothermophilus</i> 2	4.9	48 °C	11	neg	40,068	pos	✓	54	pos
			12	pos	11,415	pos	✓	47	pos
		55 °C	13,872	pos	68	pos	✓	37	pos
<i>Anoxybacillus geothermalis</i>	6.2	48 °C	558	pos	12,715	pos	-	14,036	pos
			1,352	pos	1,959	pos	-	15,390	pos
		55 °C	5,320	pos	3,024	pos	-	7,816	pos
<i>Anoxybacillus flavithermus</i>	7.1	48 °C	18	pos	5,960	pos	-	4,760	pos
			19	pos	6,702	pos	-	3,803	pos
		55 °C	1,358	pos	2,079	pos	-	1,874	pos
<i>Anoxybacillus kamchatkensis</i> subsp	3.8	48 °C	4,072	pos	20	pos	✓	20	pos
			23,153	pos	21	pos	✓	15	pos
		55 °C	143	pos	14	pos	✓	13	pos
<i>Aneurinibacillus thermoaerophilus</i>	6.6	48 °C	1,635	pos	2,709	pos	-	1,294	pos
			1,307	pos	4,483	pos	-	2,836	pos
		55 °C	436	pos	1,165	pos	-	842	pos

pos = contaminated samples, neg= contamination free samples, pos in orange = CFUs/mL < 10⁴, pos in red = CFUs/mL > 10⁴

In this study, the growth behavior of the thermophilic spore formers was examined depending on the incubation temperature, time and matrix. When samples were incubated at 55 °C, the RLU values started to decrease after 24 hours of incubation. The decreasing RLU values indicate that the organism is producing less ATP, as they are already entering the sporulation phase. However, with a product incubation at 48 °C, the sporulation timepoint could be delayed. For this reason, the metabolism in vegetative cells and, therefore, the ATP production is highest after 48 hours, which is optimal for the Innovate System detection of the thermophilic organisms, represented in maximum RLU values. Consequently, all beverages inoculated with *Geobacillus stearothermophilus*, *Anoxybacillus geothermalis*, *Aneurinibacillus flavithermus* and *Aneurinibacillus thermoaerophilus* were detected successfully after 48 hours of product incubation at 48 °C. *Aneurinibacillus thermoaerophilus* did not grow in oat drink, confirmed by the alternative and reference methods. While the sporulation of all other organisms could be delayed by the incubation temperature of 48 °C, this was not the case with *Anoxybacillus kamchatkensis* in almond drinks. However, the pH value of the almond drink decreased significantly from pH 7.8 to 5.6, which is evidence of growth of microorganisms that influenced the pH of the product. Therefore, a combined measurement of pH value and ATP-based measurement is recommended for almond drinks if *Anoxybacillus kamchatkensis* is expected. In oat and soy drinks, *Anoxybacillus kamchatkensis* was successfully detected after 48 hours at 48 °C.

Innovate™ AUTOSAMPLER III

Table 3: Results of Oat Drink

	Inoculum CFUs/sample	Temperature (°C)	Matrix: Oat Drink, pH 7.03						
			Threshold RLU: 66						
			Day 1		Day 2		Day 3		
			RLUs	ISO [pos/neg]	RLUs	ISO [pos/neg]	pH-drop > 1.5	RLUs	ISO [pos/neg]
<i>Geobacillus stearothermophilus</i> 1	9	48 °C	24	pos	13,700	pos	-	11	pos
			16	pos	75,082	pos	-	27	pos
		55 °C	28,285	pos	16	pos	✓	28	pos
<i>Geobacillus stearothermophilus</i> 2	4.9	48 °C	20	pos	71,949	pos	-	8,972	pos
			14	pos	50,521	pos	-	11,847	pos
		55 °C	48,300	pos	3,402	pos	✓	408	pos
<i>Anoxybacillus geothermalis</i>	6.2	48 °C	983	pos	3,327	pos	-	6,970	pos
			803	pos	2,086	pos	-	5,263	pos
		55 °C	1,466	pos	2,650	pos	-	3,499	pos
<i>Anoxybacillus flavithermus</i>	7.1	48 °C	12	neg	33,842	pos	-	20,703	pos
			10	neg	33,746	pos	-	23,279	pos
		55 °C	1,383	pos	18,362	pos	-	308	pos
<i>Anoxybacillus kamchatkensis</i> subsp	3.8	48 °C	53,789	pos	4,259	pos	-	626	pos
			33,893	pos	2,226	pos	-	365	pos
		55 °C	20,440	pos	533	pos	-	20	pos
<i>Aneurinibacillus thermoaerophilus</i>	6.6	48 °C	17	neg	12	neg	-	32	neg
			14	neg	10	neg	-	31	neg
		55 °C	10	neg	6	neg	-	21	neg

Table 4: Results of Soy Drink

	Inoculum cfu/sample	Temperature (°C)	Matrix: Soy Drink, pH 7.07						
			Threshold RLU: 45						
			Day 1		Day 2		Day 3		
			RLU	ISO [pos/neg]	RLU	ISO [pos/neg]	pH-drop > 1.5	RLU	ISO [pos/neg]
<i>Geobacillus stearothermophilus</i> 1	9	48 °C	110,103	pos	23,893	pos	✓	16,671	pos
			16	pos	19,694	pos	-	21,247	pos
		55 °C	110,384	pos	1,700	pos	✓	51	pos
<i>Geobacillus stearothermophilus</i> 2	8.7	48 °C	87,403	pos	1,897	pos	✓	45	pos
			10	neg	1,762	pos	-	863	pos
		55 °C	4,263	pos	3,222	pos	✓	45	pos
<i>Anoxybacillus geothermalis</i>	11	48 °C	194	pos	8,563	pos	✓	71	pos
			3	neg	2,324	pos	-	4,448	pos
		55 °C	7	neg	2,808	pos	-	4,518	pos
<i>Anoxybacillus flavithermus</i>	7.1	48 °C	1,154	pos	2,620	pos	-	3,051	pos
			89	pos	2,737	pos	-	3,414	pos
		55 °C	9	neg	172	pos	-	37,650	pos
<i>Anoxybacillus kamchatkensis</i> subsp	3.8	48 °C	5	neg	1,309	pos	-	57,677	pos
			12	pos	60,527	pos	-	15,750	pos
		55 °C	9	pos	72,341	pos	-	14,012	pos
<i>Aneurinibacillus thermoaerophilus</i>	7.3	48 °C	31,968	pos	4,872	pos	✓	257	pos
			44,571	pos	2,113	pos	✓	169	pos
		55 °C	8,011	pos	157	pos	✓	87	pos

SIGNIFICANCE:

The challenging but important detection of the most common thermophilic spore formers in plant-based beverages could be reached for 95% of products using the ATP-based detection provided by the Innovate System after two days of product incubation at 48 °C. Combining this with pH measurement leads to the opportunity to detect all spore formers after 48 hours of incubation.