Achieving AOAC RI *PTM* Certification for the Innovate™ System RapiScreen™ Dairy Kit Using Multiple Matrices and Microorganism Types

A Summary of the Validation Study Results

Introduction

Aseptic processing is a widely used method in food and beverage applications. It utilizes ultra-high temperatures to maximize the sterility of long-life shelf-stable products. However, contamination can still occur during manufacturing and production. Therefore, products must still be tested for microbial contamination.

Traditional methods for microbiological testing can take 7-15 days for results and require manual processes that are prone to technician error. In addition, results are not quantitative and require visual inspection for interpretation. In the case of pH, results are unreliable as many organisms generate little change in product acidity initially, if at all, and certain food products can mask the detection of contamination by pH due to their buffering capacity.

To minimize risk, it is vital to test final products for microorganism contamination. The Innovate™ Rapid Microbial Screening System is designed for the rapid detection of microorganisms in a range of products. 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 packaging to enrich the ATP from any contaminating microbial cells. Pre-established baselines obtained from uncontaminated products are used to determine positive results.

The Innovate™ RapiScreen™ Dairy Kit utilizes the Innovate System and adenosine triphosphate (ATP) bioluminescence, the industry standard, for rapid microbial screening of Ultra-High Temperature (UHT) and Extended Shelf-Life (ESL) dairy and plant-based dairy alternatives.

The objective of this study was to demonstrate the consistency, stability, inclusivity and robustness of this system to achieve AOAC RI^{PTM} certification for detecting microorganisms in ultra-high temperature (UHT) bovine milk, UHT plant-based drink, half and half, protein-based drink and extended shelf-life (ESL) plant-based (oat) drink matrices.

Equipment, Supplies and Reagents

Necessary materials and equipment included:

- RapiScreen Dairy Kit (KIT4001)
- Plastic, conical tubes
- Microplates
- ATP Positive Control Kit
- Sterile inoculating loops
- Pipettor and pipette tips
- Incubators (30 ± 1° C, 35 ± 1° C)
- TSA, PDA, MRS agar plates
- TSB, PDB, MRSB Broths
- Bacterial, yeast and mold strains
- Buffered Peptone Water
- Gas Pak EZ Anaerobe Gas Generating Pouch System with indicator
- Syringes, 1 mL
- Syringes, 3 mL Luer-Lok
- Precision Glide needles
- Shoo Goo Glue
- Glass screw-cap bottles, 5 mL
- Innovate System luminometer
- UHT bovine milk, 2% fat
- UHT plant-based (almond) drink
- UHT Half and Half, 10% fat
- UHT Protein-based drink (casein)
- ESL plant-based (oat) drink

Methods

Principle

The Innovate™ System is an automated benchtop luminometer capable of high throughput screening, reading 96 samples in under 30 minutes. The Innovate System works exclusively with the RapiScreen™ family. RapiScreen utilizes adenosine triphosphate (ATP) bioluminescence, where the luciferase enzyme catalyzes the consumption of microbial ATP to produce light. ATP bioluminescence can detect viable microorganisms with high sensitivity, providing an objective result much faster than visible microbial growth on agar plates, resulting in a faster detection time.

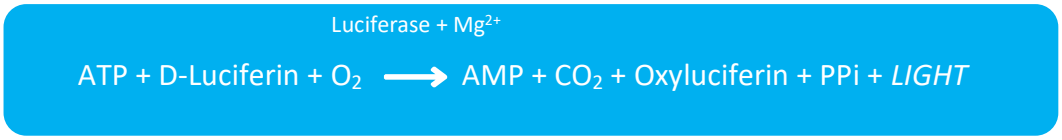

Figure 1. The Luciferase-Luciferin Reaction Produces Light

RapiScreen kits include a sample treatment step to reduce non-microbial sources of ATP prior to performing the standard bioluminescence assay. A lysis step then releases microbial ATP for the bioluminescence reaction. The assay evaluated in this study is RapiScreen Dairy, formulated to provide additional robustness and ATP clearance for testing dairy and dairy alternative UHT and ESL products.

The Innovate System luminometer controls the addition of reagents, timing of the reaction, and the recording of any generated light signal, reported in relative light units (RLUs). An enrichment step is to be performed prior to the screening assay. This involves incubation of the product for a defined period to allow any present microbes supported by the growth conditions to multiply. Unopened packaged products are typically incubated when assessing product sterility or aseptic handling, but the protocol can also feature enrichment of the product in growth media if there are concerns about the product being partly inhibitory to microbial growth.

Sample Background/Baseline Testing

Baseline studies were conducted to establish a specific RLU threshold for each matrix to ensure an accurate evaluation of RLU results on the candidate method. For each matrix, multiple non-inoculated product packs were analyzed on the Innovate System to define the expected ATP signal in a non-contaminated product. A stable baseline RLU value is needed to set the RLU positive/negative cut-off value for identifying contaminated samples, usually at triple the baseline RLU. A sample is considered positive for contamination if the average of duplicate RLU values is 3x above the established RLU threshold for the given product.

Inoculum Preparation

The organisms used were obtained from the American Type Culture Collection (ATCC, Manassas, VA), National Collection of Type Cultures (NCTC, Salisbury, United Kingdom) or routine testing cultures in the Hygiena LLC laboratory (Wild type). The strains were chosen to reflect organisms commonly found as contaminants in the food industry or as foodborne isolates. Organisms were plated from stock cultures, and then overnight cultures were prepared in the organism-appropriate broth. Target levels were achieved by serially diluting cultures in Buffered Peptone Water.

Inclusivity Study

The study examined the ability of the Innovate System Rapid Microbial Screening method (RapiScreen Dairy) to detect various organisms, including bacteria, yeasts and molds. Bacterial isolates were grown overnight while yeast and mold were incubated for 48 hours. Each isolate was diluted to a target level of 10 x LOD50 (approximately 10^4 – 10^5 CFU/50 µL). Fifty microliter aliquots of the culture were transferred in duplicate onto

the microtiter plate and placed in the Innovate System luminometer. Readings were performed on the Innovate System using the RapiScreen Dairy Kit reagents; positive (+) and negative (-) results were given based on the threshold derived from tripling broth/diluent control RLU.

Sample Inoculation & Analysis

For each matrix study, 20 replicate test portions were spiked at a fractional level of inoculation to achieve 5 - 15 positive results out of 20 portions tested, 5 test portions spiked at a high positive level of inoculation (achieving 100% detectability), and 5 non-inoculated control test portions for each matrix we analyzed.

The outside of each matrix container was disinfected with an alcohol wipe before injecting 100 µL of the appropriate dilution into each matrix container with a 1 mL insulin syringe. Following puncture and injection, each container was wiped and sealed with Shoe Goo Glue. The sealed, inoculated containers were then incubated at 30°C for 1 – 15 days.

For analysis, samples were collected from each container using a sterile syringe; then, duplicate 50 µL aliquots of each sample were transferred to the Innovate System Microtiter Plate using a sterile pipette. The Microtiter Plate was then placed in the Innovate System instrument and the RapiScreen Dairy protocol was run.

On each sampling day (Days 1, 2, 3, 5 and 7), in addition to the described standard assay procedure, confirmation plates were prepared to confirm the growth of each target microorganism by streaking a loopful of each product onto the appropriate agar medium followed by incubation at the correct temperature. The Reference Methods platings were performed only after Day 15 in accordance with the EU Directive. The results of the Candidate Method were compared to the ISO 4833-1:2013. A specific second Reference Method was also performed for each matrix, shown in Table 1. Threshold RLU values (Thr. RLUS) are provided in the third column from the left.

Table 1: Inoculation Summary of all matrixes and organisms tested (with each matrix, conditions, replicates, and reference methods evaluated in the matrix study).

Matrix	pH	Thr. RLU	Container volume (mL)	Inoculation Organism (Condition)	Inoculation Level	Replicates per method	Reference Method
UHT Bovine Milk (2% fat)	6.5	16	1,000	*Pantoea agglomerans* ATCC 27155 Heat Stressed	Non-inoculated	5	ISO 4833-1 & SMEDP
					Fractional positive	20	
					High positive	5	
UHT Bovine Milk[a] (2% fat)				*Pantoea agglomerans* CCUG 5862 Heat Stressed	Non-inoculated	5	
					Fractional positive	20	
					High positive	5	
UHT Plant-based drink (almond)	7.6	48	1,890	*Bacillus coagulans* ATCC 7050 spores	Non-inoculated	5	ISO 4833-1 & BAM Chapter 3
					Fractional positive	20	
					High positive	5	
Half and half (10% fat)	6.7	5	11	*Clostridium sporogenes* ATCC 7955 Spores	Non-inoculated	5	ISO 4833-1 & BAM Chapter 16
					Fractional positive	20	
					High positive	5	
Protein-Based Drink (casein)	6.9	15	330	*Lactobacillus fermentum* ATCC 9338 heat stressed	Non-inoculated	5	ISO 4833-1 & CMMEF
					Fractional positive	20	
					High positive	5	
ESL Plant-based drink (oat)	6.8	31	1,000	*Bacillus subtilis* ATCC 6051 Spores	Non-inoculated	5	ISO 4833-1 & BAM Chapter 3
					Fractional positive	20	
					High positive	5	

[a]Matrix tested in an independent laboratory, Q-Laboratories, Cincinnati, OH

Results

Inclusivity

When analyzed for growth, all 50 organisms (bacterial, yeast and mold) sampled showed positive results when tested on the Innovate System with the RapiScreen Dairy Kit. Background values for the broth/diluent alone were 9 RLUs (the threshold was set at 3 times that or 27 RLUs). CFU values for each organism are shown in the far right column (Table 2) and were obtained from 50 µL samples taken from cultures.

Table 2: RapiScreen Dairy Kit Inclusivity results for 50 organisms. Readings were performed on the Innovate System and positive (+) and negative (-) results were given based on broth/diluent control RLU thresholds.

No.	Genus	Species	Source	Origin	Candidate Method Result	CFU/sample aliquot
1	*Alicyclobacillus*	*acidoterrestris*	ATCC[a] 49025	Soil	+	4.00E+04
2	*Aspergillus*	*niger*	Wild Type[b]	Air Isolate	+	2.05E+03
3	*Bacillus*	*cereus*	ATCC 11778	Unknown	+	2.45E+03
4	*Bacillus*	*coagulans*	NCTC[c] 3993	Soil	+	2.45E+03
5	*Bacillus*	*licheniformis*	Wild Type	Plant-based drink	+	1.10E+04
6	*Bacillus*	*pumilus*	Wild Type	Plant-based drink	+	2.70E+04
7	*Bacillus*	*spizizenii*	NCTC 10400	Unknown	+	6.70E+04
8	*Bacillus*	*subtilis*	ATCC 6633	Unknown	+	1.42E+04
9	*Bacillus*	*thuringiensis*	Wild Type	Plant-based drink	+	2.80E+03
10	*Byssochlamys*	*fulva*	ATCC 10099	Bottled Fruit	+	5.00E+03
11	*Candida*	*albicans*	ATCC 10231	Bronchomycosis	+	1.25E+03
12	*Candida*	*orthopsilosis*	NCPF[d] 8798	Human	+	8.55E+03
13	*Cellulosimicrobium*	*cellulans*	NCTC 13518	Human	+	7.90E+03
14	*Citrobacter*	*freundii*	NCTC 9750	Unknown	+	7.53E+03
15	*Clostridium*	*perfringens*	NCTC 8237	Water	+	1.75E+03
16	*Clostridium*	*sporogenes*	ATCC 7955	Unknown	+	3.80E+03
17	*Corynebacterium*	*renale*	ATCC 10848	Human	+	3.00E+03
18	*Cronobacter*	*sakazakii*	ATCC 29544	Human	+	1.18E+04
19	*Dekkera*	*bruxellensis*	ATCC 36234	Belgian Stout	+	5.50E+03
20	*Enterobacter*	*aerogenes*	ATCC 13048	Sputum	+	9.90E+03
21	*Enterobacter*	*cloacae*	NCTC 10005	Spinal Fluid	+	1.42E+05
22	*Enterococcus*	*faecalis*	ATCC 19433	Piglet Faeces	+	5.17E+05
23	*Escherichia*	*coli*	ATCC 8739	Faeces	+	2.26E+04
24	*Geobacillus*	*stearothermophilus*	ATCC 7953	Unknown	+	7.50E+05
25	*Kluyveromyces*	*lactis*	ATCC 20185	Cheese	+	2.08E+03
26	*Kluyveromyces*	*marxianus*	NCTC 3106	Creamery	+	3.28E+03
27	*Lactobacillus*	*fermentum*	ATCC 9338	Milk	+	5.73E+04
28	*Lactobacillus*	*fructivorans*	ATCC 8288	Unknown	+	1.43E+04
29	*Lactobacillus*	*lactis*	ATCC 19435	Cheese	+	1.50E+03
30	*Lactobacillus*	*paracasei*	Wild Type	Ketchup	+	1.50E+03
31	*Lactococcus*	*lactis*	ATCC 11454	Milk	+	5.85E+04

No.	Genus	Species	Source	Origin	Candidate Method Result	CFU/sample aliquot
32	*Leuconostoc*	*mesenteroides*	ATCC 8293	Olives	+	4.00E+05
33	*Listeria*	*innocua*	ATCC 33090	Cow Brain	+	1.14E+04
34	*Listeria*	*monocytogenes*	ATCC 7644	Human	+	2.70E+03
35	*Micrococcus*	*luteus*	ATCC 4698	Human	+	3.99E+03
36	*Penicillium*	*chrysogenum*	ATCC 10106	Cheese	+	4.50E+03
37	*Pseudomonas*	*aeruginosa*	ATCC 9027	Ear infection	+	6.50E+04
38	*Pseudomonas*	*fluorescens*	ATCC 13525	Water	+	1.15E+04
39	*Pseudomonas*	*putida*	ATCC 49128	Clinical Isolate	+	3.61E+04
40	*Saccharomyces*	*cerevisiae*	ATCC 9763	Distillery	+	3.03E+03
41	*Saccharomyces*	*kudriavzevii*	ATCC 2601	Unknown	+	1.41E+04
42	*Salmonella*	Enteritidis	ATCC 13076	Unknown	+	2.54E+04
43	*Salmonella*	Newport	NCTC 14032	Unknown	+	3.70E+03
44	*Salmonella*	Typhimurium	ATCC 14028	Chicken liver	+	4.00E+04
45	*Staphylococcus*	*aureus*	ATCC 6538	Human lesion	+	1.82E+04
46	*Talaromyces*	*pinophilus*	ATCC 36839	PVC	+	2.50E+03
47	*Torulaspora*	*delbrukeii*	ATCC 10662	Unknown	+	1.80E+04
48	*Yarrowia*	*lipolytica*	ATCC 9773	Butter	+	9.50E+03
49	*Zygosaccharomyces*	*parabailii*	ATCC 56075	Unknown	+	1.78E+04
50	*Zygosaccharomyces*	*rouxii*	ATCC 2623	Grape Must	+	1.98E+04

Matrix Study

Similar methods were used to analyze the results of growing this group of organisms in various commercially available matrices to demonstrate the relevance of detection in such products at both fractional and highly positive spike levels. For these studies, inoculation levels were determined by pre-plated serial dilutions of overnight cultures. In addition, baseline threshold RLU levels were determined for each media used for growth to ensure an accurate evaluation of RLU results for each candidate method tested.

To inoculate each matrix container, the outside was disinfected with an alcohol wipe before injecting 100 µL of the diluted culture using a 1 mL syringe. Following puncture and injection, each container was wiped and sealed with Shoe Goo Glue. The sealed, inoculated containers were then placed into the 30 °C incubator. The candidate method was performed on each inoculated matrix container (20 spiked at a fractional positive level and 5 spiked at a high positive level) and the 5 non-inoculated containers on Days 1, 2, 3, 5 and 7. All containers remained in the 30 °C incubator until Day 15 per the European Directive 92/46 Annex C: Chapter 2 for UHT milk when the reference method test portion was performed.

For testing, each container was shaken before a 1 mL aliquot was removed using a 3 mL syringe. Containers were resealed and then returned to the incubator for subsequent readings. Using a sterile pipette tip, duplicate 50 µL aliquots were transferred to a microtiter plate for testing on the Innovate System, running the RapiScreen Dairy protocol. In addition, for each sampling day and matrix, a loopful of product was plated onto the appropriate agar medium and incubated to compare growth (i.e., confirmation plates).

The results for each matrix, method and inoculation level are shown in Table 3. Data demonstrates that the Innovate System could easily detect growth in all the various matrices by Days 5-7, if not sooner (2 days for UHT bovine milk).

Table 3: RapiScreen Dairy Kit results of the Spiked Matrixes and Respective Strains comparing the Candidate Method to the Reference Method. (The Innovate System read timepoint shown is indicated in column 4).

Matrix	Strain	Spiked CFU per Package[a]	Day	N[b]	Candidate Method			Reference Method			dPOD$_C$[f]	95% CI[g]
					x[c]	POD$_C$[d]	95% CI	x	PODR[e]	95% CI		
UHT bovine milk	*Pantoea agglomerans* ATCC[h] *27155*	11	2	5	5	1.00	(0.57, 1)	5	1.00	(0.57, 1)	0	(-0.47, 0.47)
		1.2		20	12	0.60	(0.39, 0.78)	13	0.65	(0.43, 0.82)	-0.05	(-0.21, 0.11)
		0		5	0	0.00	(0, 0.43)	0	0.00	(0, 0.43)	0	(-0.47, 0.47)
UHT plant-based drink (almond milk)	*Bacillus coagulans* ATCC *7050*	588	5	5	5	1.00	(0.57, 1)	5	1.00	(0.57, 1)	0	(-0.47, 0.47)
		1		20	8	0.40	(0.22, 0.61)	10	0.50	(0.3, 0.7)	-0.1	(-0.28, 0.08)
		0		5	0	0.00	(0, 0.43)	0	0.00	(0, 0.43)	0	(-0.47, 0.47)
Half and half	*Clostridium sporogenes* ATCC *7955*	6,300	5	5	5	1.00	(0.57, 1)	5	1.00	(0.57, 1)	0	(-0.47, 0.47)
		7		20	7	0.35	(0.18, 0.57)	7	0.35	(0.18, 0.57)	0	(-0.13, 0.13)
		0		5	0	0.00	(0, 0.43)	0	0.00	(0, 0.43)	0	(-0.47, 0.47)
Protein-based drink	*Lactobacillus fermentum* ATCC *9338*	19,000	7	5	5	1.00	(0.57, 1)	5	1.00	(0.57, 1)	0	(-0.47, 0.47)
		1		20	7	0.35	(0.18, 0.57)	7	0.35	(0.18, 0.57)	0	(-0.13, 0.13)
		0		5	0	0.00	(0, 0.43)	0	0.00	(0, 0.43)	0	(-0.47, 0.47)
ESL plant-based drink (oat milk)	*Bacillus subtilis* ATCC *6051*	9.6	5	5	5	1.00	(0.57, 1)	5	1.00	(0.57, 1)	0	(-0.47, 0.47)
		0.6		20	8	0.40	(0.22, 0.61)	8	0.40	(0.22, 0.61)	0	(-0.13, 0.13)
		0		5	0	0.00	(0, 0.43)	0	0.00	(0, 0.43)	0	(-0.47, 0.47)
UHT bovine milk[i]	*Pantoea agglomerans* CCUG 58262	2-10	2	5	5	1.00	(0.57, 1)	5	1.00	(0.57, 1)	0.00	(-0.47, 0.47)
		0.2-2		20	8	0.40	(0.22, 0.61)	8	0.35	(0.22, 0.61)	0.00	(-0.13, 0.13)
		0		5	0	0.00	(0, 0.43)	0	0.20	(0, 0.43)	0.00	(-0.47, 0.47)

[a] CFU = colony forming units applied to each package.
[b] N = number of test portions.
[c] X = number of positive test portions.
[d] POD$_C$ = Candidate method presumptive positive results confirmed positive divided by the total number of trials.
[e] POD$_R$ = Reference method results divided by the total number of trials.
[f] dPOD$_C$ = Difference between the candidate method and reference method POD values.
[g] 95% CI = if the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[h] American Type Culture Collection, Manassas, VA.
[i] Matrix tested in the independent laboratory, Q-Laboratories, Cincinnati, OH.

Conclusions

Discussion

The results of this study demonstrated the sensitivity, consistency, stability, and robustness of the Innovate RapiScreen Dairy Kit for five different commercial matrixes [UHT bovine milk (2%), UHT plant-based drink (almond), Half and half (10% fat), protein-based drink (casein) and ESL plant-based drink (oat)]. Across all matrixes, the Innovate RapiScreen Dairy Kit yielded positive results for microbial ATP at high positive inoculation levels and fractionally positive results at low inoculation levels comparable to the standard method. The

inclusivity study detected microbial ATP from all organisms tested when spiked at a target concentration of approximately 10 x LOD50. The product consistency and stability study met all requirements, and the fractionally positive results supported the claim of lot-to-lot consistency. The robustness study confirmed that small changes in volume, age of reagents, and delay in reading did not impact the overall results. Furthermore, an Innovate System variation study confirmed that there were no significant differences or performance issues between three different instruments (data not shown). The Innovate System consistently outperformed the Reference Method, with positive detection of growth at least 8-10 days faster.

Table 4: Summary Table of RapiScreen Dairy Kit Results Across All Tested Timepoints vs. Reference Method. (Negative controls are not displayed; all were negative.)

RapiScreen Dairy Candidate Method vs. Reference Method												
Matrix	Days										Reference Method	
	Day 1		Day 2		Day 3		Day 5		Day 7		Day 15	
	High + (n=5)	Fractional (n=20)	High + (n=5)	Fractional (n=20)	High + (n=5)	Fractional (n=20)	High + (n=5)	Fractional (n=20)	High + (n=5)	Fractional (n=20)	High + (n=5)	Fractional (n=20)
UHT Bovine Milk	0	0	5	12	5	12	5	12	5	13	5	13
UHT Plant-Based Drink[a]	0	1	5	1	5	3	5	8	5	9	5	10
Half and Half	5	0	5	0	5	0	5	7	5	7	5	7
Protein-Based Drink	4	1	5	1	5	2	5	1	5	7	5	7
ESL Plant-Based Drink	0	0	5	2	5	3	5	8	5	7[c]	5	8
UHT Bovine Milk [b]	5	8	5	8	5	8	5	8	5	8	5	8

[a]One of the samples was contaminated. See results for explanation.
[b]Matrix tested in the independent laboratory, Q-Laboratories, Cincinnati, OH.
[c]On day 7, one sample that was positive on days 3 and 5 became negative, yielding 7 of 20 samples positive. The disappearance of positivity was most likely due to sporulation, where cellular ATP per cell becomes undetectable.

The Innovate System luminometer, when paired with the RapiScreen Dairy Kit, is an easy and rapid way to detect the presence of microbial ATP in dairy and dairy alternative products. It is intended to provide quicker results than standard plating methods. The Innovate System RapiScreen Dairy Kit can reliably detect low concentrations of microbial ATP. The validation study confirmed that the Innovate System RapiScreen Dairy Kit delivers detection of contaminated product packs in 7 days or less, depending on the product, with results that are equivalent to the 15-day reference method requirement.

Time to detection is based on both the matrix type used and the growth rate of the organisms. The microorganisms used in this study panel were stressed prior to inoculation, leading to the conclusion that healthy organisms could be detected even more quickly. In the inclusivity study, the Innovate System detected various types of microorganisms, including gram-positive bacteria, gram-negative bacteria, yeast and mold. The RLU signal varied between organism types due to differences in phenotype and growth rate. The method uses product-specific sterile RLU thresholds based on background ATP to determine whether the organism has

populated the sample. The method detected all 50 inclusivity microbes tested in this study at concentrations less than 5 x 10^6 CFU/mL (2.7 x 10^5 per 50 µL test aliquot) per container.

Data obtained from matrix testing studies, which were closer to the designed use of the Innovate method when compared to the inclusivity study, further supports this claim. For all tested matrixes, the Innovate System RapiScreen Dairy method produced a 100% detection rate for the high positive spike level, low fractionally positive spike levels (25–75% positives) and negative spike levels for the control containers. The Innovate System method was more than twice as fast when 7-day incubation was considered compared to the 15-day result of the reference methods.

Final Conclusions

All positive results on the Innovate System method were significantly above the threshold obtained from the matrixes RLU baselines. Based on this study, the Innovate System using RapiScreen Dairy Kit detected microbial contaminants in the examined matrixes at least 8 days quicker than the reference method.

The Independent Matrix Study performed with the Innovate System RapiScreen Dairy Kit further substantiates the claim, successfully detecting microbial contamination in UHT bovine milk at and after 48 h of incubation and in other matrices by 5 days.

Therefore, receiving AOAC RI *PTM* certification clearly demonstrates that the Innovate System/RapiScreen Dairy Kit is an approved method for the detection of contamination in aseptically processed milk products, even those derived from plant-based sources (oat and almond).