Hygiena™ Innovate System

Claim Support Report

The Easy-to-Use Rapid Screening System

Hygiena 941 Avenida Acaso, Camarillo, CA 93012 United States
Telephone: 1-302-695-5300 • Telefax: 1-302-351-6454 • Refer to **hygiena.com/diagnostics/innovate**

Instrument: Hygiena Innovate System

Reagent kit used: RapiScreen™ Dairy Kit

Study: RapiScreen Dairy Claim Support

Date issued: November 15, 2013

Date Amended: March 30, 2020

Laboratory Technicians: Daniel Salgado and Daniel Horvath (Celsis)
Amended Laboratory Technicians: Delia Calderon, Rafael Barajas and Bernard Linke (Hygiena)

Report Author: Lori Daane (Celsis)
Amended Report Authors: Brandon Katz and Mat Lovesmith (Hygiena)

Contents

1. Introduction

Hygiena technology utilizes the efficient light-producing enzyme system of ATP bioluminescence for the rapid detection of micro-organisms. All actively respiring organisms contain adenosine triphosphate (ATP), which is used as the universal currency of free energy in biological systems – energy obtained from oxidation of foodstuffs.

Our bioluminescence luciferase enzyme hydrolyses ATP to AMP, releasing the stored energy as visible light. Luciferase can therefore be used to quickly detect the presence or absence of viable micro-organisms in a sample by examining it for their ATP.

The mechanism of the light-producing reaction is:

The reaction is very efficient; every molecule of ATP can cause emission of a photon of green light. Since all organisms rely on ATP as the main carrier of metabolic energy, ATP bioluminescence can detect living micro-organisms with great sensitivity. This provides a more rapid detection system than waiting for visible colonies to grow on agar plates.

Many Food and Beverage samples contain high levels of non-microbial ATP that needs to be removed before microbial ATP can be detected. The Hygiena RapiScreen™ Dairy reagents contain a proprietary ATP-depleting reagent (ATX). ATX is capable of isolating and depleting free and somatic ATP commonly found in these sample types. A 10-minute treatment reduces sample background readings (expressed in Relative Light Units, or RLUs) to low levels on all sample types.

The Hygiena RapiScreen Dairy Kit is designed for the rapid detection of micro-organisms in a wide range of products. In order to detect initial low levels of contaminants in these products, an enrichment step is required to ensure that there is enough ATP present for detection. Typically, product is incubated in its own packaging for a minimum of 48 hours to enrich the ATP from any contaminating microbial cells. Pre- established baselines obtained from uncontaminated product are used to determine positive results.

This report details the studies to validate the Innovate system in combination with the RapiScreen Dairy reagent kit. The studies included a wide range of products and were designed to demonstrate the following:

- Ability to produce stable baselines
- Ability to detect the presence of microbial contamination in a diverse range of products
- Ability to detect low level initial contamination (10^0-10^1 cells/spores per package)

2. Materials

2.1 Materials

- Incubators capable of 30 ± 2°C
- Sterile plastic petri dishes
- Sterile inoculating loops
- Sterile pipettes and tips
- 150 ml sterile Greiner containers
- Sterile 0.45 µm 30 mm PVDF syringe filters
- 10 ml disposable syringes
- Sterile 10 ml vials
- Alcohol wipes
- PP vials (supplied with reagent kit)
- 96- well microtiter plates (supplied with reagent kit)
- GasPak™ EZ Anaerobe Gas Generating Pouch System

2.2 Reagents and Media

All bioluminescence reagents are manufactured by Celsis according to ISO 9001 approved quality standards (Neuss, Germany). All reagents and media were used within recommended expiration dates.

Reagents and Media	Supplier	Reference No.
RapiScreen™ Dairy Kit	Hygiena	1251011
ATP Positive Control Kit	Hygiena	1291483
Tryptic Soy Agar (TSA)	BD	236950
Sabouraud Dextrose Agar (SDA)	BD	210950
α-Amylase from *Aspergillus oryzae*	Sigma	A9857-1MU
Butterfield's Buffer	3M	FTBFD9966

2.3 Microorganisms

2.3.1 Aerobic Bacteria

The following microorganisms were used for the inoculation study.

Microorganism	Reference
Bacillus cereus	ATCC 10876
Bacillus subtilis	ATCC 6633
Escherichia coli	ATCC 8739
Pseudomonas aeruginosa	ATCC 9027
Salmonella enterica sv typhimurium	ATCC 13311
Staphylococcus aureus	ATCC 6538

2.3.2 Anaerobic Bacteria

The following microorganisms were used for the inoculation study.

Microorganism	Reference
Clostridium sporogenes	ATCC 11437

2.3.3 Yeast

The following microorganisms were used for the inoculation study.

Microorganism	Reference
Candida albicans	ATCC 10231
Saccharomyces cerevisiae	ATCC 4098
Saccharomyces cerevisiae	ATCC 7753
Saccharomyces cerevisiae	NCPF 3191
Saccharomyces cerevisiae	NCPF 3178
Saccharomyces kudriavzevii	ATCC 2601
Kluyveromyces lactis	DSM 33795

2.4 Products

2.4.1 Aerobic Bacteria

The following products were selected representing a wide range of products:

Product Samples
Semi skim milk (1.5% fat)
Full fat (3.5% fat)
Chocolate milk (1.8% fat)
Coffee milk (4.3% fat)
Coffee cream (20.2% fat)
Sweetened cream (33.5% fat)
Soft serve mix vanilla
Spray dairy topping (2% fat)
Sweetened soymilk original (6% fat)
Sweetened soymilk vanilla (5% fat)
Rice drink low fat vanilla (4% fat)
Custard (4% fat)
Chocolate pudding (5% fat)
Soy drink
Unsweetened almond milk
Sweetened almond milk
Sweetened cashew milk
Sweetened oat milk
Unsweetened oat milk
Coconut milk
Unsweetened coconut milk
Hazelnut milk
Rice milk

2.4.2 Anaerobic Bacteria

The following products were selected representing a wide range of products:

Product Samples
Pumpkin spice latte
Butterscotch latte
Peppermint mocha latte
Mocha latte
Half and Half coffee creamer
Soy drink
Soy drink vanilla
Soy drink strawberry
Soy drink coffee
Soy drink chai
Soy drink cacao
Soy energy drink chocolate
Nutritional drink chocolate
Nutritional drink vanilla
Nutritional drink strawberry
Protein drink chocolate
Protein drink vanilla
Protein drink Blueberries & Cream
Protein drink Banana
Recovery drink vanilla
Recovery drink chocolate
Recovery drink strawberry

2.4.3 Yeast

The following products were selected representing a wide range of products:

Product Samples
Unsweetened almond milk
Apple juice
Beef Broth
Semi skim milk (1.5% fat)
Bechamel sauce
Orange juice
Pear juice
Lemon juice
Peach juice
Unsweetened vanilla oat milk
Chocolate milk
Red tea
Green tea
Milk tea

2.5 Instrumentation

All assays were run on the Hygiena Innovate Luminometer (serial number 2008, Driver 3.02, Innovate. im 5.09). All appropriate control procedures were performed prior to sample testing.

3. Methods

3.1 Sample Preparation for the Limit of Detection Study

100-gram product samples were aseptically weighed into 150 ml sterile containers

The spray dairy topping was sampled by first eliminating the gas from the can. Once the gas was released the bottom of the spray can was surface sterilized using 70% isopropanol. The can was then aseptically opened using a sterilized can opener. The product was then dispensed into sterile containers.

Both the custard and pudding samples were enzymatically digested prior to performing baselines or inoculation studies. This was achieved by adding 1 ml of a 20% (w/v) filter-sterilized amylase solution to the 100 g product samples. The amylase suspension was prepared in sterile water and then filter-sterilized through a 0.45 µm 30 mm PVDF syringe filter into a sterile 10 ml vial. The amylase was aseptically mixed in the product sample and allowed to sit at room temperature for 5 minutes.

3.2 Background ATP Determination and Depletion

Many dairy-based products contain high levels of non-microbial background ATP. Very high levels of non-microbial ATP may mask detection of microbial contamination therefore these products require an apyrase treatment to remove background ATP. The RapiScreen Dairy reagent kit includes an apyrase (ATX) that removes non-microbial ATP creating a stable baseline RLU value prior to testing for microbial ATP.

Product ATP baselines were determined by testing product at room temperature without a preliminary incubation. Samples were shaken thoroughly to mix the product prior to sampling and assay. Products were assayed in quadruplicate both with and without addition of ATX in order to verify background ATP levels and subsequent depletion of ATP.

The RLU values obtained were used to establish the individual cut-off limits for each product.

RLU_{sample} Less than Cut-off limit=Hygiena negative

RLU_{sample} Greater than Cut-off limit=Hygiena positive

3.3 Inoculum Preparation

QUANTI-CULT^{PLUS}® cell suspensions were used for all bacteria except Bacillus cereus in this study. Rehydration and inoculation were completed according to the Remel instructive insert. Bacillus subtilis is in spore state when rehydrated.

The Bacillus cereus spore suspension was prepared by pasteurizing a cell suspension in an 80°C water bath for 30 minutes. The cell suspension was prepared by removing several isolated colonies from a 72-hour incubated Tryptic Soy Agar (TSA) plate into 50 ml sterile ¼-strength Ringer solution. The pasteurized sample was serially diluted ten-fold in sterile ¼-strength Ringer solution and plate counts were determined on TSA.

Yeast suspensions were prepared by inoculating a single colony into Potato Dextrose Broth (PDB). The broth was then incubated at 30 ± 2°C for 24hrs. A ten-fold serial dilution was then made using Butterfield's Buffer to give the required inoculum level.

The volume of the inoculum for all bacteria were plated in duplicate onto TSA to confirm inoculation levels. Clostridium plates were sealed in GasPak™ EZ Anaerobe gas pouches prior to incubation. The volume of the inoculum for all yeast were plated in duplicate onto PDA to confirm inoculation levels. The plates were incubated at 30 ± 2°C and analyzed after 48-72 hours.

3.4 Sample Inoculation and Incubation

3.4.1 Bacteria

The method of the American Public Health Association (APHA) as described in Chapter 61 of the 5th Edition of the *Compendium of Methods for the Microbiological Examination of Foods* is followed in this study when determining incubation temperatures and times for this study to examine commercially sterile products. The *Compendium* also follows procedures references in the *Bacteriological Analytical Manual (BAM)* Chapter 21A Examination of Canned Foods. Product samples for the spiking study were set-up in triplicate and were prepared by inoculating a small number (<100) of cells or spores directly into the product containers. The product samples were then sealed and swirled to mix. All samples were incubated statically at 30 ± 2° C for 48 hours. Negative samples or samples showing low-level positive results were re-incubated and tested up to 10 days. All samples were assayed in quadruplicate.

Some samples required a 1:10 dilution in Butterfields prior to assay because the sample was solidified from the inoculated micro-organism. Hygiena recommends not testing visually contaminated product however for this study all samples were tested.

3.4.2 Yeast

The method of the American Public Health Association (APHA) as described in Chapter 61 of the 5th Edition of the *Compendium of Methods for the Microbiological Examination of Foods* is followed in this study when determining incubation temperatures and times for this study to examine commercially sterile products. The *Compendium* also follows procedures references in the *Bacteriological Analytical Manual (BAM)* Chapter 21A Examination of Canned Foods. Product samples for the spiking study were set-up in triplicate and were prepared by inoculating a small number (<100) of cells or spores directly into the product containers. The product samples were then sealed and swirled to mix. All samples were incubated statically at 30 ± 2° C for 24 hours. Negative samples or samples showing low-level positive results were re-incubated and tested up to 10 days. All samples were assayed in quadruplicate.

3.5 Limit of Detection

The limit of detection study was determined by adding between 1 to 10 cells or spores per 100-gram product sample. For this study semi-skimmed milk, coffee milk (4.3% fat), and chocolate milk were tested. A total of 5 samples per product were set-up for each micro-organism tested. Following inoculation, the product samples were sealed and swirled to mix. All samples were incubated statically at 30 ± 2° C for 48 hours. Samples testing negative were re-incubated and tested every 24 hours for up to 168 hours total incubation time. All samples were assayed in duplicate.

3.6 Hygiena Protocol

Product samples were tested using the standard Dairy protocol:

- Pipette 50 µl of product into sample well
- Place microtiter plate into luminometer
- Injection of reagents and measurement is automatically completed by instrument
- Results are expressed in Relative Light Units (RLU)

The following Innovate.im software parameters were used for testing:

Parameter	Specification
ATX Injection	60 µl
Incubation Delay (with shaking)	10 minutes
Shaking Pattern	Square
CellSolver Injection	60 µl
Extraction Delay	0.5 seconds
Background Measure	2 seconds
Delay	2 seconds
Sensilux Injection	60 µl
Delay	0.5 seconds
Sample Measure	4 seconds

3.7 Confirmation Plates

10 µl sterile loops were used to streak all samples onto TSA and SDA plates, and incubated at 30 ± 2°C for up to 72 hours. The presence of typical, pure colony growth of all organisms on plates confirmed the Hygiena Positive ATP bioluminescence result and confirmed that the organism spiked was indeed the organism that was detected.

4. Results

4.1 Background ATP Determination and Baselines

All values are in Relative Light Units (RLU)

Product	Before ATX treatment	After 10 minutes ATX incubation (baseline)	Positive Cut-off
Semi skim milk	81	9	27
Full fat milk	19	5	15
Chocolate milk	7	6	18
Coffee milk	9	12	36
Coffee cream	9	4	12
Sweetened cream	20	4	12
Soft serve mix vanilla	1024	48	144
Spray dairy topping	457	6	18
Soymilk unsweetened	1978	5	16
Sweetened soymilk original	7	6	18
Sweetened soymilk vanilla	2824	4	12
Rice Milk	2	2	7
Rice drink low fat vanilla	9238	6	18
Custard	4	3	9
Chocolate pudding	2	2	6

Unsweetened almond milk	146	4	12
Sweetened almond milk	145	14	43
Apple juice	44398	17	51
Beef Broth	3975	8	24
Semi skim milk (1.5% fat)	100	3	9
Bechamel sauce	163	5	15
Orange juice	708183	39	117
Pear juice	196111	28	84
Lemon juice	12442	5	15
Peach juice	19122	3	9
Unsweetened vanilla oat milk	10	4	12
Chocolate milk	29	14	42
Red tea	2434	5	15
Green tea	23499	3	9
Milk tea	11879	6	18
Sweetened Cashew Milk	4192	10	29
Oat milk Sweetened	210	4	12
Oat milk unsweetened	130	7	22
Coconut Milk	163	5	14
Coconut milk Unsweetened	4	5	16
Hazelnut milk	9	6	17
Half and Half Coffee Creamer	351	6	18
Pumpkin Spice Latte	25	16	48
Butterscotch Latte	26	15	45
Peppermint Mocha Latte	20	13	39
Mocha Latte	7	4	12
Soy Drink	64	4	12
Soy Drink Strawberry	355	9	27
Soy Drink Coffee	26	8	24
Soy Drink Chai	216	13	39
Soy Drink Cacao	55	4	12
Soy Drink Vanilla	181	10	30
Soy Energy Drink Chocolate	34	3	9
Nutritional Drink Chocolate	4	4	12
Nutritional Drink Vanilla	14	12	36
Nutritional Drink Strawberry	103	16	48
Protein Drink Blueberries & Cream	35	6	18
Protein Drink Banana	91	5	15
Protein Drink Chocolate	6	3	9
Protein Drink Vanilla	12	5	15
Recovery Drink Vanilla	26	11	33
Recovery Drink Chocolate	13	7	21
Recovery Drink Strawberry	7	3	9

4.2 Bacteria Inoculation Study Results (48-hour incubation)

Bacillus cereus

Product	Inoculum (Number of Cells)	Mean Assay Result (RLU)	Result	Confirmation Plate
Semi skim milk	23	54822	Positive	Growth
Full fat milk	23	51352	Positive	Growth
Chocolate milk	23	94769	Positive	Growth
Coffee milk	23	24562	Positive	Growth
Coffee cream*	23	46759	Positive	Growth
Sweetened cream*	23	37923	Positive	Growth
Soft serve mix vanilla	33	240488	Positive	Growth
Spray dairy topping	23	60911	Positive	Growth
Sweetened soymilk original	33	27644	Positive	Growth
Sweetened soymilk vanilla	23	143922	Positive	Growth
Rice drink low fat vanilla	23	131	Positive	Growth
Rice milk	9	51638	Positive	Growth
Custard	33	601763	Positive	Growth
Chocolate pudding	23	71743	Positive	Growth
Oat milk Sweetened	9	110185	Positive	Growth
Oat Milk Unsweetened	9	107011	Positive	Growth
Almond milk sweetened	9	188902	Positive	Growth
Almond milk unsweetened	9	99953	Positive	Growth
Cashew milk sweetened	9	125100	Positive	Growth
Coconut milk sweetened	9	177640	Positive	Growth
Coconut milk unsweetened	9	149688	Positive	Growth
Hazelnut milk	9	117477	Positive	Growth
Soy Drink	45	30185	Positive	Growth

***Product sample required 1:10 dilution using Butterfields buffer.**

Bacillus subtilis

Product	Inoculum (Number of Cells)	Mean Assay Result (RLU)	Result	Confirmation Plate
Semi skim milk	7	10804	Positive	Growth
Full fat milk	17	20219	Positive	Growth
Chocolate milk	7	38817	Positive	Growth
Coffee milk	7	17448	Positive	Growth
Coffee cream*	7	24297	Positive	Growth
Sweetened cream*	7	28270	Positive	Growth
Soft serve mix vanilla	33	16522	Positive	Growth
Spray dairy topping	7	13394	Positive	Growth
Sweetened soymilk original	33	72290	Positive	Growth
Sweetened soymilk vanilla	7	83966	Positive	Growth
Rice drink low fat vanilla	25	3547	Positive	Growth
Custard	7	392624	Positive	Growth
Chocolate pudding	7	14158	Positive	Growth

Escherichia coli

Product	Inoculum (Number of Cells)	Mean Assay Result (RLU)	Result	Confirmation Plate
Semi skim milk	28	2800	Positive	Growth
Full fat milk	28	3787	Positive	Growth
Chocolate milk	28	840	Positive	Growth
Coffee milk	28	592	Positive	Growth
Coffee cream*	28	33793	Positive	Growth
Sweetened cream*	28	30707	Positive	Growth
Soft serve mix vanilla	28	48660	Positive	Growth
Spray dairy topping	28	24050	Positive	Growth
Sweetened soymilk original	28	38757	Positive	Growth
Sweetened soymilk vanilla	28	32605	Positive	Growth
Rice drink low fat vanilla	28	44193	Positive	Growth
Rice Milk	15	24442	Positive	Growth
Custard	8	6246	Positive	Growth
Chocolate pudding	28	14158	Positive	Growth
Soymilk sweetened	13	17697	Positive	Growth
Soymilk unsweetened	13	54510	Positive	Growth
Hazelnut milk	15	18053	Positive	Growth

***Product sample required 1:10 dilution using Butterfields buffer**

Pseudomonas aeruginosa

Product	Inoculum (Number of Cells)	Mean Assay Result (RLU)	Result	Confirmation Plate
Semi skim milk	17	41681	Positive	Growth
Full fat milk	17	38415	Positive	Growth
Chocolate milk	17	34631	Positive	Growth
Coffee milk	17	15771	Positive	Growth
Coffee cream*	17	20294	Positive	Growth
Sweetened cream*	17	21707	Positive	Growth
Soft serve mix vanilla	6	6646	Positive	Growth
Spray dairy topping	17	8293	Positive	Growth
Sweetened soymilk original	6	26426	Positive	Growth
Sweetened soymilk vanilla	17	23894	Positive	Growth
Rice drink low fat vanilla	17	47227	Positive	Growth
Custard	17	262310	Positive	Growth
Chocolate pudding	17	7178	Positive	Growth
Soy Drink	8	550	Positive	Growth

Salmonella typhimurium

Product	Inoculum (Number of Cells)	Mean Assay Result (RLU)	Result	Confirmation Plate
Semi skim milk	19	5864	Positive	Growth
Full fat milk	19	10836	Positive	Growth
Chocolate milk	19	2711	Positive	Growth
Coffee milk	19	1342	Positive	Growth
Coffee cream*	19	5251	Positive	Growth
Sweetened cream*	19	16311	Positive	Growth
Soft serve mix vanilla	16	75040	Positive	Growth
Spray dairy topping	19	26913	Positive	Growth
Sweetened soymilk original	16	51033	Positive	Growth
Sweetened soymilk vanilla	19	18793	Positive	Growth
Rice drink low fat vanilla	19	23640	Positive	Growth
Custard	16	110160	Positive	Growth
Chocolate pudding	19	34302	Positive	Growth

Staphylococcus aureus

Product	Inoculum (Number of Cells)	Mean Assay Result (RLU)	Result	Confirmation Plate
Semi skim milk	14	128671	Positive	Growth
Full fat milk	14	25634	Positive	Growth
Chocolate milk	14	25300	Positive	Growth
Coffee milk	14	34970	Positive	Growth
Coffee cream*	14	41561	Positive	Growth
Sweetened cream*	14	46739	Positive	Growth
Soft serve mix vanilla	15	182	Positive	Growth
Spray dairy topping	14	5442	Positive	Growth
Sweetened soymilk original	27	46940	Positive	Growth
Sweetened soymilk vanilla	14	57536	Positive	Growth
Soymilk unsweetened	12	36619	Positive	Growth
Rice drink low fat vanilla	14	24789	Positive	Growth
Rice milk	15	42963	Positive	Growth
Custard	27	356338	Positive	Growth
Chocolate pudding	14	20299	Positive	Growth
Hazelnut milk	15	48583	Positive	Growth
Soy Drink	47	783	Positive	Growth

Clostridium sporogenes

Product	Inoculum (Number of Cells)	Mean Assay Result (RLU)	Result	Confirmation Plate
Pumpkin Spice Latte	2	2660	Positive	Growth
Butterscotch Latte	1	112	Positive	Growth
Peppermint Mocha Latte	1	835	Positive	Growth
Mocha Latte	43	1077	Positive	Growth
Soy Drink	1	79838	Positive	Growth
Soy Drink Strawberry	1	61289	Positive	Growth
Soy Drink Coffee	1	10513	Positive	Growth
Soy Drink Chai	1	45675	Positive	Growth
Soy Drink Cacao	1	7775	Positive	Growth
Soy Drink Vanilla	1	44201	Positive	Growth
Nutritional Drink Chocolate	43	211408	Positive	Growth
Nutritional Drink Vanilla	2	56	Positive	Growth
Nutritional Drink Strawberry	3	1324	Positive	Growth
Protein Drink Blueberries & Cream	19	3076	Positive	Growth
Protein Drink Banana	19	1658	Positive	Growth
Protein Drink Chocolate	22	50587	Positive	Growth
Protein Drink Vanilla	22	12121	Positive	Growth
Recovery Drink Vanilla	1	1635	Positive	Growth
Recovery Drink Chocolate	1	30056	Positive	Growth
Recovery Drink Strawberry	43	56391	Positive	Growth

Clostridium sporogenes – 120hrs

Product	Inoculum (Number of Cells)	Mean Assay Result (RLU)	Result	Confirmation Plate
Half and Half Coffee Creamer	1	2628	Positive	Growth
Sweetened soymilk vanilla	27	60	Positive	Growth
Soy Energy Drink Chocolate	9	3552	Positive	Growth

4.3 Yeast Inoculation Study Results (48-hour incubation)

Candida albicans

Product	Inoculum (Number of Cells)	Mean Assay Result (RLU)	Result	Confirmation Plate
Unsweetened almond milk	54	7058	Positive	Growth
Apple juice	54	3483	Positive	Growth
Beef broth	54	8021	Positive	Growth
Semi skim milk (1.5% fat)	54	649	Positive	Growth

Candida albicans- 72hrs

Product	Inoculum (Number of Cells)	Mean Assay Result (RLU)	Result	Confirmation Plate
Bechamel sauce	38	763	Positive	Growth

Saccharomyces cerevisiae

Product	Inoculum (Number of Cells)	Mean Assay Result (RLU)	Result	Confirmation Plate
Unsweetened almond milk	12	1378	Positive	Growth
Apple juice	90	2820	Positive	Growth
Beef broth	85	718	Positive	Growth
Semi skim milk (1.5% fat)	95	495	Positive	Growth
Lemon juice	33	154	Positive	Growth
Peach juice	33	44598	Positive	Growth
Chocolate milk	25	7730	Positive	Growth
Red tea	10	16	Positive	Growth
Green tea	10	13175	Positive	Growth
Milk tea	10	196	Positive	Growth
Soymilk sweetened	41	94928	Positive	Growth

Saccharomyces kudriavzevii- 120hrs

Product	Inoculum (Number of Cells)	Mean Assay Result (RLU)	Result	Confirmation Plate
Orange juice*	30	148624	Positive	Growth
Pear juice*	30	256782	Positive	Growth

* Products only tested after 5 days of enrichment

Kluyveromyces lactis- 72hrs

Product	Inoculum (Number of Cells)	Mean Assay Result (RLU)	Result	Confirmation Plate
Unsweetened vanilla oat milk	9	480	Positive	Growth

4.4 Limit of Detection (48-hour incubation)

Semi-skimmed milk

Product	Inoculum (Number of Cells)	Hygiena Result/Plate Result of 5 sample replicates				
		1	2	3	4	5
Bacillus cereus	6	+/+	+/+	+/+	+/+	+/+
Bacillus subtilis	5	+/+	+/+	+/+	+/+	+/+
Escherichia coli	3	+/+	+/+	+/+	+/+	+/+
Pseudomonas aeruginosa	4	+/+	+/+	+/+	+/+	+/+
Salmonella typhimurium	9	+/+	+/+	+/+	+/+	+/+
Staphylococcus aureus	7	+/+	+/+	+/+	+/+	+/+

Coffee cream

Product	Inoculum (Number of Cells)	Hygiena Result/Plate Result of 5 sample replicates				
		1	2	3	4	5
Bacillus cereus	6	+/+	+/+	+/+	+/+	+/+
Bacillus subtilis	5	+/+	+/+	+/+	+/+	+/+
*Escherichia coli***	3	+/+	+/+	+/+	+/+	+/+
Pseudomonas aeruginosa	4	+/+	+/+	+/+	+/+	+/+
Salmonella typhimurium	9	+/+	+/+	+/+	+/+	+/+
Staphylococcus aureus	5	+/+	+/+	+/+	+/+	+/+

***Product samples required 1:10 dilution using Butterfields buffer**

Chocolate milk

Product	Inoculum (Number of Cells)	Hygiena Result/Plate Result of 5 sample replicates				
		1	2	3	4	5
Bacillus cereus	6	+/+	+/+	+/+	+/+	+/+
Bacillus subtilis	5	+/+	+/+	+/+	+/+	+/+
Escherichia coli	3	+/+	+/+	+/+	+/+	+/+
Pseudomonas aeruginosa	10	+/+	+/+	-/-*	+/+	+/+
Salmonella typhimurium	9	+/+	+/+	+/+	+/+	+/+
Staphylococcus aureus	7	+/+	+/+	+/+	+/+	+/+

***Samples tested negative after 96 h incubation.**

5. Summary

A total of 58 dairy and non-dairy products representing a wide range of product types were tested to demonstrate that the Hygiena Innovate System along with Hygiena RapiScreen™ Dairy reagents can reliably detect initial low-level inoculum of microorganisms added to sterile product following an incubation (enrichment) period. In this study all samples were incubated statically at 30 ± 2°C for a minimum of 48 hours prior to assay.

Baseline studies showed that the RapiScreen Dairy reagents could produce low and stable baseline values. This allows for the establishment of positive/negative threshold values. RLU values above the threshold indicate a positive result. The majority (51 out of 55) of the tested products could be interpreted with a cut-off limit of less than 50 RLU. The pear and apple juice could be interpreted with a cut-off limit of less than 100 RLU while the Soft Serve Mix Vanilla could be interpreted with a cut-off limit of less than 150 RLU.

Inoculation and limit of detection studies were performed with a wide range of microorganisms.

Bacillus cereus and Bacillus subtilis were inoculated in spore-state while the remaining bacterial micro-organisms were inoculated directly from rehydrated lyophilized cultures (Remel quanti-cults). All yeast micro-organisms were inoculated using an isolated colony from agar and growing an overnight culture

The inoculation studies were performed in triplicate for each product and each inoculated product sample was assayed in quadruplicate. The target inoculation level was between 10-100 cells with actual results ranging between 1-95 cells. The results showed successful detection of most bacterial microorganisms after 48 hours enrichment. Remaining bacteria were detected after 120hrs enrichment Majority yeast microorganisms were detected after 48hrs enrichment. All yeast micro-organisms were detected after 120hrs of enrichment.

The limit of detection study was performed on 3 products (semi-skimmed milk, coffee cream, and chocolate milk). Each product was set-up x5 with a target inoculation level between 1-10 cells with actual results ranging between 3-10 cells. Each inoculated product sample was assayed in duplicate. The results showed successful detection of all inoculated micro-organisms after 48 hours enrichment. One replicate of the chocolate milk inoculated with Pseudomonas aeruginosa was negative by Hygiena and did not show growth on plates after 96 hours incubation and was interpreted as a sample that did not receive inoculation of at least one viable cell. To briefly summarize the results of this validation:

- A total of fifty-eight (58) products have been tested with six (6) different aerobic bacterial, one (1) anaerobic bacterial, and four (4) different yeast microorganisms to evaluate the Hygiena RapiScreen Dairy reagent kit on the Innovate system.

- The bacterial microorganisms tested included *Bacillus cereus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Clostridium sporogenes,* and *Staphylococcus aureus*. Both *Bacillus* species were inoculated as spores.

- The yeast micro-organisms tested included Candida albicans, Saccharomyces cerevisiae, Saccharomyces kudriavzevii, and Kluyveromyces lactis

- All products were found to give low and stable baseline RLU values

- In all products, all bacterial microorganisms with an initial contamination level between 1-95 cells were successfully detected after up to 5 days enrichment

- In all products, all yeast microorganisms with an initial contamination level between 6-95 cells were successfully detected.

- The Hygiena Rapid Detection System comprising of the RapiScreen Dairy reagents and Innovate was found to be a reliable test method with a 100% sensitivity (true positives) and 100% specificity (true negatives) after a product incubation of 2-10 days for all bacteria and yeast species tested.