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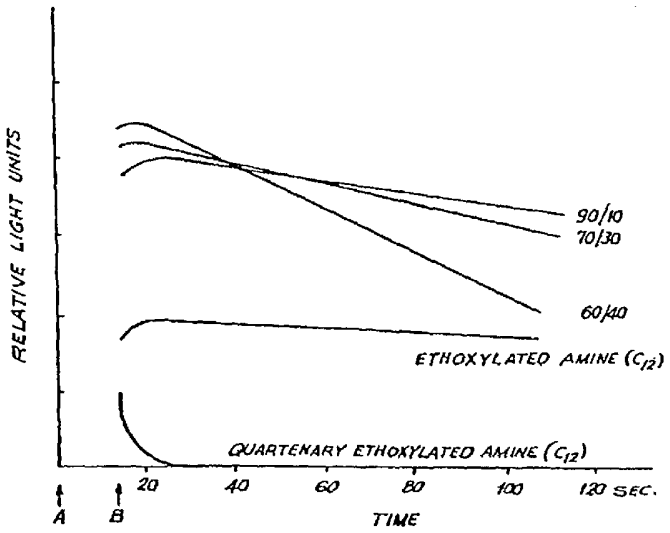
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(54) Title: USE OF NOVEL COMPOUNDS TO RELEASE NUCLEOTIDES FROM LIVING CELLS



(57) Abstract: A method for effecting a rapid, comprehensive release of cellular contents, including nucleotides and associated molecules of interest from living cells, using a novel family of quaternary ammonium compounds. Modification of the alkyl chain length of these compounds can enable selective release from different classes of living cells, allowing them to be rapidly distinguished from each other.

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USE OF NOVEL COMPOUNDS TO RELEASE NUCLEOTIDES FROM LIVING CELLS

RELATED APPLICATION

This application is based on U.S. Provisional Application No. 60/495,440 titled "Use of novel compounds to release nucleotides from living cells" filed on 15 August 2003.

FIELD OF THE INVENTION

The present invention relates to a method for effecting a rapid and comprehensive release of cellular contents, including nucleotides and other associated molecules of interest from living cells.

BACKGROUND OF THE INVENTION

Many industries and markets have a requirement to detect the presence of contamination in their products or samples. Chief among these contaminants are microbial cells, such as bacteria, yeasts or molds. These types of cells are microscopic in size, effectively impossible to detect by visible means.

Most conventional methods to detect microbial cells rely on techniques that encourage the growth of possible contaminants until the growth can be seen by the naked eye; a process that can take many days. The delays caused by this requirement are costly and even potentially dangerous.

There is therefore a need to detect microbial contamination as rapidly as possible. One approach commonly taken to effect this is to isolate and detect specific chemical markers within contaminating microbial cells. One example is the molecule Adenosine Triphosphate ("ATP"), which is universally present in all living matter, where it serves as a source of energy for processes within cells. ATP can be quickly detected using the firefly luciferase reaction – addition of ATP to a purified preparation of the firefly enzyme luciferase and its substrate luciferin will produce an instant light emission, a phenomenon known as ATP-bioluminescence. This light is proportional to the amount of ATP present, and can be measured using a sensitive light detecting instrument such as a luminometer.

Commercial systems are available, comprising luciferase-based reagent kits and luminometer instrumentation, that enable customers to quickly test their products and samples for ATP which, if found in unusual amounts, indicates the presence of microbial contamination.

One challenge to the development of tests for microbial contamination by ATP-bioluminescence is that the ATP required for detection is 'hidden' or contained within the contaminating cells. Hidden ATP is not available to trigger the luciferase/luciferin light reaction, so in this form the test will not work. To enable the test to work, ATP first has to be released from contaminating cells, in a process typically referred to as extraction.

A second challenge to the development of a successful ATP-bioluminescence test is that a majority of samples contain ATP of non-microbial origin. An example of this is bovine ATP in milk, derived ultimately from the udder cells of the cow. This non-microbial ATP may conceal or smother the microbial ATP that the test is designed to detect. To counter this, it is possible to pre-treat the sample being tested with an ATP-destroying enzyme. Such enzymes are commonly used in commercial kits, and are generally referred to as apyrase.

Treatment of a sample with apyrase is successful because the apyrase is able to remove only the ATP it finds free in solution. During this pre-treatment, ATP locked up inside microbial cells is kept safely away from the action of the apyrase; however, once release/extraction of this microbial ATP has occurred, it is vulnerable to apyrase degredation, and typically a proportion of microbial ATP is indeed lost to the action of apyrase before it can be read.

Extraction is usually accomplished by chemical means – the most common types are based around a family called Quaternary Ammonium Compounds ("QAC's"). QAC's were first described in United States Patent 4,303,752. Specific examples include quaternary ethoxylated ammonium chlorides, benzyl trimethyl ammonium chloride, cetyl trimethyl ammonium bromide and other generic cationic detergents under various trade names.

Microbial ATP extraction poses additional challenges, in that microbial cells are typically protected by robust cell walls. A successful extractant should:

- Release cell components such that they are available for subsequent detection and measurement by outside systems such as ATP-bioluminescence.

- Release cell components from a variety of different types of cells, with accordingly different wall compositions (*See, for example*, U.S. Patent No. 5,558,986 to Lundin, which is hereby incorporated by reference).
- Effect this release in as rapid manner as possible (*Id.*).
- Effect this release in a reproducible manner.
- Enable the timely, reproducible release of a maximum of cell components.
- Perform the extraction in a wide potential range of sample environments – which may include, but are not limited to, milk, cream, fruit juice, oil emulsions, detergent solutions, liquid dyestuffs and combinations thereof.
- Not interfere significantly with the stability of the released components (such as ATP) prior to subsequent detection and measurement.
- Not interfere significantly with subsequent detection and measurement systems (such as ATP-bioluminescence).

SUMMARY OF THE INVENTION

The present invention relates to a method for effecting a rapid and comprehensive release of cellular contents, including nucleotides and other associated molecules of interest from living cells, using a novel family of quaternary ammonium compounds. In addition, the invention relates to a method of using dimethyl -dialkyl- ammonium halides having varying alkyl chain lengths for selective extraction of microbial cell contents. Finally, the use of these novel compounds exhibits potential to effect the inactivation of ATP-destroying enzymes (used to deplete a sample of its non-microbial ATP).

The present invention demonstrates a superior ability to release ATP from bacteria and yeasts in dairy products. The present invention also demonstrates a superior ability to release ATP from property in case of personal care products incubated in growth media , but with the possible exception of Pseudomonas in certain cases.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 illustrates the effect of the proportion of quaternary ethoxylated amine on the reaction kinetics and level of light emulsion as a function of time after adding ionic surface active agent in bacterial sample.

Figure 2 illustrates the baselines for a prior art kit verses a kit in accordance with the present invention.

Figure 3 illustrates the spiked samples for a prior art kit verses a kit in accordance with the present invention.

Figure 4 illustrates ratios between relative light units for a prior art kit verses a kit in accordance with the present invention.

Figure 5 illustrates the average relative light units for the different organisms tested in different products for a prior art kit verses a kit in accordance with the present invention.

Figure 6 illustrates the signal / blank values for a prior art kit verses a kit in accordance with the present invention.

Figure 7 illustrates the average relative light units for the different organisms tested in different products for a prior art kit verses a kit in accordance with the present invention.

Figure 8 illustrates the signal / blank values for a prior art kit verses a kit in accordance with the present invention.

DETAILED DESCRIPTION OF THE INVENTION

It is known that surface active agents have been used to rupture (lyze) single cells in order to eliminate ATP from somatic cells (*See, for example*, U.S. Patent No. 3,745,090), but the above method has drawbacks which limit its applicability. Rupturing the cell membrane allows enzymes to be released from somatic cells and these enzymes can cause interference in the later phases of the assay of nucleotides. U.S. Patent No. 3,745,090 suggests both non-ionic detergents (octyl phenoxy polyethoxyethanol, terpenoid saponins, steroid saponins, sulfosuccinate glycosides, and fatty acid esters of sorbitol anhydrides) and ionic detergents can be used to rupture somatic cells. Some of these, particularly the ionic surface active agents, affect the permeability of the microbial cell wall and membrane and release

nucleotides (ATP, FMN and other small molecules) from microbial cells. As a result, such surfactants cannot be used for selective rupturing of somatic cells prior to measurement of microbial ATP or other nucleotides. The present invention describes methods of applying specific surface active agents for selective release of nucleotides (purine and pyridine nucleotides, and PMN) either for somatic or microbial cells. This selective release of nucleotides is accomplished without releasing enzymes from cells and the assay of nucleotides can be made without interference from undesired enzymatic hydrolysis of assayed nucleotides.

Concentration of nucleotides is measured in metabolic studies, biochemistry, clinical chemistry and bioassays. About one third of the known 2000 enzymes use purine nucleotides (ATP, ADP, AMP GTP, GMP, ITP, etc.) and pyridine nucleotides (NAD, NADH, NADP, NADPH, CTP, UTP, etc.) as substrates. In conventional methods, these substrates have been extracted from cells by destroying the cells by physical or chemical means and inactivating the enzymes in the cells by freezing, heating or with chemicals. In many conventional methods proteins have to be separated from the sample before the measurement of nucleotides. Such complicated manipulations can cause errors in the assay and make the sample preparation laborious. The present invention makes the sample preparation simple, rapid and reproducible. When these methods are used in conjunction with bioluminescent assay of metabolites, the measurement is specific to ATP, FMN, NADH OR NADPH; whichever is meant to be measured. The samples need not be deproteinized nor the nucleotides separated by chromatography or liquid extraction techniques.

Dimethyl -dialkyl- ammonium halides represent a novel class of Quaternary Ammonium compounds that can be used for efficient and effective release of cellular contents, such as nucleotides, from microbial organisms. Variation of the alkyl chain lengths of dimethyl - dialkyl- ammonium halides may affect their ability to act as nucleotide extractants. It is believed a shorter chain length of approximately 8 to 10 carbon atoms may represent a rapid, quantitative release. It is further believed that extending the chain length beyond 10 carbon atoms may allow for selectively extracting nucleotides from different microbial sources. For example, having an alkyl chain length greater than 10 carbon atoms may allow for the release of bacterial cell contents while yeast cell contents remain largely intact.

Dimethyl -dialkyl- ammonium halides also are able to inactivate apyrase enzymes while leaving luciferase enzyme molecules untouched and fully active. It is believed that this

property can be used to improve and optomise current test methods – inactivating apyrase eliminates the loss of microbial ATP that currently occurs in the interval between extracting microbial contents and adding the ATP-sensitive luciferase enzyme.

DESCRIPTION OF A PREFERRED EMBODIMENT

In the present invention, the nucleotides are released from single cells in suspension or from mono- and bilayers of cells through the cell wall and cell membrane made permeable by means of the action of surface active agents. Certain surface-active agents change the permeability of the cell membrane by affecting the integrity of the lipid and phospholipid layer in the membrane. It is possible to select certain non-ionic surface active agents which do not lyze the somatic cell but make the cell membrane permeable to small-size molecules, such as purine and pyridine nucleotides. When the cell membrane is made permeable to small molecules, nucleotides diffuse out of the cells instantaneously, but enzymes and proteins having a large molecular size will not be able to penetrate the membrane and stay inside the cells. This allows a simple and rapid extraction of nucleotides from cells without having to inactivate enzymes in the cells. Released nucleotides in extracellular solution are stable for several minutes if the solution does not have endogenous enzymes or broken cells in high enough quantities to cause a rapid, undesired enzymatic hydrolysis of nucleotides in the solution.

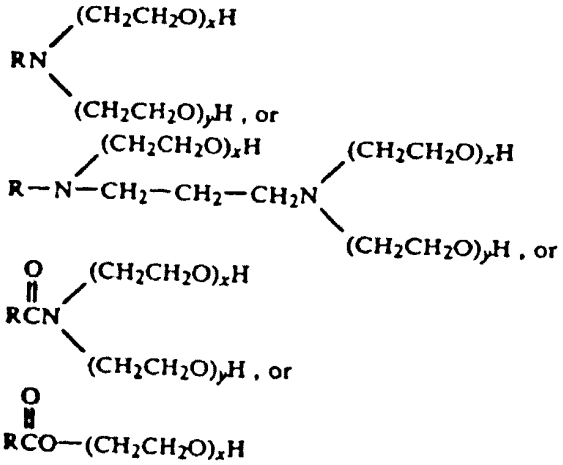
By selecting a non-ionic surfactant that does not lyze somatic cells but only changes the permeability of the membrane, does not affect microbial cell walls, and does not inhibit the bioluminescent reaction, it is possible to release nucleotides selectively from somatic cells for assaying by bioluminescent systems. However, microbial cells, such as bacteria, yeasts, fungi and slime molds have a cell wall that is resistant to chemical and environmental factors. The cell wall contains substances, such as muramic acid (a peptidoglycan) in bacteria, and chitinous substances in fungi which protect the more fragile cell membrane. Therefore, it is necessary to use stronger surface active agents, that is, ionic surface active agents to make the cell wall of microbes permeable for small molecules. The ionic surfactants are selected by their specific properties, such as the length of alkyl chain, degree of ethoxylation (lipophility or hydrophility) and presence of radicals, such as quaternary salts, to affect the permeability of the cell wall of microbial cells for releasing nucleotides. Since many ionic surfactants precipitate proteins and inactivate enzymes, it is necessary to use a surfactant that does not inactivate the luciferase enzyme or other enzymes used in the bioluminescent reaction, or the

reaction conditions have to arranged to such that the interference from the surfactant can be eliminated, for example, by dilution.

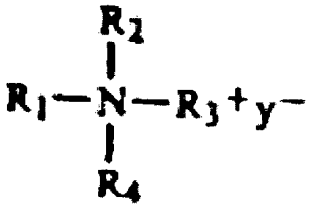
The sample preparation for releasing nucleotides from a suspension of somatic or microbial cells requires only the mixing of the surfactant in a concentration from about 0.02 to about 2% by volume of the total combined volumes of the sample suspension and surface active agent. The diffusion of metabolites from the cells through the membrane is so rapid that even ATP, which has a turnover time in the metabolism within the cell of less than one second, can be quantitatively released.

Samples having primarily microbial cells, for example activated sludge and microbial cultures, can be extracted for nucleotides without prior elimination of nucleotides of non-microbial cells. Microbial cell walls have substances, such as peptidoglycans, chitin and mucoidal substances that make the wall resistant to chemicals. Therefore, it is more difficult to release nucleotides from microbial cells than from somatic cells. According to the present invention, the application of ionic surface active agents changes the permeability of the cell wall and membrane of microbes to make them permeable for small size molecules, such as nucleotides, but not for enzymes. By "ionic surface active agents" is meant anionic or cationic surface active agents to the exclusion of non-ionic surface active agents. This selective permeability is obtained by treating microbes with ionic surface active agents, the best of which are those that contain quaternary ammonium salts and a fatty group with a chain length of 12 carbon atoms; however, any chain length of carbon atoms from 8 to 18 can be used.

Examples of suitable ionic surfactants for quantitative release of nucleotides from microbial cells for bioluminescent measurement with firefly and photobacterial systems are: ethoxylated amines, ethoxylated diamines, polyethylene glycol esters of fatty acids, and ethoxylated amides having a chemical structure of:



where R is a fatty alkyl group having 8-18 carbon atoms and x, y and z are numbers ranging from 2 to 50, and quaternary ammonium salts having a formula of:



where R_1 and R_2 are an alkyl, alkyl-aryl-alkyl, ethoxyalkyl, hydroxyalkyl, or ethoxylated alkylphenol with a 4 to 22 carbon atom chain and the ethoxylated alkyls having 2 to 15 ethoxyl groups, and R_3 and R_4 are alkyl groups having a 1 to 15 carbon atom chain and y, for example, a halogen, sulphate, sulphite or phosphate.

Combinations of the aforesaid amines and quaternary ammonium salts are particularly advantageous, since such combinations facilitate a precise release rate for the nucleotides. Such surface active agents also include hyamine chloride, that is, diisobutyl cresoxy ethoxy ethyl dimethyl ammonium chloride.

Ethoxylated amines release nucleotides quantitatively from microbes, but the quaternary ammonium salts of ethoxylated amines penetrate the cell wall of microbes faster and quaternary salts are affected less by buffers, pH, and other agents possibly encountered in the sample than are ethoxylated amines. A 0.02-0.5% solution of a mixture of 1 to 19 parts of an ethoxylated amine, and 1 to 19 parts of a quaternary ammonium salt of an ethoxylated amine,

both having a carbon atom chain length from 8 to 18, provide a complete and rapid release of nucleotides from bacteria, yeasts, fungi, and slime molds as well as from certain bluegreen, green, brown and red algae. Nucleotides are also released from somatic cells with these reagents, but due to the precipitation of some proteins by the reagents, the release can be incomplete. These reagents do not inhibit firefly luciferase in the degree that would interfere with the measurement. On the contrary, the presence of a low concentration of a quaternary ethoxylated amine (0.001-0.03%) enhances the turnover rate of the firefly luciferase and produces up to about twice as many photons per second during the first part of the reaction as is produced by the same concentration of ATP with the same luciferin-luciferase reagents in plain buffer solution.

The rate of release of ATP and the bioluminescent reaction are affected by the proportions of the ethoxylated amine and the quaternary salt of the ethoxylated amine. The release of ATP and other nucleotides from microbial cells is slow, but still quantitative, and the reaction rate of luciferase is the same as in buffer when ethoxylated amines are used for the release. By including a quaternary salt of an ethoxylated amine with the ethoxylated amine the rate of nucleotide release and the bioluminescent reaction can be increased from moderately slow to moderately fast depending on the proportion of the quaternary ethoxylated amine in the reagent.

The accompanying Figure illustrates the effect of the proportion of quaternary ethoxylated amine on the reaction kinetics and level of light emulsion as a function of time after adding ionic surface active agent in bacterial sample. Numbers refer to the percentage of ethoxylated amine and quarternary ethoxylated amine, respectively in a total concentration of 0.1% in the sample. Both ethoxylated amine and quarternary ethoxylated amine had a chain length of 12 carbons. "A" refers to the time of adding the surface active agent and "B" to the time of adding the firefly reagent. In the tests 100 microliters of 0.2% aqueous solution of the ethoxylated amine and a quaternary ethoxylated amine were pipetted to 100 microliters volume of bacterial suspension (E. coli) and the sample was mixed by shaking for fifteen seconds before being measured in a photometer where 100 microliters of luciferin-luciferase reagent was injected to the sample in a light-tight reaction chamber just prior to the measurement. The ethoxylated amine alone releases ATP from bacteria in 15 seconds and produces the same reaction kinetics as ATP in plain buffer, that is a continuous emission of

light that decays 1 to 10% per minute depending on the proportions of the components in the luciferin-luciferase reagent.

The quaternary salt of an ethoxylated amine used alone causes too fast a reaction rate to allow easy and reproducible measurement of ATP. A mixture of the ethoxylated amine and a quaternary salt of an ethoxylated amine, having both a 12 carbon chain length, gives the possibility to select a desired rate of nucleotide release and reaction kinetics. As can be seen in the Figure, the level of light emission is increased about two times by the use quaternary salt over the ethoxylated amine alone as a releasing agent. More than one half of a quaternary salt of an ethoxylated amine in the reagent causes inactivation of the luciferase enzyme; thus the proportions have to be controlled.

While ethoxylated amines do release nucleotides reasonably rapidly from bacteria, they produce a slow release from yeasts and fungi which have an even stronger cell wall than bacteria. Therefore, it is beneficial to include a quaternary salt of an ethoxylated amine as part of the release reagent. One third of a quaternary ethoxylated amine and two thirds of ethoxylated amine give a complete release of nucleotides, such as ATP and FMN from most bacteria in a few seconds, and from Mycobacteria, yeasts and fungi in 30-60 seconds.

If the sample contains microbial cells only, or if the proportion of non-microbial cells is insignificant (for example, activated sludge, soil, sediments), the release of nucleotides from the sample is accomplished by simply adding, for example a mixture of an ethoxylated amine and a quaternary ethoxylated amine in an end-concentration of 0.05-0.5% by volume of the sample. The reagent and sample are mixed and the reagent is allowed to remain in contact with the cells for a sufficient time to complete the release of nucleotides. After this the sample should be measured within five minutes to avoid any breakdown of nucleotides by the enzymes left inside the cells. These ionic reagents do inactivate some enzymes, among which is photobacterial luciferase. Therefore, the sample should be diluted 5 to 100 times after treatment and before measurement if FMN or pyridine nucleotides are measured in the sample with the bacterial bioluminescent system. The nucleotide release reagent for microbial cells, consisting of ethoxylated amines, can be adjusted to acidic and alkaline pH; thus the reduced and oxidized pyridine nucleotides can selectively be extracted from microbial cells.

The release reagent for microbial nucleotides requires a direct contact with the cell wall, in order to give a quantitative release. Therefore, the cells have to be in suspension. If cells form clumps or flocculates in the sample, they should preferably be dispersed prior to application of the reagent. The dispersion or homogenizing must not stress the cells because stress would affect the level of nucleotides in the cells.

Table 1, below, shows that a linear relationship is obtained between the number of bacterial cells and the concentration of released ATP when a 3:7 mixture of an ethoxylated quaternary amine and ethoxylated amine were used to extract this purine nucleotide from E. coli suspension:

Number of bacteria per milliliter	Relative light units for 10 second integration
150,000	1,480
500,000	4,800
1,000,000	9,580
2,000,000	18,900

Table 1

The procedure was as follows: 100 microliters of 0.2% releasing reagent was added to 100 microliters of sample and the solution was mixed for 15 seconds. The sample was placed in the light-tight reaction chamber of the photon counter. Just prior to starting a 10-second integration, 100 microliters of firefly luciferin-luciferase reagent was added to the sample in a transparent cuvette in the reaction chamber. The bacteria were grown on a liquid nutrient medium and the number of cells was determined by standard colony counting. The different dilutions were made in physiological saline solution.

The extraction efficiency of the release reagent based on a quaternary ethoxylated amine and an ethoxylated amine mixture was also tested against the boiling tris-EDTA buffer and the perchloric acid extraction methods. The samples consisted of whole blood diluted with physiological saline 200 times, a suspension of *E. coli* bacteria in concentration of one million cells per milliliter, and a suspension of green algae, *Chlorella sp.*

Sample treatments were:

- Ionic surface active nucleotide releasing reagent: To a 100 microliter aliquot of sample solution, 100 microliters of a 0.2% aqueous solution of a 3:7 part

mixture of an ethoxylated quaternary amine and ethoxylated amine was pipetted and mixed for 15 seconds (for blood and bacteria) or 60 seconds (for algae).

- Perchloric acid method: 0.1 ml 1 N perchloric acid was pipetted to 1 ml of sample and mixed for one minute. Sample was neutralized by adding 0.1 ml 1 N NaOH.

- Boiling tris-EDTA method: Nine milliliters of tris (0.02 molar)-EDTA (0.002 molar) at pH 7.4 was heated to boiling and 1 ml of sample solution was pipetted on the boiling buffer. Samples were boiled for three minutes and cooled on ice.

- Measurement of ATP by bioluminescence: 100 microliter aliquots of extracted sample solution were pipetted into transparent glass cuvettes and these were placed into the light-tight reaction chamber of a photon counter. Just prior to the measurement, 100 microliters of firefly luciferin-luciferase reagent was injected into the sample. The light emission was integrated for 10 seconds and the results read on the digital display as relative light units. The relative light units were converted to ATP by internal standardization whereby a known quantity of ATP standard was added into an aliquot of the extracted sample and measured as above. The standard was added in 10 microliters in order not to change the total volume of the sample significantly. By subtracting the reading of the sample from that of the sample + added ATP standard a conversion factor for relative light units to ATP was calculated. The results of the extraction efficiency test are given in Table 2, below:

Sample	Ionic surface active nucleotide releasing agent	Perchloric acid method	Tris-EDTA boiling buffer
Whole blood	100%	7%	80%
E. Coli suspension	100%	3%	95%
Chlorella sp., alga	100%	-	100%

Table 2

In all sample types the ionic surface active reagent gave the highest extraction efficiency. Boiling tris-EDTA buffer method extracted the same quantity of ATP from algae, but less from bacteria and blood. The low values obtained with perchloric acid method are due to co-

precipitation of ATP with the perchlorate during the neutralization step. Perchloric ion is also a strong inhibitor of the firefly bioluminescent reaction.

Examples

Appendix A shows comparative results of bacterially spiked samples between an older dairy products test, using less effective releasing agents (Dessert Kit available from Celsis, Inc., 400 West Erie, Suite 300, Chicago Illinois 60610 - 6910 USA) compared to a new test in accordance with the present invention, using a 0.8 percent aqueous solution of didodecyl dimethyl ammonium chloride as an extractant or releasing reagent. These spiked samples were then measured. As a test sample various milk based dairy products were spiked with a number of bacterial strains. 50 microliters of milk based dairy product was incubated with 25 microliters of an apyrase enzyme solution in order to reduce non-microbial ATP, followed by an injection of 50 microliters of extractant. The samples were then allowed to stand for 5 seconds and 100 microliters of Luciferase – Luciferin was injected into the sample. The light signal was then recorded and expressed as relative light units (RLU). Figure 2 illustrates the baselines for a prior art kit verses a kit in accordance with the present invention. Figure 3 illustrates the spiked samples for a prior art kit verses a kit in accordance with the present invention. Figure 4 illustrates ratios between relative light units for a prior art kit verses a kit in accordance with the present invention.

Appendix B is similar to Appendix A and gives a comparison between a current, commercially available test (Biotrace kit available from Biotrace International Plc., The Science Park, Bridgend, Wales CF31 3NA United Kingdom) and a new test in accordance with the present invention, which uses halide containing quaternary ammonium compounds as releasing agents as disclosed in this application.

Appendix C also gives a comparison between an older test (Rapid Screen Kit available from Celsis, Inc.) and a new test in accordance with the present invention, which uses halide containing quaternary ammonium compounds as releasing agents. Specifically, this Appendix shows test results for non-dairy types of samples incubated over 16 hours in a nutrient solution in order to allow microbes in the samples to multiply. The results are expressed as ratio of the light signal divided by the background noise signal – this ratio is used regularly to show differences in detection sensitivity. Figure 5 illustrates the average relative light units for the different organisms tested in different products for a prior art kit

verses a kit in accordance with the present invention. Figure 6 illustrates the signal / blank values for a prior art kit verses a kit in accordance with the present invention. Figure 7 illustrates the average relative light units for the different organisms tested in different products for a prior art kit verses a kit in accordance with the present invention. Figure 8 illustrates the signal / blank values for a prior art kit verses a kit in accordance with the present invention. This incubation technique is common for products such as shampoos and tooth pastes, which have lower numbers of microbes than other products. Incubation of such samples is a multiplying technique which allows a low number of microbes to be increased and thereby made more easily detectable. The new kit allows for much better and faster detection of bacterial strains which grow slowly such as *Burkholderia* and *Candida*.

Appendix D is similar to Appendix C except that the results are expressed as relative light units instead of signal to blank ratio as in Appendix C.

Appendix E describes the use of didodecyl dimethyl ammonium bromide as a selective releasing reagent for Gram negative bacteria in presence of yeast cells.

While the invention has been described with specific embodiments, other alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it will be intended to include all such alternatives, modifications and variations set forth within the spirit and scope of the appended claims.

Appendix A (Sheet 1 of 4)

Chicago Lab Study 3 - Concell 'Unilait' kit vs current Concell 'Dessert' kit - further baselines and spiked samples

Sheet 1 - All data, Plate Layout
Sheet 2 - Sorted data - Baselines
Sheet 3 - Sorted data - Spiked Samples
Sheet 4 - Protocols
Author - A.Hearn
Date - 8 June 03

Raw data

Controls

	unilait ctrls	dessert ctrls
Inst blk	4	5
Inst blk	4	7
ATP +ve	235603	173312
ATP +ve	225945	162699

Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Choc Rice Milk	Choc Rice Milk	Choc Soy milk	Choc Soy milk	UHT whole	UHT whole	Irish Crème	Irish Crème	French Vanilla	French Vanilla	Hazelnut	Hazelnut
B	Butter Pecan	Butter Pecan	Canela	Canela	Amaretto	Amaretto	Vanilla Haz'nut	Vanilla Haz'nut	Toffee Caramel	Toffee Caramel	Heavy whip crean	Heavy whip crean
C	Heavy whip crean	Heavy whip crean	Half & Half	Half & Half	No sample	No sample	No sample	No sample	No sample	No sample	No sample	No sample
D	Choc Rice Milk + E.coli	Choc Rice Milk + E.coli	Choc Rice Milk + E.coli	Choc Rice Milk + P.aerug	Choc Rice Milk + P.aerug	Choc Rice Milk + P.aerug	Choc Rice Milk + S.aureus	Choc Rice Milk + S.aureus	Choc Rice Milk + S.aureus	Choc Rice Milk + B.cereus	Choc Rice Milk + B.cereus	Choc Rice Milk + B.cereus
E	Choc Soy milk + E.coli	Choc Soy milk + E.coli	Choc Soy milk + E.coli	Choc Soy milk + P.aerug	Choc Soy milk + P.aerug	Choc Soy milk + P.aerug	Choc Soy milk + S.aureus	Choc Soy milk + S.aureus	Choc Soy milk + S.aureus	Choc Soy milk + B.cereus	Choc Soy milk + B.cereus	Choc Soy milk + B.cereus
F	UHT whole + E.coli	UHT whole + E.coli	UHT whole + E.coli	UHT whole + P.aerug	UHT whole + P.aerug	UHT whole + P.aerug	UHT whole + S.aureus	UHT whole + S.aureus	UHT whole + S.aureus	UHT whole + B.cereus	UHT whole + B.cereus	UHT whole + B.cereus
G	Vanilla Haz'nut + E.coli	Vanilla Haz'nut + E.coli	Vanilla Haz'nut + E.coli	Vanilla Haz'nut + P.aerug	Vanilla Haz'nut + P.aerug	Vanilla Haz'nut + P.aerug	Vanilla Haz'nut + S.aureus	Vanilla Haz'nut + S.aureus	Vanilla Haz'nut + S.aureus	Vanilla Haz'nut + B.cereus	Vanilla Haz'nut + B.cereus	Vanilla Haz'nut + B.cereus
H	Half & Half + E.coli	Half & Half + E.coli	Half & Half + E.coli	Half & Half + P.aerug	Half & Half + P.aerug	Half & Half + P.aerug	Half & Half + S.aureus	Half & Half + S.aureus	Half & Half + S.aureus	Half & Half + B.cereus	Half & Half + B.cereus	Half & Half + B.cereus

Plate 1 - unilait 5s 1											
8	9	4	1	5	5	4	8	4	3	2	2
9	11	17	14	5	7	10	10	8	7	341	296
71	75	16	14	2	1	2	1	2	2	1	1
27864	27183	34333	50105	50576	48572	74260	90455	72605	213784	237511	234508
82974	78708	74174	84175	64869	68114	92060	96096	96012	105836	119246	90674
243555	224857	248390	402434	306369	318080	71576	84569	87522	152128	139454	141273
77	76	75	11	11	12	422	430	408	12	7	44
189599	199680	187738	275010	305505	285673	46821	49803	53788	119260	125970	124947

Plate 2 - unilait 10s 1											
9	12	4	3	6	9	4	4	5	5	4	3
9	8	22	19	7	6	12	13	8	7	219	220
61	91	19	17	2	2	1	1	2	1	1	1
16073	25757	26120	39302	35181	41031	59905	59145	48816	242592	244894	249230
50475	57805	56143	74971	62833	57502	76797	96570	79788	85074	86216	96926
124891	170198	177573	300038	305244	287716	96679	104557	93703	99148	95940	86743
48	64	61	16	14	15	334	325	329	17	10	10
140709	141311	155070	277482	272485	263110	68216	63843	70248	114336	113329	102955

Plate 3 - Dessert 1											
3	85	3	1	7	4	7	7	6	7	5	7
13	15	35	41	11	12	21	23	19	17	41	25
28	45	13	9	2	1	1	1	1	1	1	1
2006	2863	3186	16332	17816	13458	17213	15953	15721	568	87	66
22896	24865	32900	47288	48056	53096	29823	32479	29255	59803	63648	54504
32198	46268	41306	81859	67620	83837	26942	26738	27753	27209	27618	23523
28	44	38	21	24	24	111	91	109	24	23	22
32872	34954	28307	55577	57980	54142	14871	16284	11984	33003	29012	30061

Plate 4 - Dessert 2											
4	4	3	5	9	9	8	7	9	9	29	7
17	16	36	35	15	15	24	20	17	18	114	20
21	29	12	10	2	1	2	4	2	2	7	2
1208	1852	2061	12488	13320	12200	14482	10841	11108	1828	46	98
17922	18301	25603	31546	40334	45315	18868	27378	22689	42320	42393	39069
24890	31962	36349	68846	67641	66497	23351	22654	28430	32276	19833	20619
31	38	36	28	24	28	79	101	91	23	29	26
22489	26341	24788	63516	70775	47996	12307	8953	13112	34922	24530	21866

Plate 5 - unilait 10s 2											
6	8	2	2	5	8	4	5	6	3	3	6
9	10	22	21	6	11	13	13	9	8	159	56
28	75	14	15	1	1	1	1	2	1	1	1
5227	11622	12577	19824	15864	14757	30680	37676	32381	196582	184029	191861
20914	23220	31910	37339	39141	44375	60383	69317	59755	125404	108192	105461
61412	73490	93481	196356	224458	222569	76987	75483	81172	91718	90092	80497
29	40	34	16	19	17	263	233	298	13	11	13
67357	84275	78391	265851	274911	296780	53085	57011	48868	85316	85533	77608

Plate 6 - unilait 5s 2											
7	7	3	4	3	9	6	6	5	5	5	3
15	10	25	24	8	9	16	15	9	9	41	42
49	110	16	14	2	1	1	1	1	1	1	1
5278	11330	12197	17594	15351	16668	34804	43370	37804	225054	200048	241477
26232	31682	43720	42823	46920	45127	57707	78513	60811	147592	123265	127190
68052	87858	99912	247019	223983	217455	69919	75679	75789	111793	109440	101701
25	39	39	17	21	19	272	224	280	27	11	14
75304	119059	118713	328278	321280	324569	51905	49360	57456	103591	96487	91668

Appendix A (Sheet 2 of 4)

Sample Baselines (sporadic 'flyers' highlighted in red; not used in average/SD/CV calculation

UNILAIT KIT 5SEC EXTRACTION

	Choc Rice Milk	Choc Soy milk	UHT whole	Irish Crème	French Vanilla	Hazelnut	Butter Pecan	Canela	Amaretto	Vanilla Haz'nut	Toffee Caramel	Heavy whip cream	Heavy whip cream	Half & Half
	8	4	5	4	4	2	9	17	5	10	8	341	71	16
Plate 1	9	1	5	8	3	2	11	14	7	10	7	296	75	14
Plate 1	7	3	3	6	5	5	15	25	8	16	9	41	49	16
Plate 6	7	4	9	6	5	3	10	24	9	15	9	42	110	14
Plate 6	**8**	**3**	**6**	**6**	**4**	**3**	**11**	**20**	**7**	**13**	**8**	**180**	**76**	**15**
Average	**1**	**1**	**3**	**2**	**1**	**1**	**3**	**5**	**2**	**3**	**1**	**161**	**25**	**1**
SD	**12**	**47**	**46**	**27**	**23**	**47**	**23**	**27**	**24**	**25**	**12**	**89**	**33**	**8**
CV														

UNILAIT KIT 10SEC EXTRACTION

	Choc Rice Milk	Choc Soy milk	UHT whole	Irish Crème	French Vanilla	Hazelnut	Butter Pecan	Canela	Amaretto	Vanilla Haz'nut	Toffee Caramel	Heavy whip cream	Heavy whip cream	Half & Half
	9	4	6	4	5	4	9	22	7	12	8	219	61	19
Plate 2	12	3	9	4	5	3	8	19	6	13	7	220	91	17
Plate 2	6	2	5	4	6	3	9	22	6	13	9	159	28	14
Plate 5	8	2	8	5	3	6	10	21	11	13	8	56	75	15
Plate 5	**9**	**3**	**7**	**4**	**5**	**4**	**9**	**21**	**8**	**13**	**8**	**164**	**64**	**16**
Average	**3**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**77**	**27**	**2**
SD	**29**	**35**	**26**	**12**	**26**	**35**	**9**	**7**	**32**	**4**	**10**	**47**	**42**	**14**
CV														

CONCELL DESSERT KIT

	Choc Rice Milk	Choc Soy milk	UHT whole	Irish Crème	French Vanilla	Hazelnut	Butter Pecan	Canela	Amaretto	Vanilla Haz'nut	Toffee Caramel	Heavy whip cream	Heavy whip cream	Half & Half
	3	3	7	7	6	5	13	35	11	21	19	41	28	13
Plate 3	85	1	4	7	7	7	15	41	12	23	17	25	45	9
Plate 3	4	3	9	8	9	29	17	36	15	24	17	114	21	12
Plate 4	4	5	9	7	9	7	16	35	15	20	18	20	29	10
Plate 4	**4**	**3**	**7**	**7**	**8**	**6**	**15**	**37**	**13**	**22**	**18**	**29**	**31**	**11**
Average	**1**	**2**	**2**	**1**	**2**	**1**	**2**	**3**	**2**	**2**	**1**	**11**	**10**	**2**
SD	**16**	**54**	**33**	**7**	**19**	**18**	**11**	**8**	**16**	**8**	**5**	**38**	**33**	**17**
CV														

	Choc Rice Milk	Choc Soy milk	UHT whole	Irish Crème	French Vanilla	Hazelnut	Butter Pecan	Canela	Amaretto	Vanilla Haz'nut	Toffee Caramel	Heavy whip cream	Heavy whip cream	Half & Half
	8	3	6	6	4	3	11	20	7	13	8	180	76	15
UNILAIT KIT 5SEC EXTRACTION	9	3	7	4	5	4	9	21	8	13	8	164	64	16
UNILAIT KIT 10SEC EXTRACTION	4	3	7	7	8	6	15	37	13	22	18	29	31	11
CONCELL DESSERT KIT														

Appendix A (Sheet 3 of 4)

Spiked Sample Data

UNILAIT KIT 5SEC EXTRACTION

	E.coli					P.aeruginosa					S.aureus					Bacillus cereus				
	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half
	27864	82974	243555	77	189599	50105	84175	402434	11	275010	74260	92060	71576	422	46821	213784	105836	152128	12	119260
Plate 1	27183	78708	224857	76	199680	50576	64869	306369	11	305505	90455	96096	84569	430	49803	237511	119246	139454	7	125970
Plate 2	34333	74174	248390	75	187738	48572	68114	318060	12	285673	72605	96012	87522	408	53788	234508	90674	141273	44	124947
Plate 3	5278	26232	68052	25	75304	17594	42823	247019	17	328278	34804	57707	69919	272	51905	225054	147592	111793	27	103591
Plate 6	11330	31682	87858	39	119059	15351	46920	223983	21	321280	43370	78513	75679	224	49360	200048	123265	109440	11	96487
Plate 6	12197	43720	99912	39	118713	16668	45127	217455	19	324569	37804	60811	75789	280	57456	241477	127190	101701	14	91668
Plate 6	19698	56248	162104	55	148349	33144	58671	285887	15	306719	58883	80200	77509	339	51522	225397	118967	125965	19	110321
Average	11585	25304	85139	23	50911	18218	16436	70801	4	22115	23179	17482	7060	91	3748	15911	19383	20801	14	14589
SD	59	45	53	42	34	55	28	25	29	7	39	22	9	27	7	7	16	17	73	14
CV																				

UNILAIT KIT 5SEC EXTRACTION

	E.coli					P.aeruginosa					S.aureus					Bacillus cereus				
	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half
	16073	50475	124891	48	140709	39302	74971	300038	16	277482	59905	76797	96679	334	68216	242592	85074	99148	17	114336
Plate 2	25757	57805	170198	64	141311	35181	62833	305244	14	272485	59145	96570	104557	325	63643	244894	86216	95940	10	113329
Plate 2	26120	56143	177573	61	155070	41031	57502	287716	15	263110	48816	79788	93703	329	70248	249230	96926	86743	10	102955
Plate 2	5227	20914	61412	29	67357	19824	37339	196356	16	265851	30680	60383	76987	263	53085	196582	125404	91718	13	85316
Plate 5	11622	23220	73490	40	84275	15864	39141	224458	19	274911	37676	69317	75483	233	57011	184029	108192	90092	11	85533
Plate 5	12577	31910	93481	34	79391	14757	44375	222569	17	296780	32381	59755	81172	298	48866	191861	105461	80497	13	77608
Plate 5	16229	40078	116841	46	111352	27660	52694	256064	16	275103	44767	73768	88097	297	60212	218198	101212	90690	12	96513
Average	8298	16725	49188	14	38369	12148	14905	46990	2	11932	13072	13861	11687	41	8576	30329	15203	6633	3	15781
SD	51	42	42	31	34	44	28	18	11	4	29	19	13	14	14	14	15	7	22	16
CV																				

CONCELL DESSERT KIT (PLATE 3)

	E.coli					P.aeruginosa					S.aureus					Bacillus cereus				
	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half
	2006	22896	32198	28	32872	16332	47288	81859	21	55577	17213	29823	26942	111	14871	568	59803	27209	24	33003
Plate 3	2863	24865	48268	44	34954	17816	48056	67620	24	57980	15953	32479	26738	91	16284	87	63648	27618	23	29012
Plate 3	3186	32900	41306	38	28307	13458	53096	83837	24	54142	15721	29255	27753	109	11984	66	54504	23523	22	30061
Plate 3	1208	17922	24890	31	22489	12488	31546	68846	28	63516	14482	18868	23351	79	12307	1828	42320	32276	23	34922
Plate 4	1852	18301	31962	38	26341	13320	40334	67641	24	70775	10841	27378	22654	101	8953	46	42393	19833	29	24530
Plate 4	2061	25603	36349	36	24788	12200	45315	66497	28	47996	11108	22689	28430	91	13112	98	39069	20619	26	21866
Plate 4	2196	23748	35829	36	28292	14269	44273	72717	25	58331	14220	26749	25978	97	12918	449	50290	25180	25	28899
Average	717	5528	8158	6	4798	2274	7481	7908	3	7922	2660	5051	2392	12	2536	704	10378	4745	3	4961
SD	33	23	23	16	17	16	17	11	11	14	19	19	9	13	20	157	21	19	11	17
CV																				

	E.coli					P.aeruginosa					S.aureus					Bacillus cereus				
	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half
	19698	56248	162104	55	148349	33144	58671	285887	15	306719	58883	80200	77509	339	51522.16667	225397	118967.1667	125964.833	19.16666667	110320.5
UNILAIT KIT	6816	20600	56762	26	51055	11811	22056	97935	10	104885	20521	28423	26637	121	18026	75419	43122	43576	11	38433
UNILAIT KIT	2196	23748	35829	36	28292	14269	44273	72717	25	58331	14220	26749	25978	97	12918.5	448.8333333	50289.5	25179.6667	24.5	28899
CONCELL DESSERT KIT																				

CONCELL DESSERT KIT

	E.coli					P.aeruginosa					S.aureus					Bacillus cereus				
	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half	Choc Rice Milk	Choc Soy milk	UHT whole	Vanilla Haz'nut	Half & Half
	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Ratio Unilait 10s	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Ratio Unilait 5s : Dessert																				

EXHIBIT A (Sheet 4 of 4)

	Unilait kit
1	
Reagents	
	ATX Lot No.: 14010503 Ref. No.: 321613000
Apyrase reagent	Dilutor ED Lot No. 05200301, Ref.no. 030501235
Apyrase buffer	Cellsolver PD Lot. No.: 05200302; Ref. No.: 090502175
cellsolving reagent	Sensilux DE200 tests Cat.No.: 321714000 Lot. No.: 11051002
light reagent	DilutorPM Lot. No.: 05210303 Ref.no: 090503575
light reagent buffer	
Instrument and settings	
sample volume	50 µL
Instrument	M 596
Instrument serial no.	801545
RLU coefficient	0, 24
Protocol name	Unilait
Protocol type	well by well
Vol inj 1	25
Vol inj 2	50
Vol inj 3	100
Timing scheme 1st / 2nd inj	Incub
Incubation time	10 minutes
Delay 2nd / 3rd inj	5 seconds
Delay 3rd inj / meas	0.5
Measuring time	2 seconds
Shaking duration (sec.)	15
Shaking interval (sec.)	30
Shaking speed (steps / s)	5000
injector speed	3500
total volume:	225µL
room temperature	23°C
	Concell Dessert
2	
Reagents	
	ATX Lot No.: 14061002 Ref. No.: 321613000
Apyrase reagent	Dilutor ED Lot No. 15031002, Ref.no. 321726235
Apyrase buffer	Cellsolver PD, Ref. 321736175, lot.no. 13021102
cellsolving reagent	SensiluxDE 200 tests Cat.No.: 321714000 Lot. No.: 11051002
light reagent	DilutorPM Lot. No.: 12010203 Ref.no: 321723575
light reagent buffer	
Instrument and settings	
sample volume	50 µL
Instrument	M 596
Instrument serial no.	801545
RLU coefficient	0, 24
Protocol name	dessert1
Protocol type	well by well
Vol inj 1	25
Vol inj 2	50
Vol inj 3	100
Timing scheme 1st / 2nd inj	Incub
Incubation time	10 minutes
Delay 2nd / 3rd inj	7 seconds
Delay 3rd inj / meas	0.5
Measuring time	2 sec.
Shaking duration (sec.)	15
Shaking interval (sec.)	30
Shaking speed (steps / s)	5000
injector speed	3500
total volume:	225µL
room temperature	23°C

CONFIDENTIAL

EXHIBIT B (1 sheet)

European dairy Products

Sample	New Kit	New Kit	Competitor Kit
	2 sec extr.		
Milk, 3.5% fat	M596	M596	M596
Blank	10	11	11
Pseudomonas aeruginosa	24	208096	27920
Ratio	2	18918	2538
-1	15	91225	10210
Ratio	2	8293	928
-2	16	4709	537
Ratio	2	428	49
-3	32	368	63
Ratio	3	33	6
Staphylococcus aureus	67144	713264	32783
Ratio	6714	64842	2980
-1	11215	53894	5964
Ratio	1122	4899	542
-2	1151	3357	557
Ratio	115	305	51
-3	114	242	78
Ratio	11	22	7
Bacillus stearothermophilus	57512	401707	56518
Ratio	5751	36519	5138
-1	3421	18170	1878
Ratio	342	1652	171
-2	401	755	251
Ratio	40	69	23
-3	64	124	56
Ratio	6	11	5
Escherichia coli	75719	156671	44983
Ratio	7572	14243	4089
-1	19454	94886	17439
Ratio	1945	8626	1585
-2	1860	7377	1401
Ratio	186	671	127
-3	181	603	145
Ratio	18	55	13
Madibic, Volume extra			
Blank	14	41	30
Pseudomonas aeruginosa	103488	267670	62198
Ratio	7392	6529	2073
-1	14597	15988	4351
Ratio	1043	390	145
-2	1530	1021	317
Ratio	109	25	11
-3	149	266	123
Ratio	11	6	4
Staphylococcus aureus	77104	86614	16722
Ratio	5507	2113	557
-1	4517	4260	932
Ratio	323	104	31
-2	437	416	171
Ratio	31	10	6
-3	101	214	84
Ratio	7	5	3
Bacillus stearothermophilus	256900	65258	13445
Ratio	18350	1592	448
-1	24042	3097	782
Ratio	1717	76	26
-2	4119	335	156
Ratio	294	8	5
-3	347	138	67
Ratio	25	3	2
Escherichia coli	29010	146836	43660
Ratio	2072	3581	1455
-1	13785	17897	3853
Ratio	985	437	128
-2	1758	1159	361
Ratio	126	28	12
-3	222	189	92
Ratio	16	5	3

8/11/2004

Appendix C (Sheet 1 of 2)

		RapiScreen Kit	New Kit, containing novel extractant
strains	**Sample**	**signal / blank**	
Aspergillus niger	*A. n.* Eucerin	2	2
Candida albicans	*C. albi.* Eucerin	1 637	4 265
Pseudomonas aeruginosa	*Ps. ae.* Eucerin	26 330	10 952
Staphylococcus aureus	*St. au.* Eucerin	26 330	46 468
Aspergillus niger	*A. n.* Toothpaste	1	1
Candida albicans	*C. albi.* Toothpaste	17	99
Pseudomonas aeruginosa	*Ps. ae.* Toothpaste	9 234	10 461
Staphylococcus aureus	*St. au.* Toothpaste	74	136
Aspergillus niger	*A. n.* Listerine	1	3
Candida albicans	*C. albi.* Listerine	989	2 879
Pseudomonas aeruginosa	*Ps. ae.* Listerine	28 090	13 205
Staphylococcus aureus	*St. au.* Listerine	28 090	57 937
Aspergillus niger	*A. n.* Nyquil	2	2
Candida albicans	*C. albi.* Nyquil	1 096	2 839
Pseudomonas aeruginosa	*Ps. ae.* Nyquil	28 885	12 767
Staphylococcus aureus	*St. au.* Nyquil	28 885	54 407
Candida albicans	*C. albi.* Cleansing lotion	733	2 693
Pseudomonas aeruginosa	*Ps. ae.* Cleansing lotion	21 645	11 774
Staphylococcus aureus	*St. au.* Cleansing lotion	21 645	48 544
Burkholderia cepacia	*B. cep.* Cleansing Lotion	8	2212
Aspergillus niger	*A. n.* Eucerin	18	1504
Burkholderia cepacia	*B. cep.* Eucerin	77	79
Burkholderia cepacia	*B. cep.* Listerine	10	1114
Aspergillus niger	*A.n.* Toothpaste	18	1721
Burkholderia cepacia	*B. cep.* Toothpaste	87	62
Burkholderia cepacia	*B. cep.* Nyquil	149	1526

		Rapid Screen Kit	Rapid Screen Kit III
strains	**Sample**	**Average RLU**	
	TAT Control*	2 386	2 084
	Eucerin Control	3 798	2 152
Aspergillus niger	*A. n.* Eucerin	6 948	4 725
Candida albicans	*C. albi.* Eucerin	6 218 673	9 178 709
Pseudomonas aeruginosa	*Ps. ae.* Eucerin	99 999 999	23 568 014
Staphylococcus aureus	*St. au.* Eucerin	99 999 999	99 999 999
	Toothpaste Control	1 661	765
Aspergillus niger	*A. n.* Toothpaste	1 325	608
Candida albicans	*C. albi.* Toothpaste	28 233	75 494
Pseudomonas aeruginosa	*Ps. ae.* Toothpaste	15 337 186	8 002 780
Staphylococcus aureus	*St. au.* Toothpaste	123 165	104 164
	TAT Control	3 713	1 949
	Listerine Control	3 560	1 726
Aspergillus niger	*A. n.* Listerine	4 849	4 333
Candida albicans	*C. albi.* Listerine	3 521 922	4 969 957
Pseudomonas aeruginosa	*Ps. ae.* Listerine	99 999 999	22 791 965
Staphylococcus aureus	*St. au.* Listerine	99 999 999	99 999 999
	TAT Control	3 359	1 594
	Nyquil Control	3 462	1 838
Aspergillus niger	*A. n.* Nyquil	6 304	3 864
Candida albicans	*C. albi.* Nyquil	3 794 861	5 217 875
Pseudomonas aeruginosa	*Ps. ae.* Nyquil	99 999 999	23 465 081
Staphylococcus aureus	*St. au.* Nyquil	99 999 999	99 999 999
	Cleansing Lotion Control**	4 620	2 060
Candida albicans	*C. albi.* Cleansing lotion	3 387 935	5 548 598
Pseudomonas aeruginosa	*Ps. ae.* Cleansing lotion	99 999 999	24 254 605
Staphylococcus aureus	*St. au.* Cleansing lotion	99 999 999	99 999 999

Appendix C (Sheet 2 of 2)

		Rapid Screen Kit	Rapid Screen Kit III	RS 3 vs. RS 1				
strains	**Sample**	**signal / blank**			AspN	Candida	Psdm	Staph
Aspergillus niger	*A. n.* Eucerin	2	2	1.2	1.2			
Candida albicans	*C. albi.* Eucerin	1 637	4 265	2.6		2.6		
Pseudomonas aeruginosa	*Ps. ae.* Eucerin	26 330	10 952	0.4			0.4	
Staphylococcus aureus	*St. au.* Eucerin	26 330	46 468	1.8				1.8
Aspergillus niger	*A. n.* Toothpaste	1	1	1.0	1.0			
Candida albicans	*C. albi.* Toothpaste	17	99	5.8		5.8		
Pseudomonas aeruginosa	*Ps. ae.* Toothpaste	9 234	10 461	1.1			1.1	
Staphylococcus aureus	*St. au.* Toothpaste	74	136	1.8				1.8
Aspergillus niger	*A. n.* Listerine	1	3	1.8	1.8			
Candida albicans	*C. albi.* Listerine	989	2 879	2.9		2.9		
Pseudomonas aeruginosa	*Ps. ae.* Listerine	28 090	13 205	0.5			0.5	
Staphylococcus aureus	*St. au.* Listerine	28 090	57 937	2.1				2.1
Aspergillus niger	*A. n.* Nyquil	2	2	1.2	1.2			
Candida albicans	*C. albi.* Nyquil	1 096	2 839	2.6		2.6		
Pseudomonas aeruginosa	*Ps. ae.* Nyquil	28 885	12 767	0.4			0.4	
Staphylococcus aureus	*St. au.* Nyquil	28 885	54 407	1.9				1.9
Candida albicans	*C. albi.* Cleansing lotion	733	2 693	3.7		3.7		
Pseudomonas aeruginosa	*Ps. ae.* Cleansing lotion	21 645	11 774	0.5			0.5	
Staphylococcus aureus	*St. au.* Cleansing lotion	21 645	48 544	2.2				2.2
				Average	**1.3**	**3.5**	**0.6**	**2.0**

B.cepacia and 48 Hour Mold Results

		Rapid Screen Kit	Rapid Screen Kit III
strains	**Sample**	**Average RLU**	
	TAT Control	2 180	1 474
	Cleansing Lotion Control	2 601	1 092
Burkholderia cepacia	*B. cep.* Cleansing Lotion	20 408	2 415 979
	Eucerin Control	3 538	1 558
Aspergillus niger	*A. n.* Eucerin	64 059	2 343 051
Burkholderia cepacia	*B. cep.* Eucerin	273 429	122 759
	Listernine Control	2 988	1 335
Burkholderia cepacia	*B. cep.* Listerine	30 414	1 486 930
	Toothpaste Control	580	254
Aspergillus niger	*A.n.* Toothpaste	10 712	437 025
Burkholderia cepacia	*B. cep.* Toothpaste	50 397	15 771
	Nyquil Control	2 861	1 124
Burkholderia cepacia	*B. cep.* Nyquil	427 695	1 715 364

B.cepacia and 48 Hour Mold Results

		Rapid Screen Kit	Rapid Screen Kit III	RS 3 vs. RS 1		
strains	**Sample**	**signal / blank**			Burkh	AspN
				282	282	
Burkholderia cepacia	*B. cep.* Cleansing Lotion	8	2212	83		83
Aspergillus niger	*A. n.* Eucerin	18	1504	1	1	
Burkholderia cepacia	*B. cep.* Eucerin	77	79	109	109	
Burkholderia cepacia	*B. cep.* Listerine	10	1114	93		93
Aspergillus niger	*A.n.* Toothpaste	18	1721	1	1	
Burkholderia cepacia	*B. cep.* Toothpaste	87	62	10	10	
Burkholderia cepacia	*B. cep.* Nyquil	149	1526	**Average**	**81**	**88**

Appendix D (1 sheet)

Preparation of organisms

1. strains were taken from plate and diluted in peptone water for CFU determination with McFarland standard or microscope

Burkholderia cepacia (ATCC 25416)	inoculation with 500 CFU
Pseudomonas aeruginosa (ATCC 10145)	inoculation with 620 CFU
Pseudomonas aeruginosa (ATCC 9027)	inoculation with 640 CFU
Candida albicans (ATCC 10231)	inoculation with 260 CFU
Aspergillus niger	inoculation with 200 Ascospores

2. Inoculated into media with the PCP product (1 % ; use of TAT Broth with Tween;) grown after inoculation 48 h

3. Assayed pereformed on Celsis Advance Luminometer

4. during the whole experiment the prepared cultures were stored in the refrigerator in order to prevent further growth

Kits examined

1) Rapid Screen Kit II from Celsis no.: 1230834 (Rapid Screen) with LuminHance

2) New Kit

200 µL New releasing reagent No.: 9020505

100 µL Luciferin-Luciferase Reagent : LuminATE (rHS) , No.: 11020103 dissolved in LuminATE buffer for rHS; No.: 8120008

device protocols used

	RapiScreen II	New Kit
Instrument	0	0
Instrument serial no.:	0	0
Control	0	0
Protocol name:	PCP 5	PCP 1
sample volume in µL	50	50
Vol inj 1	50	
Vol inj 2	200	200
Vol inj 3	100	100
measuring time	0	0
measurement protocol	3StepT.cel.	0
cal factor	0	0
delay 1	900 sec.	0
delay 2	0	0
delay 3	0	0
background measuring time	0	0
temperature	0	0
temperatur control	0	0
RLU - coefficient	0.586	0.586

average RLU measured

product no.: 18	Toothpaste; ingredients: aqua; hydrated silica, sorbitol and/or glycerin; pentasodium triphosphate; sodium laurylsulfate; flavor;		
strain tested	dilution	Kit tested	
		RapiScreen II	New Kit
Burkholderia cepacia (ATCC 25416)	10^{0}	2 503 153	10 544 611
	10^{-4}	9 188	13 376
	10^{-6}	3 847	5 190
Aspergillus niger	10^{0}	770 943	740 642
	10^{-2}	10 706	10 549
	10^{-4}	1 963	1 961

product no.: 19	Antiseptic Mouthwash", ingredients: Thymol 0,064 %; Eucalyptol 0,092 %; methyl salicylate 0,06%; Menthol: 0,042 %; water,		
strain tested	dilution	Kit tested	
		RapiScreen II	New Kit
Burkholderia cepacia (ATCC 25416)	10^{0}	795 841	4 490 095
	10^{-4}	10 312	15 581
	10^{-6}	5 345	7 745
Aspergillus niger	10^{0}	50 699 963	52 525 182
	10^{-2}	15 254	18 464
	10^{-4}	4 964	6 090

product no.: 20	Cold Medicine; ingredients: alcohol, citric acid, FD & blue no.1; FD & C red no. 40; flavor; high fructose corn syrup; polyethylenglycol,		
strain tested	dilution	Kit tested	
		RapiScreen II	New Kit
Burkholderia cepacia (ATCC 25416)	10^{0}	1 912 992	7 205 929
	10^{-4}	13 511	18 206
	10^{-6}	7 523	9 407
Aspergillus niger	10^{0}	1 400 108	1 645 284
	10^{-2}	25 930	29 062
	10^{-4}	6 386	6 942

product no.: 21	Hand Cleanser; ingredients: purified water; Glycerin; Caprylic / Capric Triglycerides; Cetyl Ricinoleate; Isohexadecane; TEA - Cocoyl		
strain tested	dilution	Kit tested	
		RapiScreen II	New Kit
Burkholderia cepacia (ATCC 25416)	10^{0}	651 344	3 324 474
	10^{-4}	10 734	14 508
	10^{-6}	5 066	6 940
Aspergillus niger	10^{0}	99 999 999	12 296 746
	10^{-2}	34 015	42 119
	10^{-4}	5 706	6 256

product no.: 22	Moisturizing Lotion; ingredients: water; mineral oil; PEG - 7 Hydrogenated castor oil; Isohexadecone; Sodium Lactate; Urea;		
strain tested	dilution	Kit tested	
		RapiScreen II	New Kit
Burkholderia cepacia (ATCC 25416)	10^{0}	490 305	2 334 294
	10^{-4}	14 942	29 380
	10^{-6}	11 028	22 589
Aspergillus niger	10^{0}	2 379 615	99 999 999
	10^{-2}	40 061	51 453
	10^{-4}	8 184	10 448

Appendix E

	Explanation	RLU	% vs DTAB
Bacteria	Dodecyl trimethyl 1.35%	985963	100%
	Dodecyl trimethyl 0.50%	918394	93%
	Trioctyl 0.05 %	929203	94%
	Trioctyl 0.20 %	872688	89%
	Didodecyl 0.05 %	847920	86%
	Didodecyl 0.20 %	888448	90%
	Dodecyl trimethyl 1.35%	1189402	100%
	Dodecyl trimethyl 0.50%	949932	80%
	Trioctyl 0.05 %	950118	80%
	Trioctyl 0.20 %	896398	75%
	Didodecyl 0.05 %	1046059	88%
	Dodecyl trimethyl 1.35%	226997	100%
	Dodecyl trimethyl 0.50%	204146	90%
	Trioctyl 0.05 %	191689	84%
	Trioctyl 0.20 %	174756	77%
	Didodecyl 0.05 %	152311	67%
	Didodecyl 0.20 %	148147	65%
Yeast's	Dodecyl trimethyl 1.35%	1336856	100%
	Dodecyl trimethyl 0.50%	1247739	93%
	Trioctyl 0.05 %	1148033	86%
	Trioctyl 0.20 %	1263582	95%
	Didodecyl 0.05 %	5845	0%
	Didodecyl 0.20 %	19186	1%
	Dodecyl trimethyl 1.35%	232206	100%
	Dodecyl trimethyl 0.50%	217253	94%
	Trioctyl 0.05 %	173872	75%
	Trioctyl 0.20 %	191694	83%
	Didodecyl 0.05 %	0	0%
	Didodecyl 0.20 %	0	0%

Dodecyl trimethyl ammonium bromide 0.50 to -1.35% aqueous solution (DTAB)

Trioctyl methyl ammonium chloride 0.05 to 0.20 % aqueous solution

Didodecyl dimethyl ammonium bromide 0.05 to 0.20 %

Extraction time 3 sec
Measurement with Sensilux 25 2sec
All aqueous soln contained 1.5 mM EDTA

WHAT IS CLAIMED IS:

1. A method for releasing cellular contents comprising using a halide containing quaternary ammonium compounds.

2. The method of claim 1 further including releasing molecules of interest from living cells.

3. The method of claim 1 further including releasing nucleotides from living cells.

4. The method of claim 1 further comprising applying ionic surface active agents containing a fatty group with a chain length of from 8 to 18 carbon atoms.

5. The method of claim 4 further comprising applying ionic surface active agents containing a fatty group with a chain length of 12 carbon atoms.

6. A method for releasing cellular contents comprising modifying the alkyl chain length of ionic surface active agents to enable selective release from different classes of living cells and applying the ionic surface active agents having different chain lengths to effect to change the permeability of the cell wall and membranes.

7. The method for releasing cellular contents of claim 6 further comprising applying anionic surface active agents to the exclusion of non-ionic surface active agents.

8. The method for releasing cellular contents of claim 6 further comprising applying cationic surface active agents to the exclusion of non-ionic surface active agents.

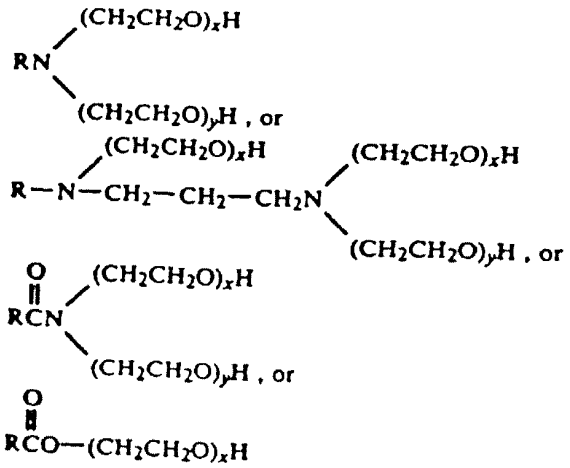
9. The method for releasing cellular contents of claim 6 further comprising applying ionic surface active agents containing quaternary ammonium salts.

10. The method for releasing cellular contents of claim 6 further comprising applying ionic surface active agents containing a fatty group with a chain length of from 8 to 18 carbon atoms.

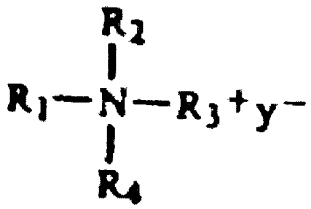
11. The method for releasing cellular contents of claim 10 further comprising applying ionic surface active agents containing a fatty group with a chain length of 12 carbon atoms.

12. The method for releasing cellular contents of claim 6 further comprising applying ionic surfactants selected from the group comprising: ethoxylated amines; ethoxylated diamines; polyethylene glycol esters of fatty acids; ethoxylated amides having a

chemical structure of:



where R is a fatty alkyl group having 8-18 carbon atoms and x, y and z are numbers ranging from 2 to 50; quaternary ammonium salts having a formula of:



where R_1 and R_2 are an alkyl, alkyl-aryl-alkyl, ethoxyalkyl, hydroxyalkyl, or ethoxylated alkylphenol with a 4 to 22 carbon atom chain and the ethoxylated alkyls having 2 to 15 ethoxyl groups, and R_3 and R_4 are alkyl groups having a 1 to 15 carbon atom chain and y is selected from the group comprising a halogen, sulphate, sulphite, phosphate and combinations thereof; and combinations thereof.

13. The method for releasing cellular contents of claim 6 further comprising applying hyamine chloride.

14. The method for releasing cellular contents of claim 13 further comprising applying diisobutyl cresoxy ethoxy ethyl dimethyl ammonium chloride.

15. The method for releasing cellular contents of claim 6 further comprising applying a mixture of an ethoxylated amine and a quaternary salt of an ethoxylated amine.

16. The method for releasing cellular contents of claim 15 further comprising applying a 0.02-0.5% solution of a mixture of 1 to 19 parts of an ethoxylated amine, and 1 to 19 parts of a quaternary ammonium salt of an ethoxylated amine, both having a carbon atom chain length from 8 to 18.

17. The method for releasing cellular contents of claim 15 further comprising applying a mixture of ethoxylated amine and a quaternary salt of an ethoxylated amine, having both a 12 carbon chain length.

18. The method for releasing cellular contents of claim 15 further comprising applying a mixture of one third of a quaternary ethoxylated amine and two thirds of ethoxylated amine.

19. The method for releasing cellular contents of claim 15 further comprising applying a mixture of a mixture of an ethoxylated amine and a quaternary ethoxylated amine in an end-concentration of 0.05-0.5% by volume of the microbe sample.

20. A substance for effecting a selective release of cellular contents comprising a dialkyl ammonium halide.

21. The substance for effecting a selective release of cellular contents of claim 20 further wherein the dialkyl ammonium halide comprises an alkyl chain lengths of approximately 8 to 10 carbon atoms.

22. The substance for effecting a selective release of cellular contents of claim 20 further wherein the dialkyl ammonium halide comprises an alkyl chain lengths of approximately 10 carbon atoms or greater.

23. A substance for effecting a selective release of cellular contents comprising a dimethyl ammonium halide.

24. The substance for effecting a selective release of cellular contents of claim 23 further wherein the dimethyl ammonium halide comprises an alkyl chain lengths of approximately 8 to 10 carbon atoms.

25. The substance for effecting a selective release of cellular contents of claim 23 further wherein the dimethyl ammonium halide comprises an alkyl chain lengths of approximately 10 carbon atoms or greater.

Figure 1

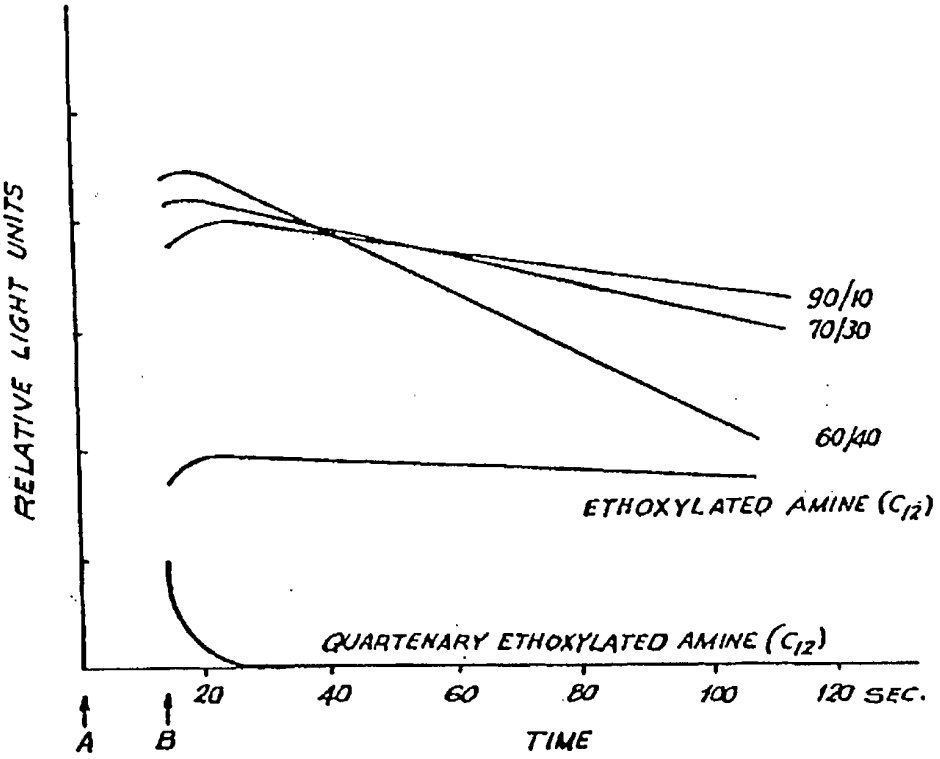
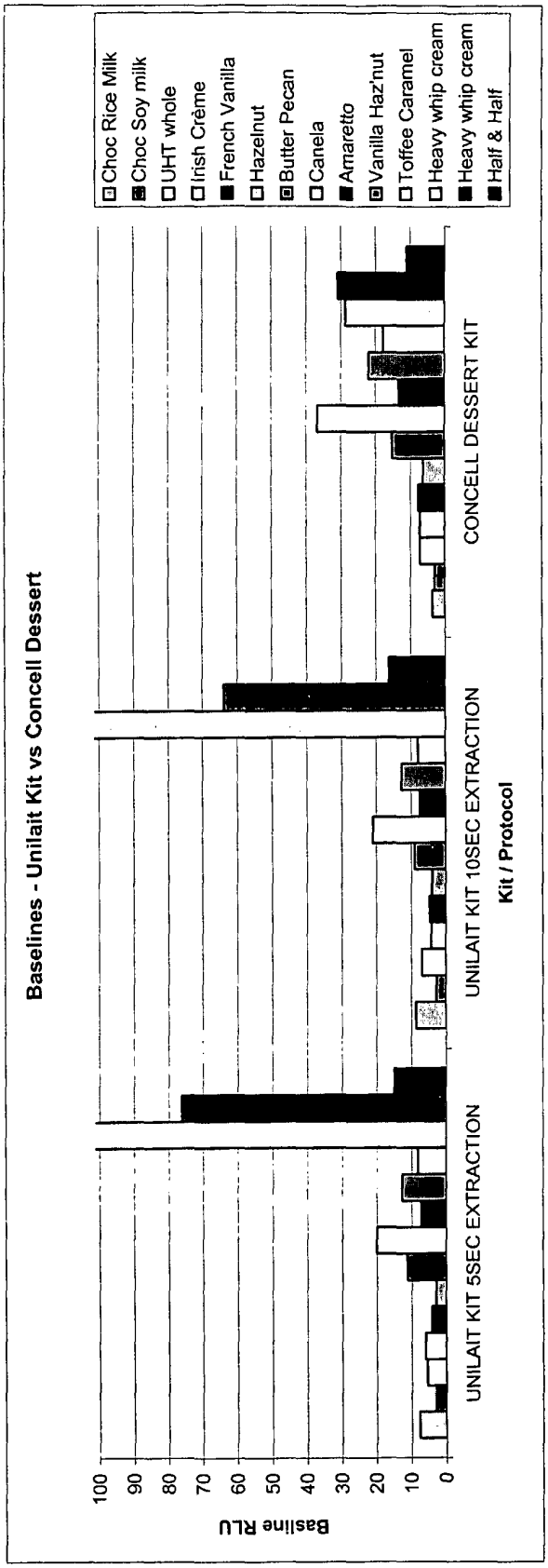

RELATIVE LIGHT UNITS
90/10
70/30
60/40
ETHOXYLATED AMINE (C_{12})
QUARTENARY ETHOXYLATED AMINE (C_{12})
20
40
60
80
100
120 SEC.
A
B
TIME

FIGURE 2


Baselines - Unilait Kit vs Concell Dessert
Choc Rice Milk
Choc Soy milk
UHT whole
Irish Crème
French Vanilla
Hazelnut
Butter Pecan
Canela
Amaretto
Vanilla Haz'nut
Toffee Caramel
Heavy whip cream
Heavy whip cream
Half & Half
Baseline RLU
100
90
80
70
60
50
40
30
20
10
0
UNILAIT KIT 5SEC EXTRACTION
UNILAIT KIT 10SEC EXTRACTION
CONCELL DESSERT KIT
Kit / Protocol

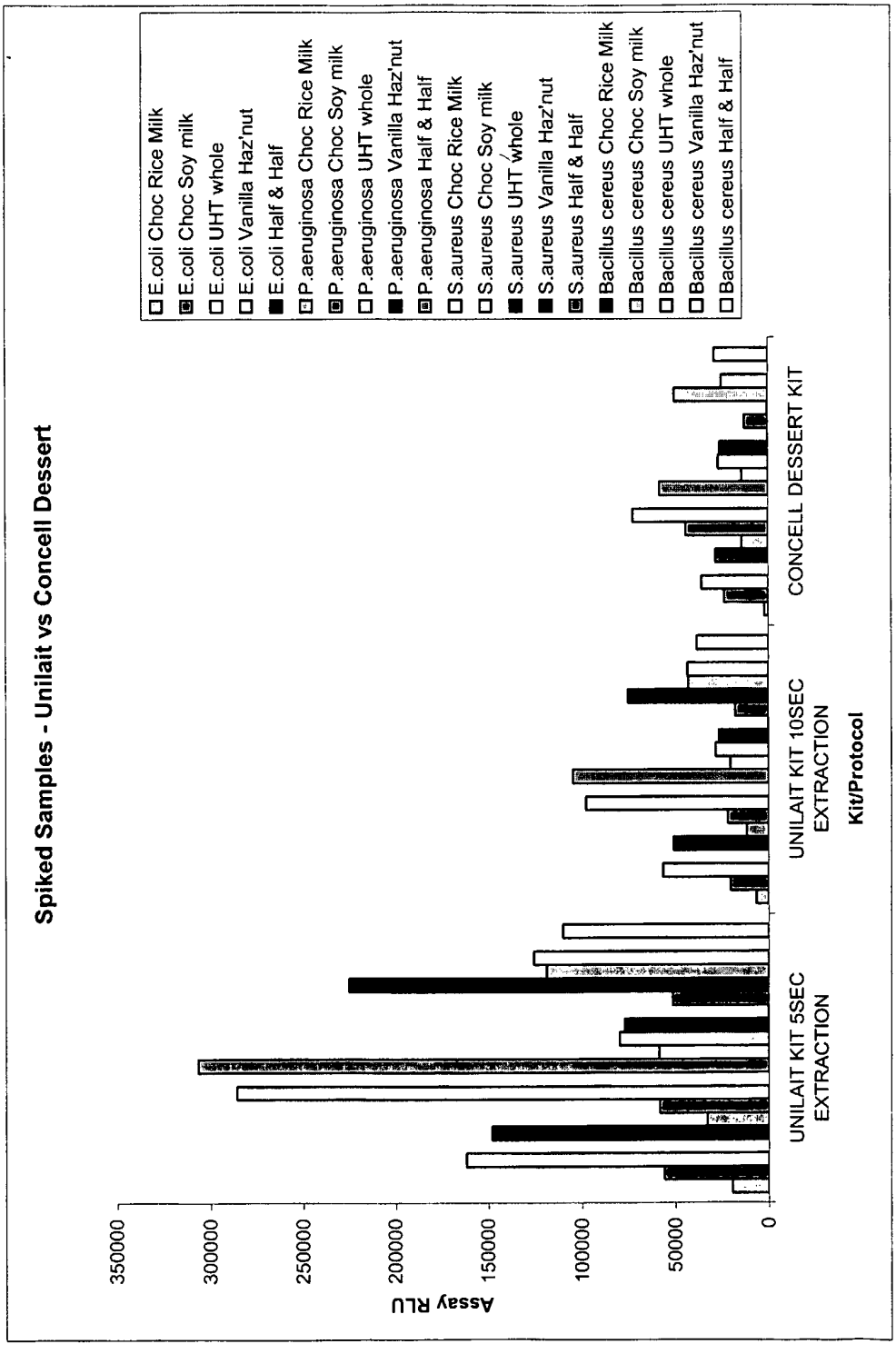

Spiked Samples - Unilait vs Concell Dessert
Assay RLU
350000
300000
250000
200000
150000
100000
50000
0
UNILAIT KIT 5SEC EXTRACTION
UNILAIT KIT 10SEC EXTRACTION
CONCELL DESSERT KIT
Kit/Protocol
E.coli Choc Rice Milk
E.coli Choc Soy milk
E.coli UHT whole
E.coli Vanilla Haz'nut
E.coli Half & Half
P.aeruginosa Choc Rice Milk
P.aeruginosa Choc Soy milk
P.aeruginosa UHT whole
P.aeruginosa Vanilla Haz'nut
P.aeruginosa Half & Half
S.aureus Choc Rice Milk
S.aureus Choc Soy milk
S.aureus UHT whole
S.aureus Vanilla Haz'nut
S.aureus Half & Half
Bacillus cereus Choc Rice Milk
Bacillus cereus Choc Soy milk
Bacillus cereus UHT whole
Bacillus cereus Vanilla Haz'nut
Bacillus cereus Half & Half

FIGURE 3

FIGURE 4

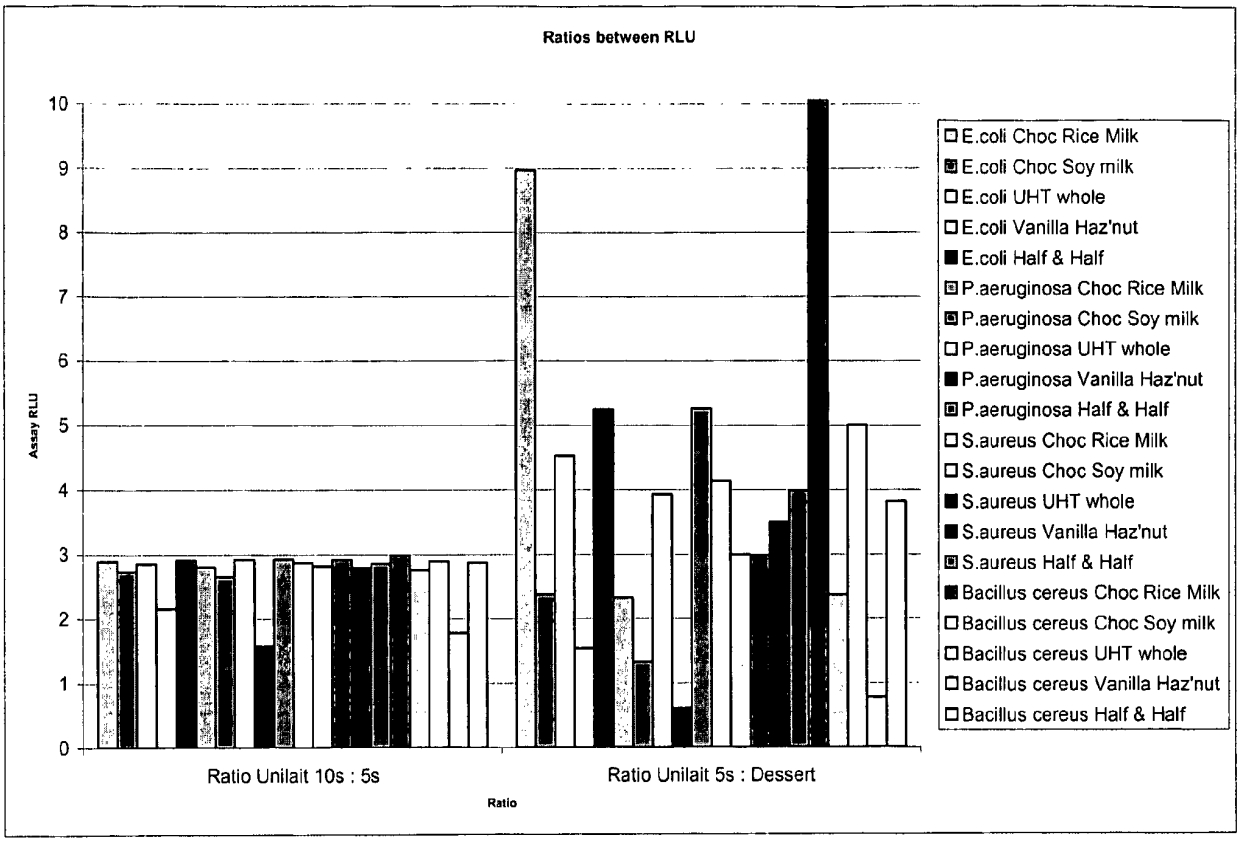
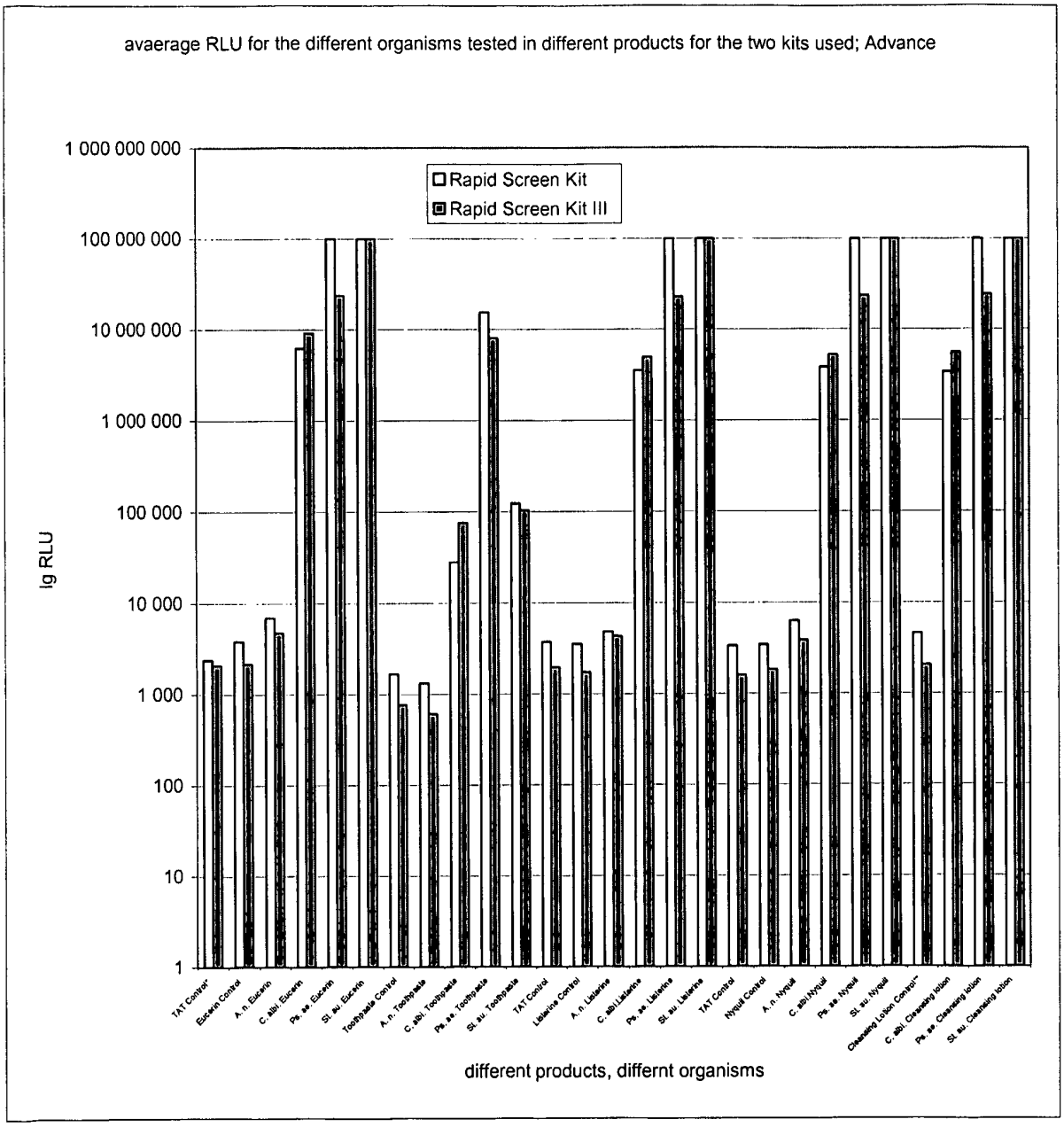

Ratios between RLU
Assay RLU
10
9
8
7
6
5
4
3
2
1
0
Ratio Unilait 10s : 5s
Ratio Unilait 5s : Dessert
Ratio
E.coli Choc Rice Milk
E.coli Choc Soy milk
E.coli UHT whole
E.coli Vanilla Haz'nut
E.coli Half & Half
P.aeruginosa Choc Rice Milk
P.aeruginosa Choc Soy milk
P.aeruginosa UHT whole
P.aeruginosa Vanilla Haz'nut
P.aeruginosa Half & Half
S.aureus Choc Rice Milk
S.aureus Choc Soy milk
S.aureus UHT whole
S.aureus Vanilla Haz'nut
S.aureus Half & Half
Bacillus cereus Choc Rice Milk
Bacillus cereus Choc Soy milk
Bacillus cereus UHT whole
Bacillus cereus Vanilla Haz'nut
Bacillus cereus Half & Half

FIGURE 5


avaerage RLU for the different organisms tested in different products for the two kits used; Advance
Rapid Screen Kit
Rapid Screen Kit III
lg RLU
1 000 000 000
100 000 000
10 000 000
1 000 000
100 000
10 000
1 000
100
10
1
different products, differnt organisms

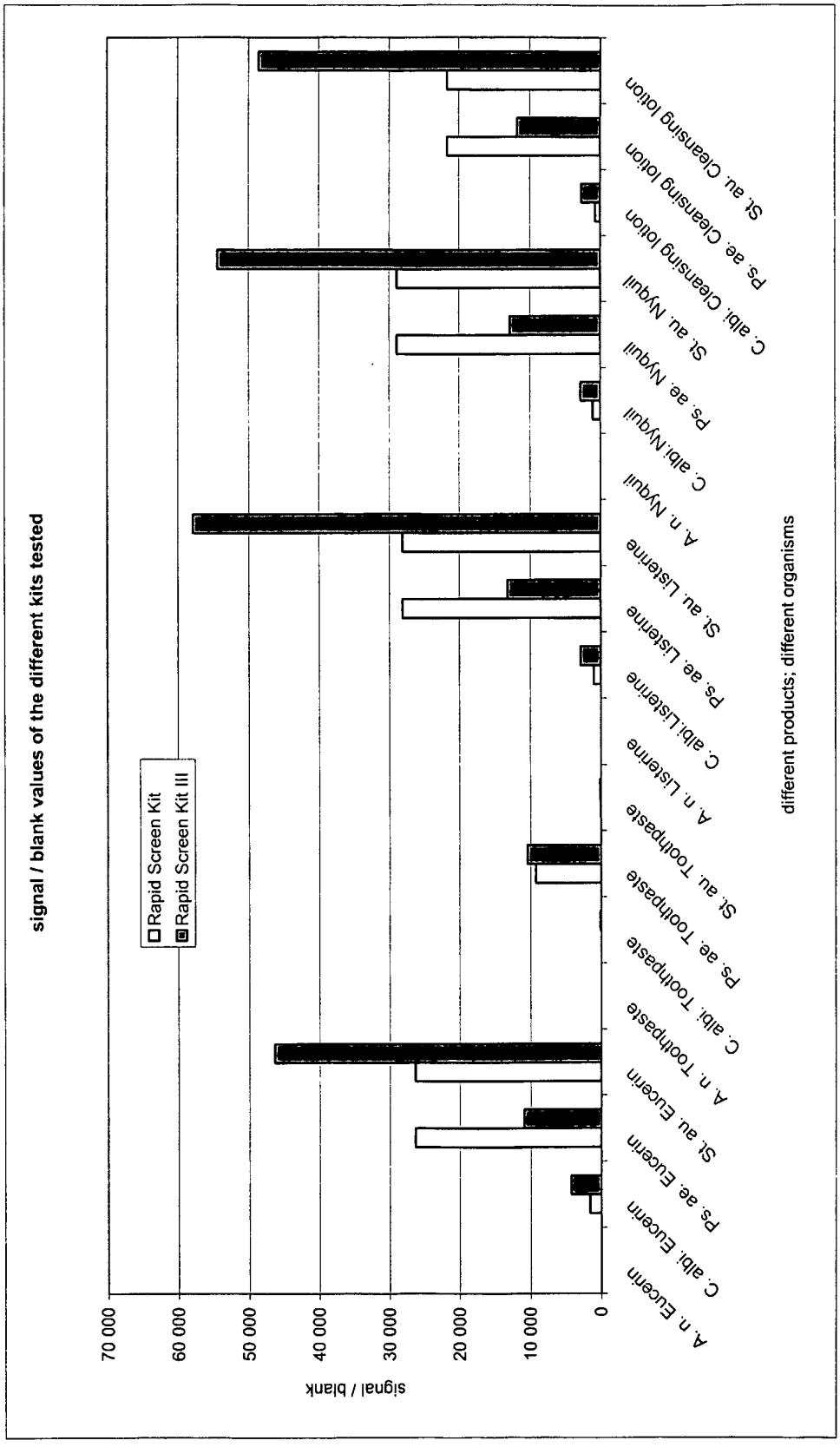

signal / blank values of the different kits tested
Rapid Screen Kit
Rapid Screen Kit III
signal / blank
70 000
60 000
50 000
40 000
30 000
20 000
10 000
0
A. n. Eucerin
C. albi. Eucerin
Ps. ae. Eucerin
St. au. Eucerin
A. n. Toothpaste
C. albi. Toothpaste
Ps. ae. Toothpaste
St. au. Toothpaste
A. n. Listerine
C. albi. Listerine
Ps. ae. Listerine
St. au. Listerine
A. n. Nyquil
C. albi. Nyquil
Ps. ae. Nyquil
St. au. Nyquil
C. albi. Cleansing lotion
Ps. ae. Cleansing lotion
St. au. Cleansing lotion
different products; different organisms

FIGURE 6

FIGURE 7

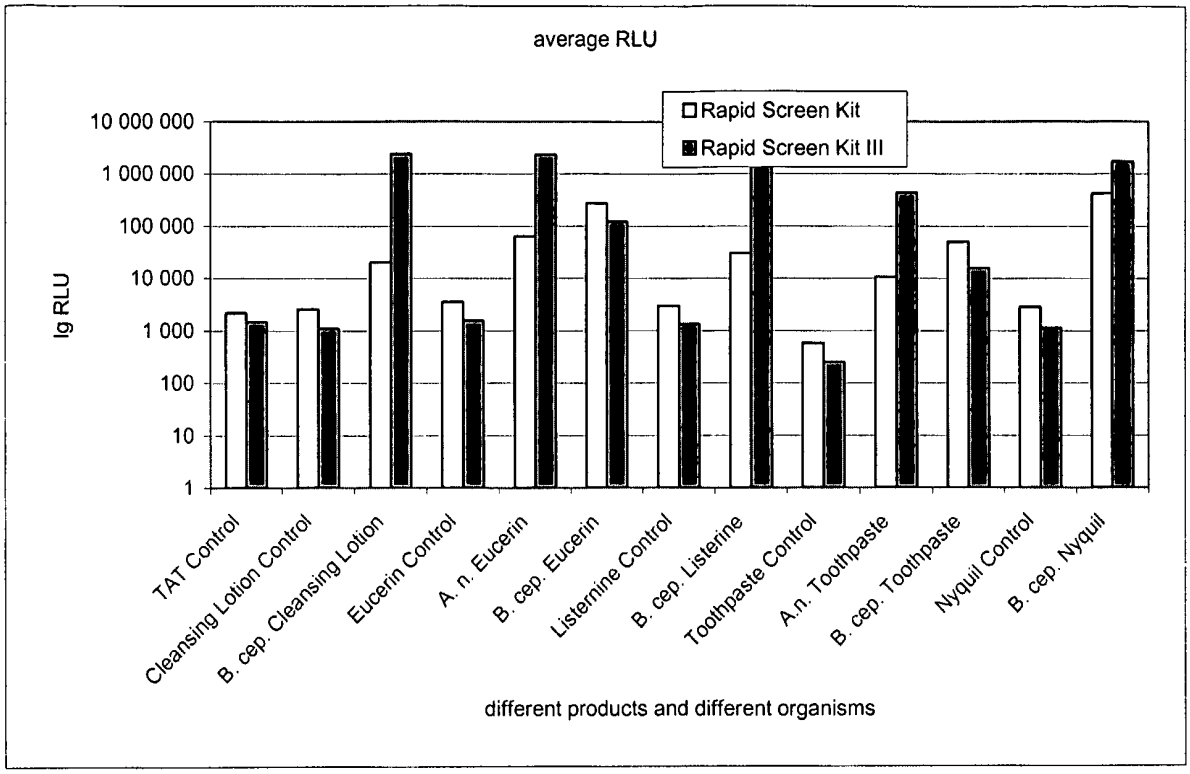
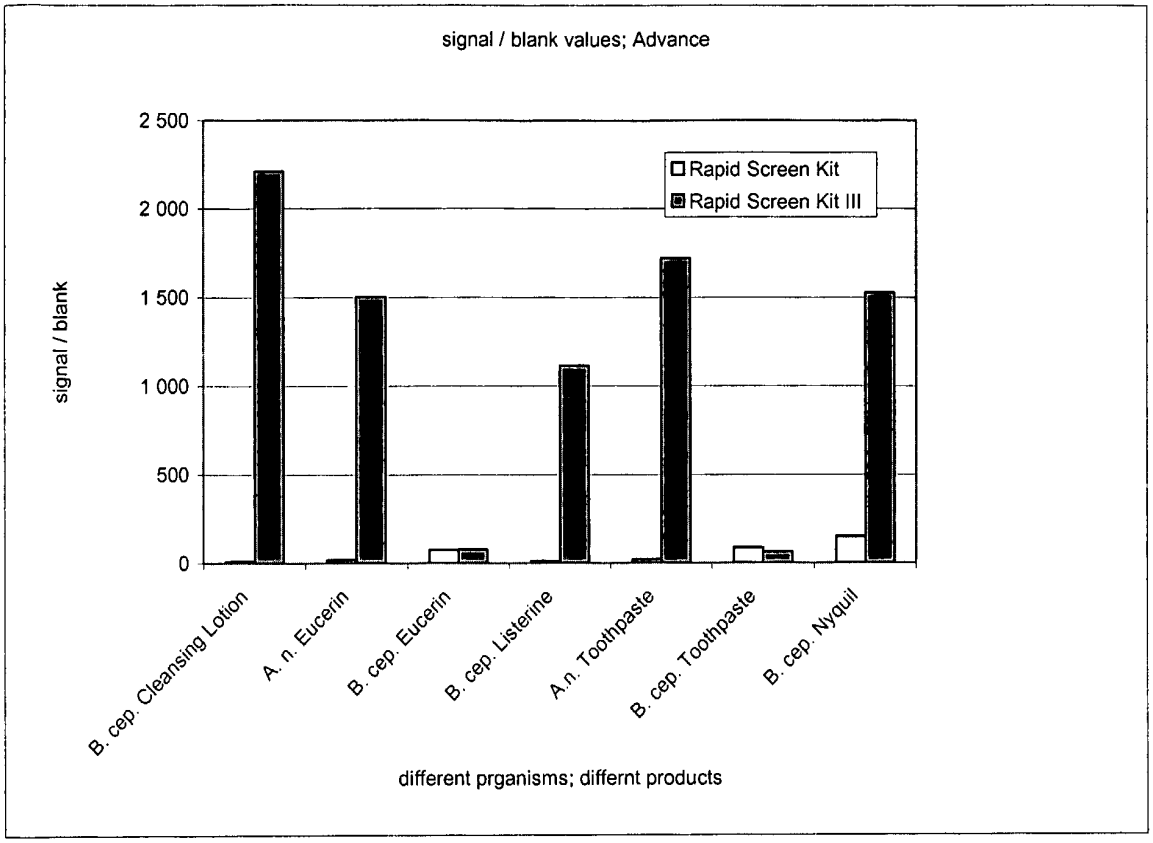

average RLU
Rapid Screen Kit
Rapid Screen Kit III
lg RLU
10 000 000
1 000 000
100 000
10 000
1 000
100
10
1
TAT Control
Cleansing Lotion Control
B. cep. Cleansing Lotion
Eucerin Control
A. n. Eucerin
B. cep. Eucerin
Listernine Control
B. cep. Listerine
Toothpaste Control
A.n. Toothpaste
B. cep. Toothpaste
Nyquil Control
B. cep. Nyquil
different products and different organisms

FIGURE 8


signal / blank values; Advance
2 500
2 000
1 500
1 000
500
0
signal / blank
Rapid Screen Kit
Rapid Screen Kit III
B. cep. Cleansing Lotion
A. n. Eucerin
B. cep. Eucerin
B. cep. Listerine
A.n. Toothpaste
B. cep. Toothpaste
B. cep. Nyquil
different prganisms; differnt products