# Technews

**National Dairy Development Board**

**For Efficient Dairy Plant Operation**

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## Technological Advancement in Methods of Microbiology in Milk Analysis- Part 2 (Indirect methods)

This bulletin includes technical information based on latest developments on products, systems, techniques etc. reported in journals, companies' leaflets and books and based on studies and experience. The technical information in different issues is on different areas of plant operation. It is hoped that the information contained herein will be useful to readers.

The theme of information in this issue "**Technological Advancement in Methods of Microbiology in Milk Analysis - Part 2 (Indirect Methods)"** It may be understood that the information given here is by no means complete.

***In this issue:***

## INTRODUCTION

Rapid microbial detection becomes increasingly essential in companies like dairy, food and beverage areas. Faster microbiological methods are required to contribute to a better control of raw materials as well as finished products. Rapid microbiological methods can also provide a better reactivity throughout the manufacturing process. Implementing rapid technologies would allow companies for cost saving and would speed up products release.

Despite clear advantages, traditional methods are still widely used. Current methods require incubation of products in liquid or solid culture media for routinely 2 to 10 days before getting the contamination result. This necessary long incubation time is mainly due to the fact that stressed microorganisms found in complex matrices require several days to grow to visible colonies to be detected.

Although these techniques show advantages like simplicity, the use of inexpensive materials and their acceptability to the regulatory authorities, the major drawback is the length of time taken to get microbiological results. Thus, to face the growing demand for rapid detection methods, various alternative technologies have been developed. Considering these limitation of conventional methods several new approaches are highlighted in this document.

## I. Methods based on microbial cell components

### 1. ATP-Bioluminescence

The principle behind ATP- Bioluminescence is process of light emission from organisms and represents a chemical conversion of energy into light. The bioluminescence mechanism involve Luciferase enzyme is a multistep process which mainly requires Luciferin substrate, Oxygen ($O_2$), Magnesium cation ($Mg^{++}$) and ATP.

ATP bioluminescence using luciferine/luciferase relies on luciferine oxidation by the luciferase and the integrated light intensity is directly proportional to ATP contents. Luciferase converts in presence of ATP and Magnesium firefly D-luciferin into the corresponding enzyme-bound luciferil adenylate. The luciferil adenylate complex is then the substrate of the subsequent oxidative reaction leading to oxyluciferin. The light emission is a consequence of a rapid loss of energy of the oxyluciferine molecule from an excited state to a stable one. This reaction induces the emission of photons with an efficient quantum yield of about 90%.

1) D-luciferin + luciferase + ATP $\xrightarrow{Mg^{++}}$ Luciferil adenylate complex + PPi

2) Luciferil adenylate complex $\xrightarrow{O_2}$ Oxyluciferin + AMP+ $CO_2$ + light

Fig. 1. Chemical reactions of the ATP-bioluminescence based on luciferin/luciferase system (PPi: inorganic pyrophosphate, CO2: Carbon Dioxide). Photons of yellow-green light (550 to 570 nm) are emitted.

**Milliflex rapid microbiological detection and enumeration system**

RMDS offers a way to detect and quantify living microorganisms grown on a membrane. By combining ATP-bioluminescence and sensitive detection system, the microbial detection is obtained more rapidly than traditional method. In order to detect a colony or a micro-colony on a membrane by ATP-bioluminescence, the first step is to release ATP from cells. This critical step is achieved by nebulizing automatically an ATP-releasing solution onto the membrane.

ATP extraction is made on micro colonies grown on membrane which represents an advantage compared to chemical or physical extraction in liquid. Once ATP is released from lysed cells, it becomes accessible to bioluminescent reaction. A second solution is then automatically nebulized onto the same membrane. This solution brings to lysed cells all components, except ATP, involved in the Luciferin/Luciferase bioluminescence chemical reaction. A spray station is used to uniformly apply small volumes of reagents onto the membrane. As soon as bioluminescent reagents are sprayed onto the membrane, the bioluminescence reaction starts and photons are emitted. The membrane is then transferred manually from the spray station to the detection system.

The Milliflex Rapid detection system combines the use of a highly sensitive CCD (Charged Coupled Device) camera to monitor light emitted from microorganisms and an image analysis software to analyse the signal and give the number of microorganisms counted. The figure 2 shows the detection tower components and their function.

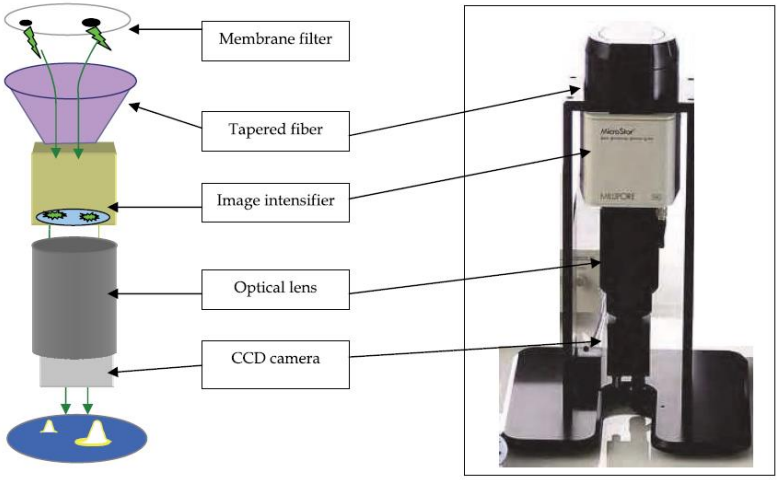


Fig. 2. Milliflex detection tower components: RMDS collects, amplifies, and registers on a CCD camera the light activity of bioluminescent reaction. Photons emitted by microorganisms go through the tapered fiber in order the light to be concentrated and becomes compatible with the size diameter of the CCD camera. In the intensifier, photons hit a photocathode and each photon is converted into cloud of electrons. Then electrons hit a phosphorous screen and are converted back into photons. The CCD camera records light every 30 times per second.

Data collected by the CCD camera are analysed and treated by software to build an image of the membrane loaded on the top of the detection tower. The image indicates the place where light is emitted.

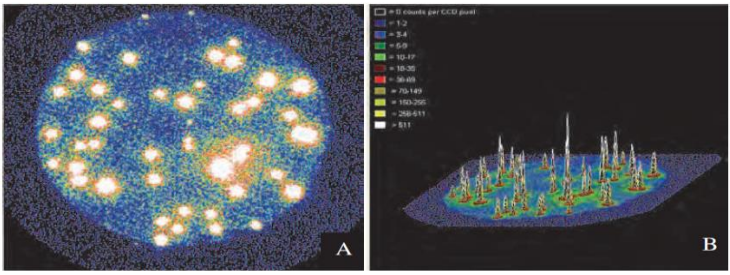


Fig. 3. Example of image given by RMDS software. Picture show the image of the membrane with spots (A) or peaks in 3 dimensions (B) representing exactly the place on the membrane where light is emitted. The result in colony forming unit is directly given by the system.

## 2. Limulus Lysate test (Endotoxin Test)

Limulus Amoebocyte Lysate (LAL) test is in vitro test to detect and measure bacterial endotoxins. Endotoxins are products of metabolism associated with outer membrane of gram negative bacteria especially Pseudomonas, E. coli and Serratia and are most significant source of pyrogens.

The LAL reagent is derived from blood cells (amoebocytes) of a horse shoe crab – Limulus Polyphemus. The oxygen-carrying pigment in horseshoe crab blood is a protein called hemocyanin. Blood is removed from the crab's body, the blood cells are separated from the serum using centrifugation and are

then placed in distilled water, which causes them to swell up and burst ('lyse'). This releases the chemicals from the inside of the cell (the 'lysate'), which is then purified and freeze-dried.

To test a sample for endotoxins, it is mixed with lysate and water; if coagulation occurs endotoxins are present.

**Endotoxin Limit (EL)**

The quantities of endotoxins are expressed in defined Endotoxin Unit (EU)

1 EU= 1 IU IU- International Unit. EL= K/M

Where,

K- Maximum No. of IU of endotoxin which the patient may receive without suffering toxic reactions (minimum pyrogenic dose)

M- Maximum dose of the drug substance per kg per hour.

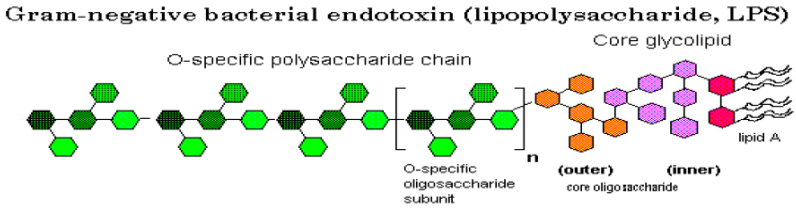


Fig.4: Structure of Endotoxin

**Requirements of LAL test**

a) **LAL Test Reagent:** It is freeze dried (LAL Powder) and reconstituted before test. It can also be reconstituted with buffer, a beta glucan inhibiting buffer. LAL powder

can be stored at 2 to 8°C and prolonged exposure to 25°C should be avoided.

b) **LAL Reagent Water (Non LAL active):** It is used to reconstitute LAL reagent and prepare samples, controls and endotoxin standards.

c) **The Endotoxin Reference Standard:** It is a purified endotoxin of Escherichia coli calibrated with International standard

d) **Control Standard Endotoxin:** It is suitably standardized against Endotoxin Reference Standard and is used for routine bacterial endotoxin testing.

e) **LAL test conditions:** The test should be carried out in aseptic environment to avoid microbial contamination. The containers used in the test should be sterilized in oven at 250°C or above for not less than 60 minutes in order to remove surface endotoxins.

**Gel Clot method**

In presence of endotoxin a proenzyme in amoebocyte lyasate is converted into an active form. The active enzyme then cleaves a clotting protein, also found within the Limulus Amoebocyte. The cleaved fragments self-aggregate to form a clot. Optimum gel clot occurs for a mixture pH 6.0 to 8.0. Tenth of a millimetre of LAL is incubated with an equal volume of sample for one hour at 37°C. After incubation period reaction tubes are inverted. If clot remains intact after inversion, the test is positive.

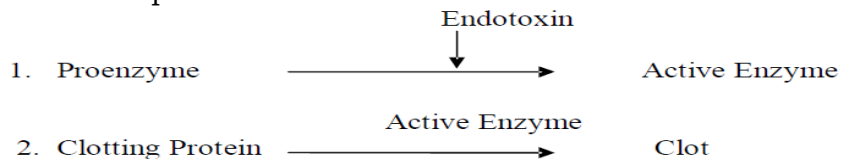


Fig.5: Reaction sequence of Gel Clot Method

Maximum sensitivity of the test is 0.03 EU/mL. Gel clot method is the least expensive method as no optical reader is required. It is the method of choice for opaque samples, suspensions and coloured samples. Equipment used in Gel Clot Method is inexpensive. It is a manually read test. Reagents of different sensitivity used are 0.5, 0.25, 0.125, 0.06 and 0.03 EU/mL.

## 3. Bacteriophage-based detection methods

Bacteriophages present ideal tools which can be used for bacterial detection. These viruses have co-evolved with their bacterial hosts to recognize and infect their target cells with an extraordinary specificity that can be harnessed for various rapid detection formats. The complete infection cycle of a virulent phage usually takes only 1–2 h and, by multiplication inside the host cell, offers an inherent amplification step, which in many detection assays makes it possible to shorten or completely dispense with lengthy pre-enrichment procedures. In addition, phages are easy and inexpensive to produce, robust (e.g., they show low susceptibility to variations of temperature and pH, organic solvents, and proteases), and are able to distinguish between live and dead cells (i.e., only multiply in viable cells).

### a. Lux Gene Luminescence

The application of *lux* genes, one starts with bacteriophages that are specific for the bacterium of interest and thus takes advantage of the highly specific relationship that exists between phages and their hosts.

If *Y. enterocolitica* is the bacterium of interest, one selects a phage that will infect the widest range of strains and yet not infect closely related species. To this phage, the *lux* genes are inserted by recombination methods, which amounts to about 2 kb of DNA. By themselves, these transduced phages are not luminous because they lack all components necessary to produce light. When added to their specific host bacteria, the *lux* gene-bearing phages enter and multiply, and thus cause the host cells to luminesce by the increased production of more *lux* genes.

The light-emitting reaction requires the components in the following equation:

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + \text{light}$$

Fig.6: Light emitting reaction.

Where FMNH2 is reduced flavin mononucleotide and RCHO is a long-chain aliphatic aldehyde such as dodecanal. The emitted light can be measured by luminometry as in the ATP assay.

**b. Detection on the Basis of Phage-Induced Lysis**

Lysis of the host cell "from within" constitutes the last step in the lytic multiplication cycle of most phages, and is commonly mediated by two components of the lytic cassette of the phage: the holin, which creates pores in the cytoplasmic membrane, and the endolysin, which accesses the peptidoglycan through these pores and degrades its substrate, resulting in destabilization

of the murein sacculus and rapid lysis. Besides liberation of the progeny phage particle, host cell lysis also results in the release of other intracellular components, some of which may be used as markers for measuring the lysis event. This can be exploited in a variety of bacterial detection methods, in which the bacteriophage provides the necessary specificity.

**c. Measurement of impedance or conductivity**

The growth of microorganisms causes changes in conductivity of the growth medium, mostly through transformation of large uncharged metabolites (such as carbohydrates) to smaller charged molecules (e.g., acids). Bacteriophage are suitable tools for specific impedimetric detection of bacteria, since addition of phage to a sample results in retardation of changes in impedance if the target organism is present.

One prominent example for employing a phage for impedimetric detection of foodborne pathogens is a study by Chang et al., who were able to detect E. coli O157:H7 cells through the absence of changes in conductivity of a MacConkey-sorbitol medium in presence of the phage AR1, which is specific for this pathogen. The method proved highly sensitive and specific (99.4% of non-O157:H7 E. coli strains tested yielded a negative result).

**d. Detection by the Phage Amplification Assay**

The use of unmodified phage particles for generation and enumeration of plaques within a bacterial lawn is certainly the most obvious and direct way of utilizing these viruses for bacterial detection. In the so-called Phage Amplification Assay samples to be tested for the presence of certain target pathogens are mixed with bacteriophages specific for these bacteria, which are then allowed sufficient time to infect their host cells.

The subsequent addition of a virucide to the sample ensures survival only of those phages currently engaged in the infection process, whereas all extracellular phage particles are inactivated. Since the number of target cells present in a sample is rarely sufficient to produce a bacterial lawn when plated directly, the sample is then mixed with an ample amount of helper cells (of a propagating strain for the respective phage) following neutralization of the virucide. Plating of the mixture in a soft agar overlay results in formation of plaques as a consequence of phage liberated from lysed target cells infecting surrounding helper cells. The number of plaques directly corresponds to the number of target cells initially present in the sample, and the nature of the assay ensures that only living cells are detected.

## II. Methods based on growth and metabolic activity

### 1. Flow Cytometery

A fluorescently stained sample is illuminated by a laser light. The forward scatter detector is in line with the laser beam. The side scatter detector measures light that is of the same color as the laser beam, but scattered at a 90º angle. The other detectors detect colors different of the laser beam that might be emitted by the sample.

The size and granularity of the cells is indicated by the light scatter which can be used in differentiating cells, such as yeast from bacterial cells. The auto fluorescent properties of cells, such as the presence of photosynthetic pigments, have been used in identifying and classifying algae. FCM has been utilized in serological discrimination of bacteria, fungi, viruses and parasites.

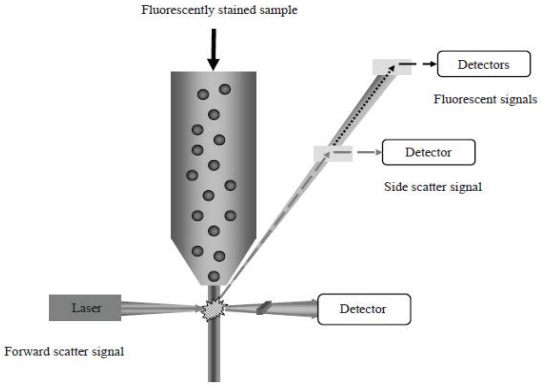


Fig.7: Flow Cytometer.

In molecular biology, FCM can be used for DNA fragment analysis of bacteria and viruses, and detection of clones and mutants by reporters encoded by genes. In food microbiology, FCM is advantageous since it can be used in differentiating between dead, viable, and VNC (Viable but not culturable) cells by using fluorescent dyes that indicate the membrane integrity, membrane potential, respiration, intracellular pH, and enzyme activity of cells, directly from food, such as milk, juice, wine, vegetable products and meat products

**2. Radiometry**

The radiometric detection of microorganisms is based on the incorporation of a $^{14}C$-labeled metabolite in a growth medium so that when the organisms utilize this metabolite, $^{14}CO_2$ is released and measured by use of a radioactivity counter. For organisms that utilize glucose, $^{14}C$-glucose is usually employed. For those that cannot utilize this compound, others such as $^{14}C$-formate or $^{14}C$-glutamate are used.

The overall procedure consists of using capped 15-mL serum vials to which are added anywhere from 12 to 36 mL of medium containing the labelled metabolite. The vials are made either aerobic or anaerobic by sparging with appropriate gases and are then inoculated. Following incubation, the headspace is tested periodically for the presence of $^{14}CO_2$. The time required to detect the labelled $CO_2$ is inversely related to the number of organisms in a product.

The detection of non-fermenters of glucose by this method is possible when metabolites such as labelled formate and/or glutamate are used. It has been shown that a large number of foodborne organisms can be detected by this method in 1-6 hours. The radiometric detection of 1-10 coliforms in water within 6 hours was achieved by employing $^{14}$C-lactose with incubation at 37°C in a liquid medium. It is conceivable that a differentiation can be made between fecal *E. coli* and total coliforms by employing 45.5°C incubation along with 37°C incubation.

**3. Impedance**

Impedance is the apparent resistance in an electric circuit to the flow of alternating current corresponding to the actual electrical resistance to a direct current. When microorganisms grow in cultural media, they metabolize substrates of low conductivity into products of higher conductivity and thereby decrease the impedance of the media. When the impedance of broth cultures is measured, the curves are reproducible for species and strains, and mixed cultures can be identified by use of specific growth inhibitors. The technique has been shown capable of detecting as few as 10-100 cells. Cell populations of $10^5$-$10^6$/mL can be detected in 3-5 hours and $10^4$-$10^5$/mL in 5-7 hours.

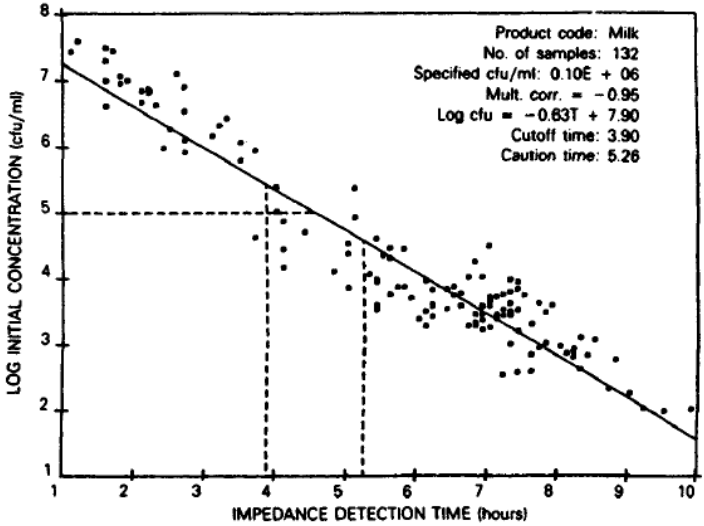


Fig.8: Scattergram relating IDT to APC on 132 samples of raw milk. Samples containing > 105 mesophiles per milliliter were detected within 4 hours. Courtesy of Ruth Firstenberg-Eden.

The microbiological quality of pasteurized milk was assessed by using the impedance detection time (IDT) of 7 hours or less, which was equivalent to an aerobic plate count (APC) of $10^4$/mL or more bacteria. Of 380 samples evaluated, 323 (85%) were correctly assessed by impedance. Using the same criterion for 27 samples of raw milk, 10 hours were required for assessment.

## 4. Microcalorimetry

This is the study of small heat changes: the measurement of the enthalpy change involved in the breakdown of growth substrates. The heat production

that is measured is closely related to the cell's catabolic activities.

There are two types of calorimeters: batch and flow. Most of the early work was done with batch type instruments. The thermal events measured by microcalorimetry are those from catabolic activities, as already noted. One of the most widely used microcalorimeters for microbiological work is the *Calvet* instrument, which is sensitive to a heat flow of 0.01 cal/h from a 10 mL sample. With respect to its use as a rapid method, most attention has been devoted to the identification and characterization of foodborne organisms. Microcalorimetric results vary according to the history of the organism, inoculum size, fermentable substrates, and the like.

The latter method is one in which a microcalorimeter is filled with a flow-through calorimetric vessel. By the use of a chemically defined medium containing seven sugars, thermograms were produced by nine lactic acid bacteria (belonging to the genera *Enterococcus, Leuconostoc,* and *Lactobacillus)* distinctive enough to recommend the method for their identification. All cultures were run at 37°C except *"S. cremoris,"* which was run at $30^0$C, and results were obtained within 24 hours. This method has been used to study spoilage in canned foods, to differentiate between the *Enterobacteriaceae*, to detect the presence of *S. aureus.*

## References


1. Use of ATP Bioluminescence for rapid Detection and enumeration system Renaud chollet and sébastien ribault *Merck-millipore France,* Feb-2012 pp – 100 – 104

2. A review of bioluminescent ATP techniques in rapid microbiology;J Biolumin Chemilumin. Jul-1989; 4(1): 375-80.

3. McElroy, W. D. The energy source for bioluminescence in an isolated system. Proceedings of the National Academy of Sciences USA, 1947, 342–345.

4. International Research Journal of Pharmacy - An overview of limulus amoebocyte lysate (lal) test by Mulay Sushruta*, Khale Anubha, Mar-2011, pp- 67-71

5. Application of bacteriophages for detection of foodborne pathogens by Mathias Schmelcher* and Martin J Loessner, Feb-2014

6. Modern Techniques in Detection, Identification and Quantification of Bacteria and Peptides from Foods Dissertationes bioscientiarum molecularium Universitatis Helsingiensis in Viikki JANETTA HAKOVIRTA Department of Applied Chemistry and Microbiology Division of Microbiology, May-2008, pp 11-14.

7. Modern Food Microbiology, sixth edition -Physical. Chemical, Molecular & Immunological methods by James M. Jay Professor Emeritus Wayne State University Detroit, Michigan Adjunct Professor University of Nevada Las Vegas, 2000, pp- 208-213

8. Fluorescence-Based Bioassays for the Detection and Evaluation of Food Materials Kentaro Nishi, Shin-Ichiro Isobe, Yun Zhu and Ryoiti Kiyama*, Oct-2015

9. An overview of Limulus Amoebocyte Lysate (LAL) test Mulay Sushruta*, Khale Anubha M.E.S. Instititute's H. K. College Of Pharmacy, Pratikshanagar, Jogeshwari (W) Mumbai, India, Feb-2011 pp- 1-5.

**TECHNOLOGICAL ADVANCEMENT IN METHODS OF MICROBIOLOGY IN MILK ANALYSIS- PART 2 (INDIRECT METHODS)**

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