PRACTICAL USE AND DEVELOPMENT OF BIOMÉRIEUX TEMPO® SYSTEM IN MICROBIAL FOOD SAFETY

by

YOUSEF SAEED ALSAADI

B.S., Sultan Qaboos University, 1996
M.S., Sultan Qaboos University, 2008

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Food Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2014

Abstract

In the food industry, coliform testing is traditionally done by the time consuming and labor intensive plate count method or tube enumeration methods. The TEMPO® system (bioMérieux, Inc.) was developed to improve laboratory efficiency and to replace traditional methods. It uses a miniaturization of the Most Probable Number (MPN) method with 16 tubes with 3 dilutions in one single disposable card. It utilizes two stations: the TEMPO® Preparation station and the TEMPO® Reading station. In this study, the Oxyase® (Oxyase®, Inc.) enzyme was added to TEMPO® CC (Coliforms Count), TEMPO® AC (aerobic colony count) and TEMPO® EC (*E. coli* Count) methods. Water samples of 1 ml with 0.1 ml of Oxyase® enzyme were compared to samples without the Oxyase® enzyme using the TEMPO® system. Samples were spiked with different levels of coliforms (10, 10^2, 10^3 and 10^4 CFU/ml), stomached (20 sec), and pipetted into the three different TEMPO® media reagents (4 ml) in duplicate and then automatically transferred into the corresponding TEMPO® cards by the TEMPO® preparation station. Counts were obtained using the TEMPO® reading station after 8, 12, 16, 22 and 24 hours at an incubation temperature of 35°C. Results from 20 replicates were compared statistically. Using TEMPO® tests, high counts in food samples (>6 $\log_{10}$ CFU/ml) can be read in 6±2 hours of incubation using the time-to-detection calibration curve. The TEMPO® system reduces reading time (reading protocol should be changed). There is no need to wait for 22 hours of incubation only 12 hours is required. Oxyrase® enzyme is not needed for the TEMPO® system.

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In the food industry, coliform testing is traditionally done by the time consuming and labor intensive plate count method or tube enumeration methods. The TEMPO® system (bioMérieux, Inc.) was developed to improve laboratory efficiency and to replace traditional methods. It uses a miniaturization of the Most Probable Number (MPN) method with 16 tubes with 3 dilutions in one single disposable card. It utilizes two stations: the TEMPO® Preparation station and the TEMPO® Reading station. In this study, the Oxyase® (Oxyase®, Inc.) enzyme was added to TEMPO® CC (Coliforms Count), TEMPO® AC (aerobic colony count) and TEMPO® EC (*E. coli* Count) methods. Water samples of 1 ml with 0.1 ml of Oxyase® enzyme were compared to samples without the Oxyase® enzyme using the TEMPO® system. Samples were spiked with different levels of coliforms (10, 10^2, 10^3 and 10^4 CFU/ml), stomached (20 sec), and pipetted into the three different TEMPO® media reagents (4 ml) in duplicate and then automatically transferred into the corresponding TEMPO® cards by the TEMPO® preparation station. Counts were obtained using the TEMPO® reading station after 8, 12, 16, 22 and 24 hours at an incubation temperature of 35°C. Results from 20 replicates were compared statistically. Using TEMPO® tests, high counts in food samples (>6 log $_{10}$ CFU/ml) can be read in 6±2 hours of incubation using the time-to-detection calibration curve. The TEMPO® system reduces reading time (reading protocol should be changed). There is no need to wait for 22 hours of incubation only 12 hours is required. Oxyrase® enzyme is not needed for the TEMPO® system.

Table of Contents

List of Figures

List of Tables

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I am grateful to my wonderful kids: sons Abdulrahman and Rashed, and daughters Aysha and Maryam, for their forever invaluable love, patience, understanding and compassionate encouragement from their hearts.

Dedication

I dedicate this dissertation to my beloved parents (Saeed and Sandyah).

They raised me, supported me, taught me, and loved me.

Chapter 1 - Introduction

In the food industry, indicator organisms testing are performed by a labor-intensive Plate Count Method. Indicator organisms are used for assessment of different issues:

1. Sanitation, hygiene, and environmental conditions in the food processing plants.
2. Quality to evaluate the spoilage of food
3. Safety to give indication about the presence of pathogens or fecal contamination.
4. Simple, cheap, easy to use and efficient testing method.

The food industry routinely enumerates microbial flora, which indicates the microbiological load in the entire production process, from raw materials to finished products. These quality indicators are of major importance in establishing a Hazard Analysis Critical Control Point (HACCP) approach, a food safety management system, and guarantee the commercial value of food products from the time they leave the factory to their expiration date. Quality indicators routinely enumerated are: Total Plate Count, Total Coliforms, *Enterobacteriaceae*, *Escherichia coli*, *Staphylococcus aureus*, yeast and molds, etc. Lactic Acid Bacteria is also routinely tested on certain food products. The TEMPO® system from bioMérieux company (Hazelwood, MO) provides testing capabilities for all of these microbial quality indicators.

The TEMPO® system (bioMerieux) automates testing for quality indicator organisms. The technology behind TEMPO® is based on an established microbiological method, called Most Probable Number (MPN) method. Through automation, and miniaturization TEMPO® takes the older, labor-intensive MPN method and standardizes numerous

preparation steps, interpretation, and test results. The outcome is a fast, accurate method with more reliability than the traditional process.

Traditional methods for the enumeration of quality indicators such as *E. co*li in foods are laborious and material intensive. In addition, quality assurance in the food industry requires rapid test methods that allow fast reaction to detect possible risks and contamination levels (Kawasaki et al., 2003). For these reasons, several alternative rapid methods have been developed recently for the enumeration of quality indicators in foods. These methods are generally based on the utilization of chromogenic or fluorogenic substrates for the detection of specific enzyme activities (Manafi et al., 1991). Some of these methods allow identification to be performed directly on the isolation plate or in the broth.

Due to their many advantages, particularly their ease of use, the popularity of ready-to-use systems for the enumeration of hygiene indicator microorganisms is increasing (Ferrati et al., 2010). TEMPO® (bioMérieux, Marcy l'Etoile, France; bioMerieux, Durham, U.S.A.) is an automated enumeration system (see Figure 1.1) based on the most probable number (MPN), and is the most recent of these systems for quality indicators in foods and environment. All current available TEMPO® tests are shown in Table 1.1 with their time incubation requirements. For example, TEMPO® EC (*E. coli*), which is similar to the ISO 16649-2 method based on B-glucuronidase activity, is a test for the 24 hours enumeration of *E. coli* in food. The TEMPO® EC test consists of a card (Figure 1.2) and a vial of culture medium specific to this test. The TEMPO® filler transfers the inoculated medium into the card that contains three sets of 16 wells (small, medium and large wells) with a one-log

difference in volume for each set of wells (volumes 2.25 µl, 22.5 µl, and 225 µl). *Escherichia coli* present in the card hydrolyses the substrate in the culture medium during incubation causing a fluorescent signal to appear, which is detected by the TEMPO® reader.

Figure 1.1 bioMerieux TEMPO® System at 202 Call Hall, Kansas State University, Kansas

Table 1.1 Current available TEMPO® tests and their incubation time (2014)

TEMPO® test	Test description	Food Products	Environmental samples	Incubation time at 35° C
TEMPO BC	Enumeration of *Bacillus cereus*	YES	NO	22-27 hours
TEMPO AC	Viable aerobic mesophilic total flora	YES	YES	24- 48 hours
TEMPO TVC	Aerobic mesophilic total flora	YES	YES	40 – 48 hours
TEMPO EB	Enumeration of *Enterobacteriaceae*	YES	YES	22-27 hours
TEMPO TC	Enumeration of Coliforms as in ISO method	YES	YES	22-27 hours
TEMPO CC	Enumeration of Coliforms as in BAM method	YES	YES	22-27 hours
TEMPO EC	Enumeration of *Escherichia coli*	YES	YES	22-27 hours
TEMPO STA	Enumeration of *Staphylococcus aureus*	YES	YES	24-27 hours
TEMPO LAB	Enumeration of Lactic acid bacteria	YES	YES	40 – 48 hours
TEMPO YM	Enumeration of Yeasts and molds	YES	YES	72 – 76 hours (25°C)

Depending on the number and type of the positive wells, the TEMPO® system calculates the number of *E. coli* present in the original sample according to the calculations based on the MPN method (Raugel 1999, 53-85) .

The TEMPO® system (bioMerieux, Hazelwood, MO) was developed to improve laboratory efficiency and to replace traditional methods. It uses a miniaturization of the Most Probable Number (MPN) method with an ingenius approach16 tubes with 3 dilution in one single disposable card. It utilizes two stations: TEMPO® Preparation station and TEMPO® Reading station

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PRINCIPLE

The TEMPO® test consists of a vial of culture medium and a card, which are specific to this test. The plastic card (Figure 1.2) contains 3 sets of 16 wells (small, medium, and large wells) with a one-log difference in volume for each set of wells (volumes 2.25 µl, 22.5 µl, and 225 µl). Each set is connected between wells 2.25 µl, 22.5 µl, and 225 µl. The card is designed to simulate the Most Probable Number (MPN) method. Essentially each liquid sample tested is distributed to 16 sets of 3 dilutions of sample (1; 10; 100). The data of the sample will be reported automatically as MPN of the sample. After filling, the card is hermetically sealed in order to avoid any risk of contamination during subsequent handling. Depending on the number and type of the positive wells in the 3 log dilution range, the TEMPO® system calculates the level of contamination of the original sample according to a calculation based on the MPN.

Figure 1.2 TEMPO® card with wells of different sizes

Front side

Back side

Chapter 2 - Literature Review

2.1 Food Safety

Food is important part to everyone's life, but if contaminated the food consumed can cause illness and even death. Food safety is a very important, and critical, topic all the time, all over the world. Pesticides, herbicides, chemical additives, and spoilage are all of concern, but food scientists, food processors, and consumers focus mostly on microbiological food safety and quality. Microorganisms pose a challenge to the food industry and most food processes are designed with microbial quality in mind. Microorganisms are too small to be seen with the naked eye and have the ability to reproduce rapidly. Many of them produce toxins and can cause infections, sickness, and even death. For all of these reasons, microbial food safety is given more attention recently, national, and international.

Each year, 48 million people in United States (or 1 in 6 Americans) become sick from and 3,000 die of foodborne diseases (CDC, 2014). Even a 10% reduction in foodborne illness would save 5 million Americans from being sick each year. Prevention of a single fatal case of *E. coli* O157 infection would save an estimated $7 million (CDC, 2014).

The list of the 10 worst food and water outbreaks in United State history is shown in Table 2.1., sorted by the number death cases. (Food Safety News 2014a)

Table 2.1 Historical top ten deadliest food and waterborne outbreaks in United States of America

No.	Pathogens	Year	Food or water implicated	Source	Outbreak places	Number of people Sick	Deaths
1	Typhoid fever	1924-25	Oysters	Long Island, NY	New York, Chicago, and Washington, D.C.	> 1,500	150
2	Typhoid fever	1903	A public water source	Ithaca, NY	New York	1,350	82
3	*Streptococcus*	1911	Raw milk	Boston	Boston	NA	48
4	*Listeria*	2011	"Rocky Ford" cantaloupes	Colorado	28 states	146	36
5	*Listeria*	1985	Mexican cheese	Los Angeles		NA	28
6	*Streptococcus*	1922	Raw milk	Portland, OR		NA	22
7	*Listeria*	1998	Ball Park hot dogs & Sara Lee deli meats	Michigan processing Plant	Michigan	NA	21
8	Botulism	1919	Canned olives	California	3 states	NA	19
9	*Salmonella Typhimurium*	2008-09	Peanut butter $ paste		46 states	714	9
10	*Listeria*	2002	Sliced turkey meats from Pilgrim's Pride		Multiple states	NA	8

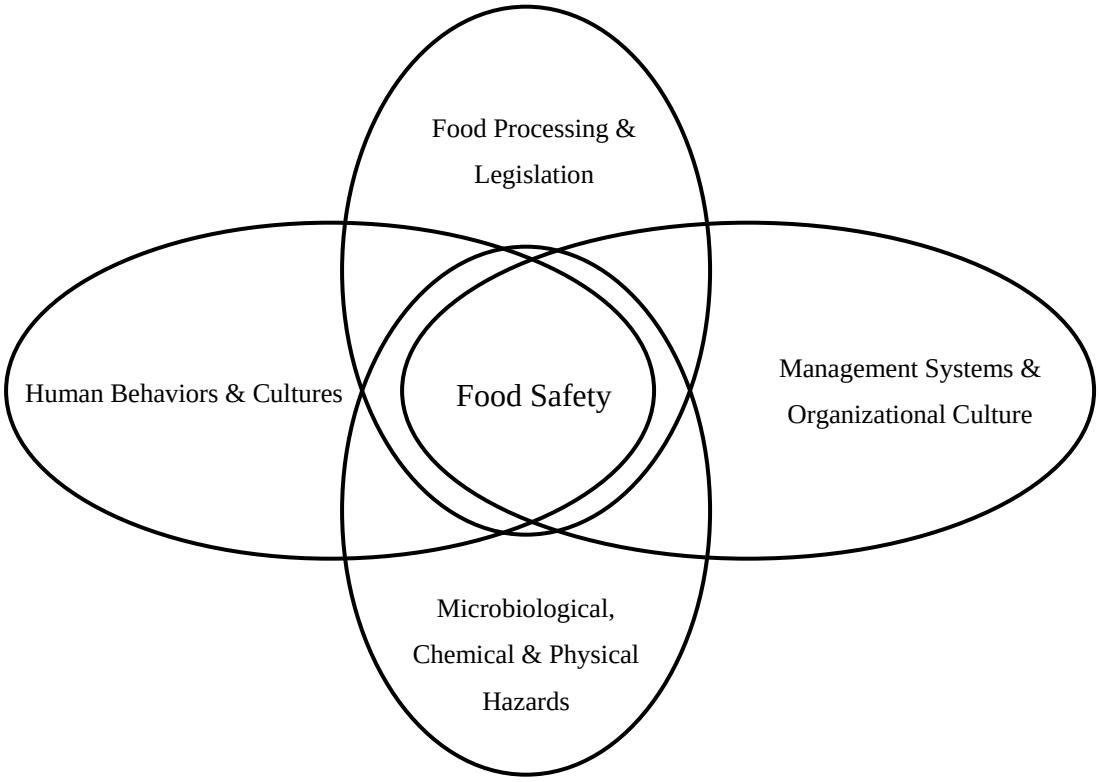
NA= Not Available

Adapted from Food Safety News (2014)

Food safety is a multidisciplinary issue that is affected by many factors (Figure 2.1). Documented food management systems (e.g., HACCP or ISO 22000) explain how things "should be correctly done" but what food handlers "actually do" is more related to the food safety organizational culture (Griffith 2000, 235-256; Griffith 2006, 6-15). This is a complete integration of the individual food handlers' knowledge, attitudes and practices with the organizational culture and standards at food chain business. Food safety

organizational culture and standards are influenced by many factors, including the facilities available (e.g. for hand-washing), as well as the availability of time to implement these food safety practices. Improvements work of all factors will improve the whole food safety system. The major emphasis in this research is to improve microbiological testing for the identification and enumeration of microbiological hazards.

Figure 2.1 Food safety is a multidisciplinary issue adapted from (Griffith, 2006)



2.2 Indicator Organisms

Percy Frankland is the first scientist to introduce the concept of indicators testing of aerobic viable counts back in 1885 by starting the routine examination of water in London using Robert Koch's solid gelatin media plate to count all bacteria (Skinner 1977).

Mainly, indicator species are used because current test methods for pathogens can be costly and time-consuming, making it highly inefficient to test large batches of product or environmental samples. Indicator species, on the other hand, can be tested for with relative ease, and act as an early-warning system that can signal contamination issues and the need for further diagnostic testing. Depending on their application, the major factors considered in classifying an organism as an indicator may include:

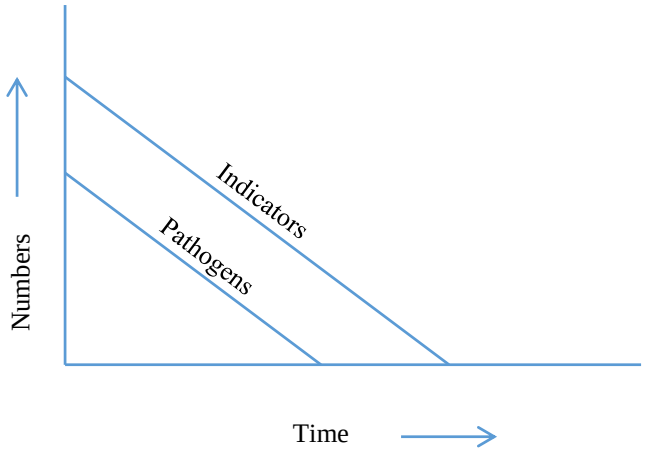
1. Strong association with fecal contamination
2. Co-habitation with pathogens of concern
3. Simple, easy to use and efficient testing procedure

Major roles of indicator organisms in food testing are the following:

1. Quality: to assess and indicate spoilage (e.g., mold growth).
2. Safety: to indicate environmental conditions hospitable to the growth of pathogens or the presence of fecal contamination in the environment. For example, a food processor may conduct routine testing either in-house or through an external laboratory for *E. coli* and coliforms, a common indicator species that originates in fecal matter, in their rinse water (Global Food Safety Resource 2014)

Using indicator organisms in the food process control measurements is better than pathogens since pathogens presence is very low compared to indicators organisms (see Figure 2.2).

Figure 2.2 Ideal relationships between indicator organisms and pathogens. Pathogens exist in lower numbers than indicators during any given time.



Adapted from (Jay, et al., 2005)

2.2.1 Aerobic Colony Counts

The aerobic colony count (ACC) is sometimes called the colony count (CC), total viable count (TVC), aerobic plate count (APC), or plate count (PC). It is also known as the Heterotrophic Plate Count, and it is frequently used for process control of raw materials, selection and qualifying of suppliers, and process control of processed foods. The estimation of total viable bacterial counts is useful to estimate the sterility of food products. It will give how efficacious was the commercial sterilization process.

The ACC is perhaps the simplest and most widely used quality indicator in the U.S. and Europe. This method is designed to provide enumerative results based on the ability of microbes in the sample to grow on a nutrient-rich medium incubated under aerobic and

mesophilic conditions. The ACC is frequently used to monitor a large number of food types for compliance with standards or guidelines set by various agencies, for compliance with purchase specifications, to qualify suppliers of raw materials, and to monitor adherence to Good Manufacturing Practices system (GMPs). Total counts can be used as indices of sanitary quality, organoleptic quality, and evidence of temperature abuse. However, it must be recognized that reliable interpretation of the ACC of a specific food depends on intimate knowledge of the expected microbial population at the point along the farm-to-fork chain at which the sample was collected. Overall, the ACC of raw refrigerated perishable foods will exceed that of shelf-stable foods and indicates the combined impact of source, sanitation, and time-temperature storage. Because the ACC does not differentiate microbes from one another, it can be of limited value in determining spoilage profiles, although in some instances (i.e., highly perishable fresh foods) the ACC can be a good indicator of shelf-life or ingredient suitability. In addition, ACC with fermented food products gives a false positive since it cannot differentiate the fermenting bacteria from others (Jay, et al., 2005; Garcia, 2009).

2.2.2 *Entereobacteriacae*

Increasing emphasis on a total quality management approach in food production, HACCP plans, and Risk Assessment procedures have enhanced the role that quality indicators such as Total Viable Count, Coliforms, *Escherichia coli* and *Enterobacteriaceae* have in monitoring the hygienic and commercial quality of food.

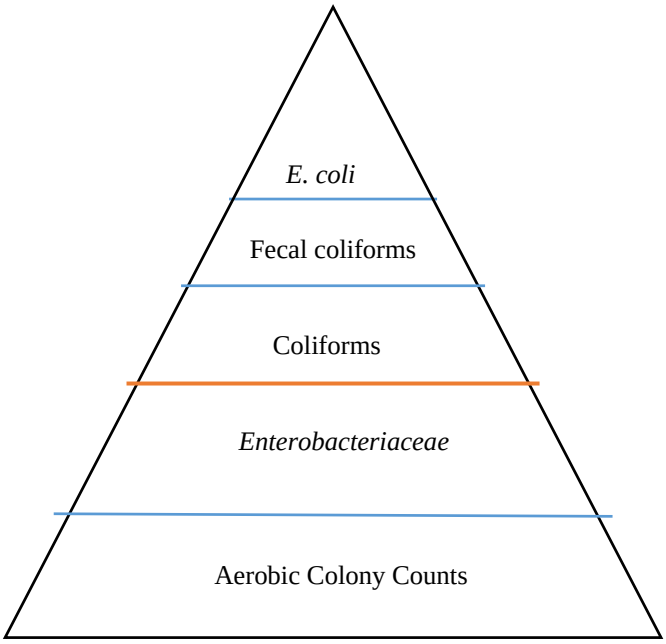
The key hygiene parameter in the latest European regulation on microbiological criteria for food, EC 2073/2005 is *Enterobacteriaceae* enumeration (bioMérieux Industry

2014) . The *Enterobacteriaceae* family includes important food spoilage agents and certain intestinal pathogens such as *Salmonella* spp., and *Shigella* spp. This makes it a very important indicator for hygiene, quality and safety of foods (bioMérieux Industry 2014). Figure 2.3 shows the relationship of different indicator organisms that makes *Enterobacteriaceae* the best choice since it includes all other indicators, and it is the current trend in food testing.

In a food factory, *Enterobacteriaceae* count is used to monitor and assist in evaluating the level of hygiene in clean/dry processing environments. Overall, *Enterobacteriaceae* monitoring plans are used for internal control only and are generally not subject to review by local legislation.

In such clean/dry environments, routine sampling for *Enterobacteriaceae* (or coliforms) should be performed with the aim of locating: (i) sources of potential bacterial contamination, (ii) points of entry of potential bacterial contamination, and (iii) places where multiplication of bacteria has occurred. The design of these sorts of monitoring programs must recognize the importance of the critical control points (CCPs) identified in HACCP and provide monitoring of the relevant environmental factors which impact these CCPs.

Figure 2.3 Relationship between indicators organisms groups



2.2.3 Coliforms

Coliforms are facultative aerobic bacilli-in-shape bacteria that commonly inhabit the intestines of humans and other vertebrates. Coliforms are an indicator of product quality and safety. Coliforms are the most popular indicator group used in the food industry and are a functional sub-group of the *Enterobacteriaceae*. They are defined on cultural characteristics, as aerobic or facultatively anaerobic Gram-negative asporogenous rods which ferment lactose with production of acid and gas at 35°C + 0.5 within 48 hours. They are frequently used for process control of raw materials, selection and qualifying of suppliers, and process control of processed foods.

2.2.4 Fecal Coliforms

Fecal coliforms are a functional subgroup of the coliforms, and are defined by their cultural characteristics as members of the *Enterobacteriaceae* family which also produce acid and gas in EC broth at 44.5°C (Jay, et al., 2005) . The term "thermotolerant colifoms" is sometimes used in place of fecal coliforms. The major discriminating feature here is the ability to grow at elevated temperatures, which was originally thought to separate organisms of fecal origin from other coliform organisms; this has since been disproven (Kornacki and Johnson, 2001) . The major genera represented in the fecal coliform group are *Enterobacter*, *Citrobacter*, and *Klebsiella*, although the majority of the fecal coliforms are strains of *E. coli*.

2.2.5 *Escherichia coli*

More than a century ago, a microbiologists discovered that human feces contained bacteria which, if present in water or food, can make them unsafe. In 1885, Theodor Escherich, a German pediatrician, observed 2 types of organisms present in feces, one of which he named *Bacterium coli* (*B. coli*, which was renamed *Escherichia coli*) and the concept that the presence of *B. coli* implied contamination. The concept of "indicators" had already been suggested in 1880 by van Fritsch based on his observations of *Klebsiellae* in human feces that were also present in water (Berg 1978; National Health and Medical Research Council 2003).

Most *E. coli* types are harmless; some types can cause diseases. The worst known type of *E. coli*, known as *E. coli* O157:H7, causes bloody diarrhea and can sometimes cause kidney failure and even death. *E. coli* O157:H7 produces a toxin called Shiga toxin and is

known as a Shiga toxin-producing *E. coli* (STEC). There are many other types of STEC, and some can cause severe diseases as much as *E. coli* O157:H7.

One severe complication associated with *E. coli* infection is hemolytic uremic syndrome (HUS). It can destroy red blood cells in the human body by production of toxic substances, causing kidney injury. In such cases, intensive care is a must, in addition to kidney dialysis, and transfusions (FoodSafety.gov, 2014) .

Some believe that *E. coli* is a better indicator of fecal contamination originating from warm-blooded animals since animals almost always have high levels (10^5-10^9 CFU/g) of *E. coli* in their feces. Further discrimination of the *E. coli* from within the fecal coliform group has historically been based on a combination of biochemical tests referred to as IMViC, which stands for Indole (ability to produce indole from metabolism of tryptophan); Methyl red (ability to ferment glucose, producing substantial acid as detected by the pH indicator dye methyl red); Vogues-Proskauer reaction (production of 2,3 butanediol and/or acetoin from glucose metabolism); and Citrate (use of citrate as a sole carbon source). As is the case for the fecal coliforms, it has since been determined that the IMViC profile will not accurately identify all *E. coli* strains (Kornacki and Johnson 2001).

While low levels of fecal coliforms and *E. coli* may be present on raw foods (e.g., produce, nuts and grains, meat, poultry, and seafood), high levels are indicative of substantial fecal contamination. The fecal coliforms and *E. coli* should never be present in highly processed ready-to-eat foods and their presence indicates that the product has been subjected to an unhygienic environment (fecal contamination).

2.3 Environment Monitoring

Environmental and in-process testing of indicator organisms accounts for 25% of total test volume in the food industry in the world, while in Asian food plants just 9% of test samples are collected in process and in the production environment. More microbiology environmental testings are done in other regions to support proactive HACCP programs among other reasons. The end-products food microbiology testings accounts for testing of 44% to 59% of test volume in all regions in the world (Hawkins 2014b) .

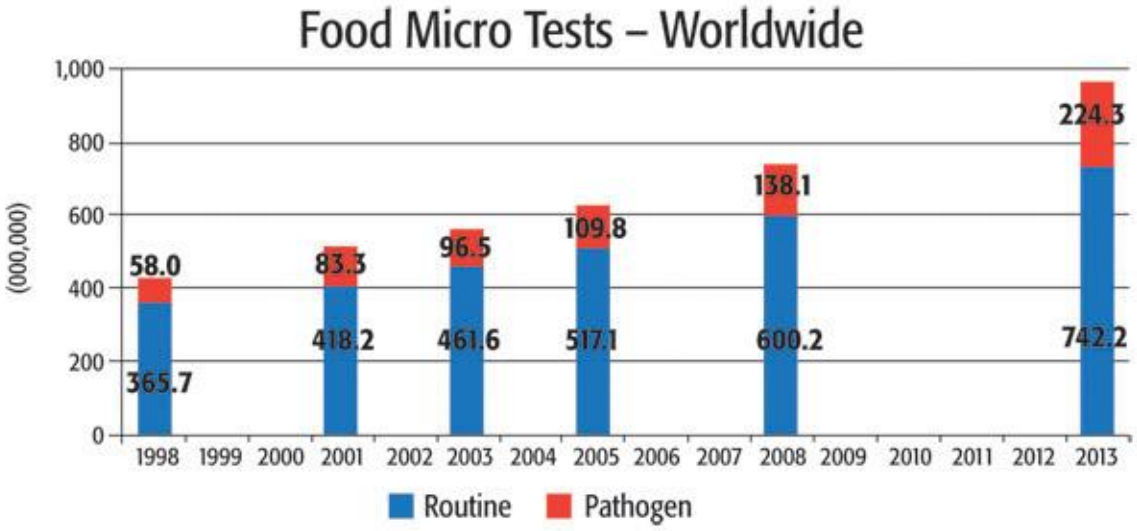
Pathogens testing of environmental and in-process samples accounts for 44% of samples in USA, while in Asian food plants just 8% are tested (Hawkins 2014a; Hawkins 2014b)

2.4 Food and Water Testing

According to Microbiology Testing in the Global Food Industry Report (*Food Micro—8*), by Strategic Consulting, the food industry conducted 966.5 million microbiology tests in 2013 to ensure the food safety in the world. Strategic Consulting's research shows a 128% increase in worldwide microbiology test volumes over the last 15 years. In addition, testing for specific foodborne pathogens (e.g. *Salmonella* and *E. coli*) has grown at an even faster rate. In 2014, pathogen testing represents 23.2% of total food microbiology tests conducted, while in 1998 pathogen testing represented just 13.7% of all such tests. The remaining, indictors testing represent 76.8% of all conducted food microbiology tests in the world (Weschler 2014). In North America, indicators testing accounts for 76% of test volume, and in the European Union and Asia it accounts for 81%

and 72% of test volume respectively (Hawkins 2014a; Hawkins 2014b; Weschler 2014). Figure 2.4 shows that 742.2 million tests were done for indicator organisms in the world compared to 224.3 million for pathogens tests during 2013.

Figure 2.4 Worldwide food microbiology test volumes (1998-2013)



Routine = indicator-organism testing

Adapted from (Woodstock 2013)

Figure 2.5 Pathogen samples collection places in food factories worldwide

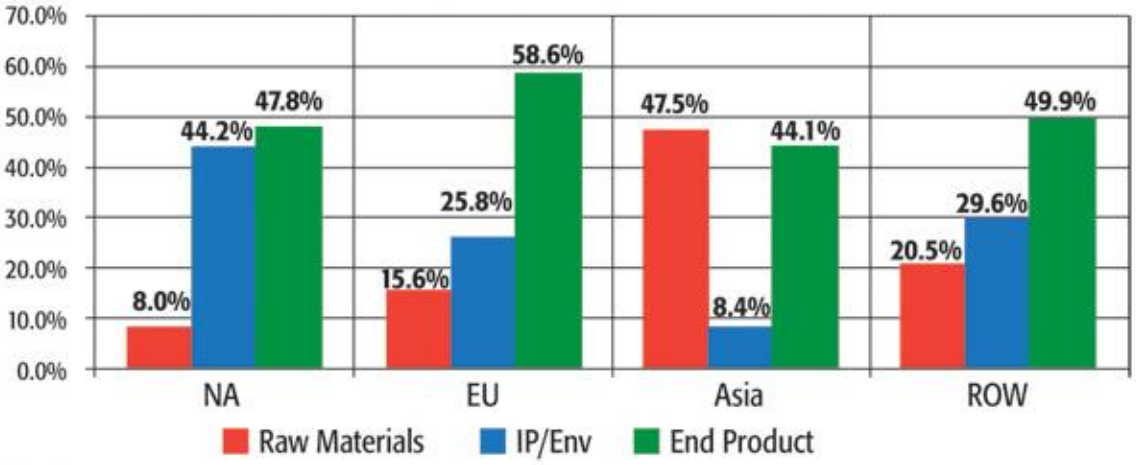


Figure 2.5 shows collection places where food samples are collected for pathogen analysis. It accounts for almost half or more of all samples are from end products (Hawkins 2014a; Hawkins 2014b; Weschler 2014; Anonymous2014; Anonymous2013, n/a; Woodstock 2013)

Figure 2.6 Test volumes by region 2008-2013

F

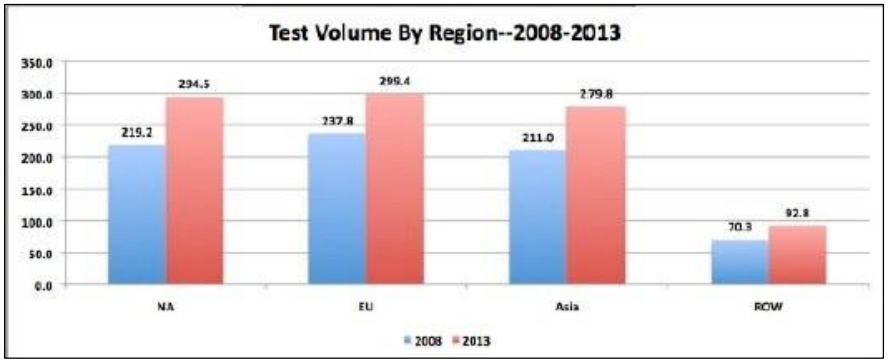


Figure 2.6 compare tests volume done during 2008 and 2013. All microbial food testing has increased during 2013 compared to 2008 in all region of the world (Weschler 2014)

2.5 Food Recalls

Food recalls due to indicator organisms are far less compared to ones due to pathogen contamination. Some selected recent food recalls due to indicators organisms are shown in the Table 2.2. These were voluntary recalls: the first one was a yoghurt cup intended for baby consumption. The second one was almond cake recalled from 23 countries after Chinese customs officials found a batch of 1,800 cakes contaminated with high levels of coliforms that did not meeting the Chinese hygiene standard.

Table 2.2 Selected recent food recalls due to indicator organisms

No.	Company	Food Product	Date	Places Sold in	Reasons	Reference
1	Stonyfield Farm 10 Burton Drive Londonderry, NH 03053	Yobaby Peach/Pear yoghurt cups	April 25, 2014	Alabama, Virginia, Tennessee, Florida, Georgia, North Carolina, South Carolina, Pennsylvania, Maryland, New Jersey, Delaware, Oregon, Washington, Montana, Idaho, Alaska, and California	Coliform contamination *Klebsiella pneumoniae*	(Food Safety News 2014b)
2	Ikea Sweden	Almond cake with chocolate and butterscotch	March 05, 2013	Australia, Austria, Belgium, Bulgaria, China, Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Italy, The Netherlands, Norway, Poland, Romania, Russia, Slovakia, Sweden, Switzerland, Taiwan, and the United Arab Emirates.	Coliform contamination	(Collins 2013)

2.6 Oxyrase® Enzyme

The stories of Oxyrase® started by using a sterile suspension of bacterial membrane fragments and their associated enzymes as reagents for elimination of dissolved oxygen were first reported by (Adler and Crow, 1981). After the addition of membrane fractions, the redox potential in a medium is reduced to -200 to -300 mV, becoming completely anaerobic within about a minute. The partially purified membrane fragments from *Escherichia coli* are now commercially available as Oxyrase® from Oxyrase, Inc., Mansfield, OH.

In 1987, H.I. Adler, with N.D. Crow and his former student J. Copeland, formed the Oxyrase Inc., company in order to promote the use of these membrane fragments in anaerobic microbiology. Adler served as Vice President for Research of Oxyrase until his death; even during his dreadful illness, he continued to consult with the company.

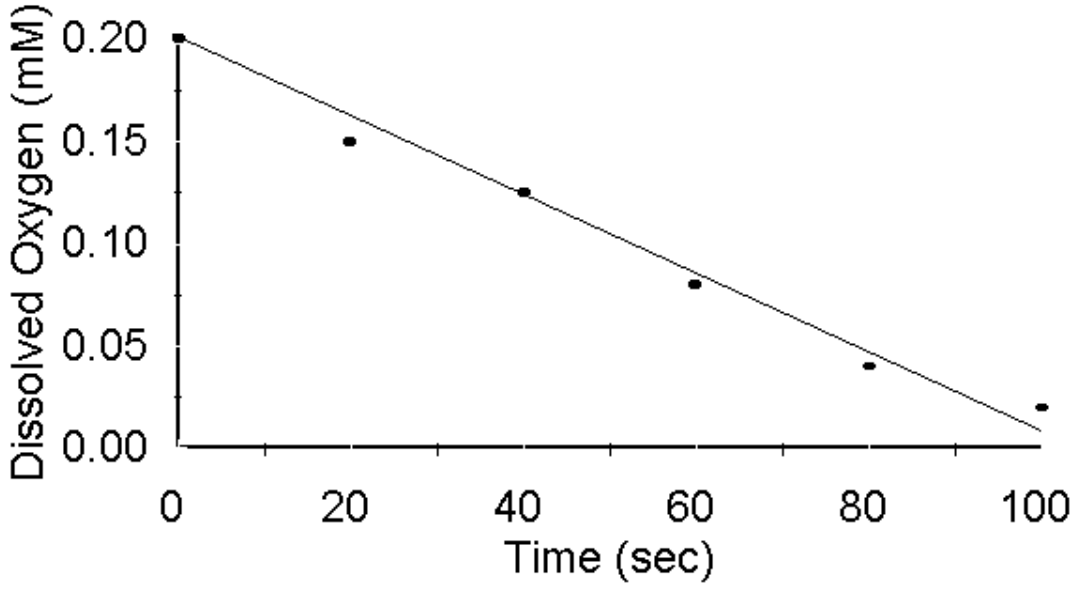
A unit of Oxyrase® per milliliter of liquid reduces dissolved oxygen at the rate of 1% per second at 37°C, pH 8.4 in 40 mM phosphate buffer and 50 mM sodium lactate in an air saturated solution (Hoskins and Davidson, 1988; Ali and Fung, 1990; Yu and Fung, 1990a; Yu and Fung, 1990b; Fung, and Tuitemwong, 1994; Patel and Beuchat, 1995; Patel et al., 1995; Thippareddi et al., 1995; Wonglumsom, et al., 2000; Wonglumsom et al., 2001; Wonglumsom and Fung, 2001) .

Oxyrase® is a naturally antioxidant enzyme system that removes oxygen from its environment, selectively, and efficiently. It is used for culturing anaerobes and Oxyrase® provides a bio-tech approach to making media for isolating and growing anaerobes.

Oxyrase® for Broth is formulated Oxyrase® to be used in broth media and other Oxyrase® for Agar to be used in agar media. Pre-poured plates with different formats of Oxyrase® primarily for use in Clinical Microbiology also available. The inclusion of Oxyrase® in the final plate product confers added benefits to plated media such as long shelf-life.

Oxyrase® enzyme is made to have a minimum of 30 Oxyrase Units per ml over its shelf life, if kept as specified in their directions (see Appendix A). An Oxyrase Unit is defined as that amount of activity that reduces dissolved oxygen, at 37C and pH 8.4 in a phosphate buffer with lactate as substrate, at the rate of 1% per second (see Figure 2.7). Under these conditions, in about 4 minutes 0.3 unit of Oxyrase® can reduce dissolved oxygen at saturation to very low levels measured in ppb (Oxyrase Inc. 2014).

Figure 2.7 Oxyrase® enzyme activity of one unit under standard conditions



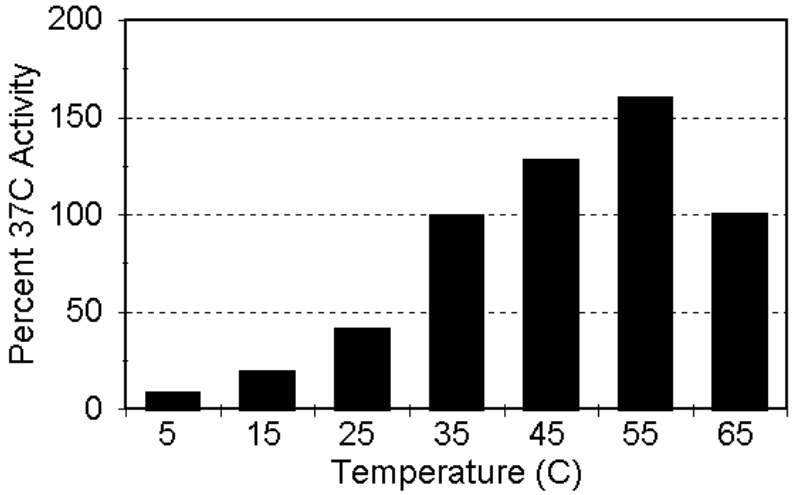
Oxyrase enzyme is active over a wide temperature range of 5°C to 65°C (see Figure 2.8). It can be kept at 40C for hours without substantial loss of activity. It is heat inactivated above 55°C.

Oxyrase® operates over a wide pH range of 6.8 to 9.4 (see Figure 2.9). The optimum pH is about 8.0. As the pH moves away from optimum, activity decreases. The lower activity level can be compensated for by increasing time to complete oxygen removal or by increasing Oxyrase® enzyme concentration. Stability of Oxyrase® enzyme can be maintained by storing at constant - 20°C or lower. It may be thawed and refrozen several times without loss of activity (Oxyrase Inc. 2014) .

Oxyrase® for Broth makes growing anaerobes easy. All you have to do is add Oxyrase® for Broth to broth medium. Oxyrase® for Broth makes broth medium anaerobic. No need to boil broth tubes again.

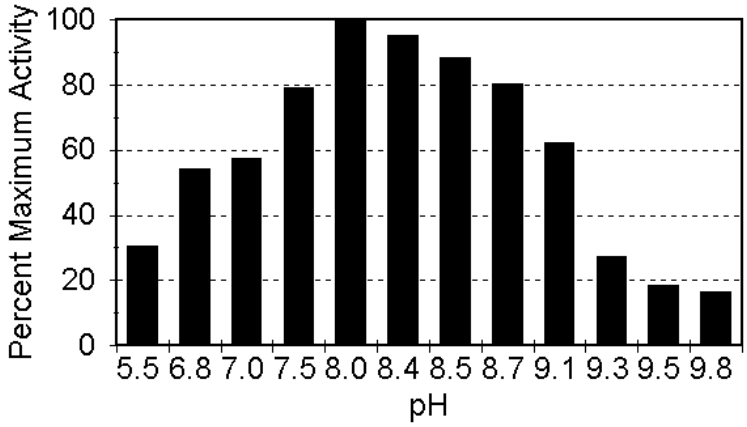
Oxyrase® for Broth is a medium supplement that contains the Oxyrase® Enzyme System and a blend of substrates to maximize the Oxyrase® activity in virtually any broth medium. Oxyrase® for Broth is available in a convenient Dropper Bottle making it easy to add one drop per ml of broth medium in the tube.

Figure 2.8 Oxyrase® enzyme temperature activity profile at pH 8.4



Adapted from (Oxyrase Inc. 2014) .

Figure 2.9 Oxyrase® enzyme pH activity profile at temperature of 37° C



Adapted from (Oxyrase Inc. 2014) .

The Oxyrase® enzyme has been used with many different organisms including pathogens Table 2.3 shows a list of food pathogens that has been proved by scholars that Oxyrase® enhance growth and increase recovery of injured cells.

Table 2.3 Food pathogens shown growth enhancement using Oxyrase® enzyme

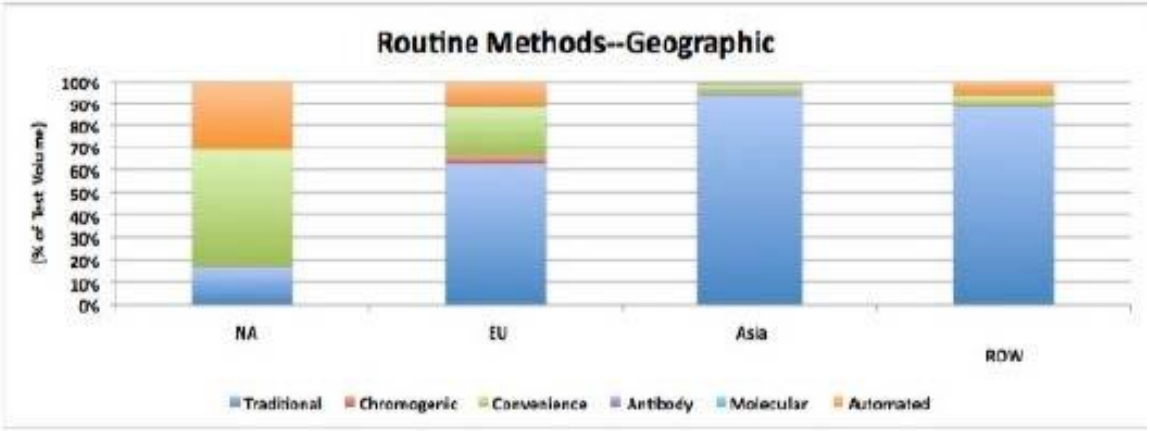
No.	Organisms
1.	*Listeria spp.*
2.	*Listeria monocytogenes*
3.	*Campylobacter spp.*
4.	*Campylobacter jejuni*
5.	*Escherichia coli*
6.	*Escherichia coli* O157:H7

All previous studies with Oxyrase® enzyme were used with traditional microbial method. In this study, Oxyrase® enzyme will be used with rapid methods as a way for further improvement of rapid methods.

2.7 Current Rapid Methods Used

In North America, it is only around 15% of all test volume of indicators organisms are conducted using traditional methods. Chromogenic,

Figure 2.10 How indicator organisms (routine) testing are conducted in the world



1) Increasing Food Microbiology Testing Worldwide

Food safety testing at food processing facilities around the world is increasing for a number of reasons. Public concern is a key driver, and every time the public reads about another food recall, the concern grows. Active media coverage of food safety issues is a prime catalyst.

Also driving growth in food safety test volumes are increasing regulations in many countries and regions. The Food Safety Modernization Act 2011 (FSMA) in the U.S.A., and a heightened food safety action plan for China are just two examples, albeit critical ones given the volume of food production in the two countries.

Not surprisingly, food processing companies are proactively increasing testing efforts in order to avoid the huge costs associated with food recalls, to their bottom lines, their brand names, and to avoid litigation.

Growth in food microbiology testing will not be even across all geographic regions, however. Testing in North America and Asia will grow rapidly but Europe should see slower growth.

2) Growing Use of Rapid Microbial Methods (RMMs)

Thirty years ago, all microbiology testing utilized traditional methods for analysis. Beginning in 1980, newer microbiology methods have been introduced that are easier to use and faster, and as a result, more cost effective overall.

Many food plants have embraced RMMs as the best way to meet their increased testing needs, but not uniformly across all geographies. While the use of RMMs is increasing everywhere, it is quite striking how different the regions are in the level of their adoption of RMMs.

The Kansas State University International Rapid Methods and Automation in Microbiology Workshop, directed and initiated by Dr. Fung, has attracted more than 4,500 microbiologists from 60 countries and 46 states to the program in the past 30 years (1980 to 2010).

Table 2.4 Selected rapid test kits

No.	Test Kit Name	Manufacturer	Organisms
1.	SimPlate for TPC	BioControl Systems, Inc.	aerobic bacteria
2.	Sanita-kun Total Plate Count	JNC Corporation	aerobic bacteria
3.	RIDA Count Aerobic Count	R-Biopharm AG	aerobic bacteria
4.	Compact Dry Total Count	Nissui Pharmaceutical Co., Ltd	aerobic bacteria
5.	DOX 60F/30F TVC	Bio-Theta, Ltd.	aerobic bacteria
6.	Soleris/ MicroFoss Coliform Test	Neogen Corporation/ FOSS A/S	Coliforms
7.	Sanita-kun Coliforms	JNC Corporation	Coliforms

Adapted from (AOAC International 2014)

Table 2.4 shows some of rapid microbiological tests that have been validated by the Association of Analytical Communities International (AOAC) with their manufacturing companies.

2.8 TEMPO® Technique

TEMPO® technique developed and marketed by bioMérieux, Inc. (Figure 2.12). It automates testing for quality indicator organisms. The technology behind TEMPO® is based on an established microbiological method called Most Probable Number (MPN). Through automation, TEMPO® takes the older, labor-intensive MPN method and standardizes numerous preparation steps, interpretation, and test results. The outcome is a fast, accurate method with more reliability than the original process.

Traditional methods for the enumeration of quality indicators such as *E. co*li in foods are laborious and material intensive. In addition, quality assurance in the food industry requires rapid test methods that allow faster responses to any possible risks (Kawasaki et al., 2003) . For these reasons, several alternative rapid methods have been developed recently for the enumeration of quality indicators in foods. These methods are generally based on the utilization of chromogenic or fluorogenic substrates for the detection of specific enzyme activities (Manafi et al., 1991) . Some of these methods allow identification to be performed directly on the isolation plate or in the broth.

The ease of use and the ready-to-use systems are the main advantages for the enumeration of hygiene indicator microorganisms, which is increasing in popularity (Ferrati et al., 2010) . TEMPO® (bioMérieux, Marcy l'Etoile, France) is an automated enumeration system based on the most probable number (MPN) method, and is the most recent of these systems for quality indicators in foods and environment samples. Table 2.5 shows the current available TEMPO® tests and their validation. TEMPO® EC (*E. coli*), which is similar to the ISO 16649-2 method based on b-glucuronidase activity, is a test for the 24 h enumeration of *E. coli* in food. The TEMPO® EC test consists of a card (Figure

2.11) and a vial of culture medium specific to this test. The TEMPO® filler transfers the inoculated medium into the card that contains three sets of 16 wells (small, medium and large wells) with a one-log difference in volume for each set of wells. *Escherichia coli* present in the card hydrolyses the substrate in the culture medium during incubation causing a fluorescent signal to appear, which is detected by the TEMPO® reader. Depending on the number and type of the positive wells, the TEMPO® system calculates the number of *E. coli* present in the original sample according to the calculations based on the MPN method (Raugel 1999, 53-85) .

Figure 2.11 Front side of TEMPO® CC card

Figure 2.12 TEMPO® System components

A. TEMPO® Filler **B. TEMPO® PC & Software** **C. TEMPO® Reader**

Table 2.5 Current available TEMPO® tests and their Validation (2014)

TEMPO test	Test description	Time to result	AOAC validation	AFNOR/ISO 16140 validation
TEMPO BC	*Bacillus cereus*	24 hours	N° 071401	Pending
TEMPO AC	Aerobic mesophilic total flora	24 to 48 hours	Certificate N° 121204	BIO 12/35 - 05/13
TEMPO TVC	Aerobic mesophilic total flora	40 – 48 hours	Certificate N°120602[a]	BIO 12/15 - 09-05
TEMPO EB	*Enterobacteriaceae*	24 hours	Certificate N° 050801	BIO 12/21 - 12/06
TEMPO TC	Coliforms (ISO)	24 hours		BIO 12/17 - 12/05
TEMPO CC	Coliforms (BAM)	24 hours	Certificate N° 060702	
TEMPO EC	*Escherichia coli*	24 hours	Certificate N°060803[b]	BIO 12/13 - 02/05
TEMPO STA	*Staphylococcus*	24 hours	Certificate N° 120901	BIO 12/28 - 04/10
TEMPO LAB	Lactic acid bacteria	40 – 48 hours		
TEMPO YM	Yeasts and molds	72 – 76 hours	Certificate N° 060702	

[a] = AOAC Official Method - N°2008.10

[b] = AOAC Official Method - N°2009.02

2.9 Research Objectives

2.9.1 Research Focus

The purpose of this study was to improve the current TEMPO® method for

1. Total viable counts
2. *E. coli* counts
3. Coliforms counts

by addition of Oxyrase® enzyme to shorten the incubation time.

2.9.2 Research Questions

1. Does the use of Oxyrase® enzyme in the TEMPO® test for total viable counts shorten the required testing incubation time?
2. Does the use of Oxyrase® enzyme in the TEMPO® test for *E. coli* shorten the required testing incubation time?
3. Does the use of Oxyrase® enzyme in the TEMPO® test for coliforms shorten the required testing incubation time?

2.9.3 Research Aim

The aim of this research was to speed up TEMPO® tests for *E. coli*, total viable counts, and coliforms to less than 24 hours.

Chapter 3 - Materials and Methods

3.1 Bacterial cultures and inoculum preparation

Six different strains of coliforms were used in this study (see Table 3.1) and all were obtained from Microbiologics Inc., St. Cloud, MN. The bacteria were maintained on Brain Heart Infusion Broth (BHI) at 4° C and subcultured every 2 weeks. Coliforms from stock cultures were subcultures on Plate Count Agar. The 48-hours cultures were transferred into 0.1 % sterile peptone water (Difco Laboratories, Sparks, MD) and were diluted serially (1:10) in peptone water to achieve an initial bacterial count of about 1-2 log CFU/ml as low-level inoculum, 2-3 log CFU/ml medium-level inoculum and 3-5 log CFU/ml high-level inoculum using Mcfarland equivalence turbidity standards.

Table 3.1 Coliform cocktail cultures used

No.	Bacteria	Strain
1	*Enterobacter cloacae*	ATCC® 35030™
2	*Enterococcus faecalis*	ATCC® 29212™
3	*Enterococcus faecalis*	ATCC® 51299™
4	*Escherichia coli*	ATCC® 51813™
5	*Klebsiella pneumoniae*	ATCC® 4352™
6	*Klebsiella pneumoniae*	ATCC® 10031™

3.2 Statistical analysis

The microbial counts data were analyzed as a Completely Randomized Design (CRD) with repeated measures using the PROC MIXED procedure of SAS® (SAS Institute Inc., Cary, NC), with the bacterial counts as the experimental unit. The effects of treatment either with or without Oxyrase® enzyme, reading times, and their interactions were considered to be fixed effects. The SAS® software used version 9.4 for windows. Significant difference was determined at $P < 0.05$. Differences of Least Square Means were used with Tukey adjustment for all comparisons of the treatments and reading times.

3.3 Oxyrase® enzyme in TEMPO® EC, AC, and CC tests

Figure 3.1 Protocol of the TEMPO® method in this study

3ml of sterile distilled water was added to TEMPO® vials (without & with Oxyrase® was added at this stage 3 drops/vial 0.1ml/1ml broth)

↓

Required 1ml with desired culture were prepared

↓

Vortex

↓

The related cards were assigned then entered in the system

↓

Cards were filled using TEMPO filler unit then incubated

↓

Cards were read after 8, 12, 16, 22, and 24 hours of incubation and results were recorded

3 samples with 3 different levels (low, medium, and high) of inoculation for each treatment with and without Oxyrase® for each test were performed.

3.4 Oxyrase enzyme in TEMPO® CC test for coliform counts in ground beef samples

Ground beef samples were inculcated with coliform cultures to get to desired concentration of low (1-2 log), medium (2-3 log) and high (3-5 log) using Mcfarland turbidity standards.

3.5 Time-to-detection in TEMPO® CC test for coliform counts

Vials with very high concentrations of coliforms were made with serial dilution. Cards were incubated and read every half hour. Results were recorded when reaching the maximum range of detection. Calibration (time-to-detection) curve was constructed so that it can predict the bacterial concentration when reaching the maximum range of the TEMPO® system.

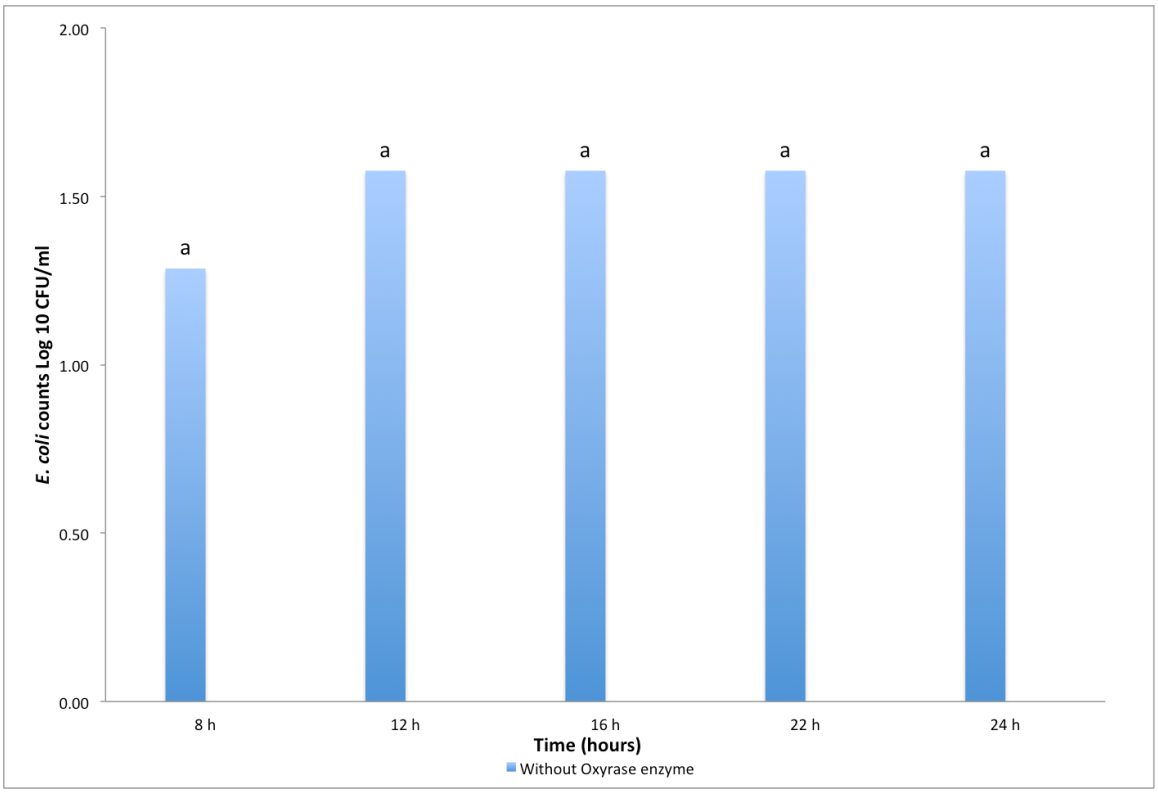
3.6 Relative fluorescence units & pH when using TEMPO® CC

Blank negative samples were used without coliforms (cultures) by pipetting 4 ml of sterile distilled water into TEMPO® CC vials. Then 3 drops of Oxyrase® enzyme were added using plastic Pasture dropper to the vials. Then both sets with Oxyrase® and without Oxyrase® were pH measured using pH meter. Other vials were filled into TEMPO® CC cards using TEMPO® filler unit after entering their details in the TEMPO® system using TEMPO® preparation software. Then data for these tests are exported and emailed to bioMerieux headquarters in France for fluorescence interpretation.

Chapter 4 - Results and Discussion

4.1 *E. coli* counts using TEMPO® EC and Oxyrase® enzyme

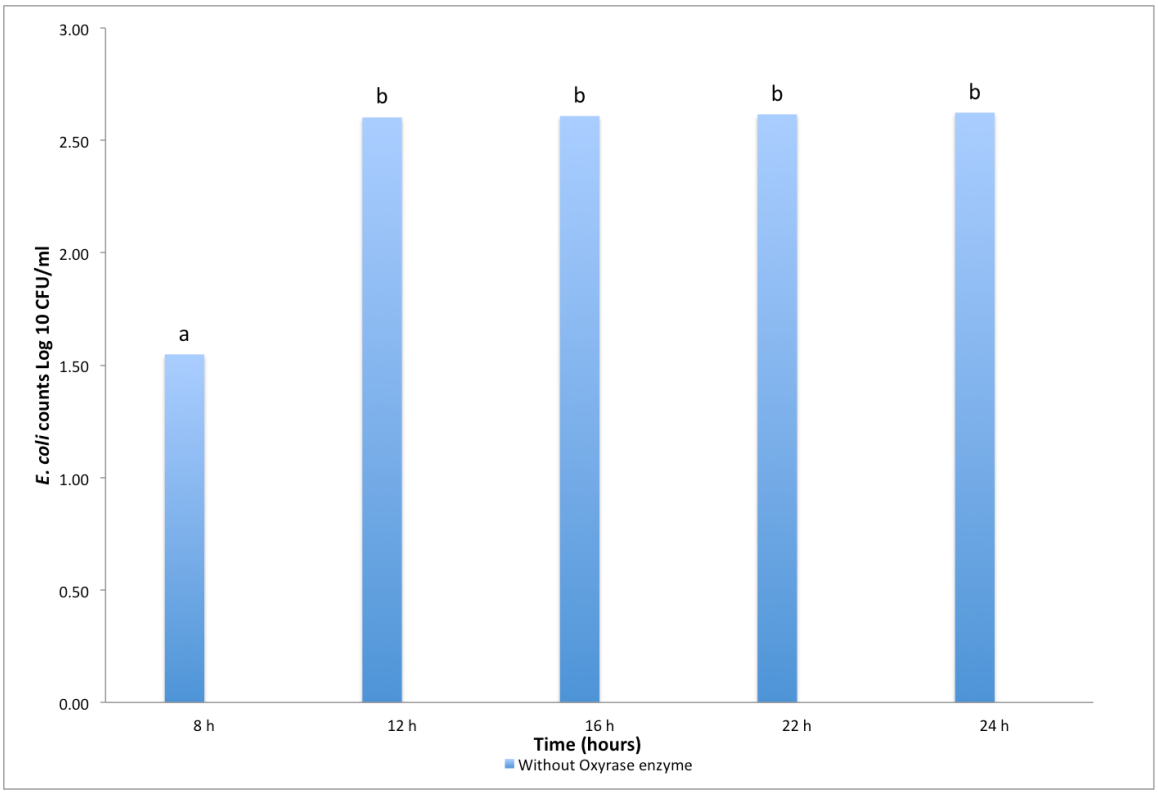
Figure 4.1 Least Square Means of *E. coli* counts (low concentration) using TEMPO® EC without Oxyrase® enzyme at different times



Note: means with different letters on bars are significantly different (P<0.05). Standard Error= 0.17 results of treatment with Oxyrase® is not shown because of false positive.

Using the low concentration (1-2 Log $_{10}$ CFU/ml) resulted (Figure 4.1) in no significant difference when reading of the TEMPO® EC cards at 8 hours or 24 hours of incubation. That means whenever low concentration can be read at 8 hours and there is no need to wait for the full incubation period. Results with Oxyrase® enzyme were giving a false positive (i.e., indicates that TEMPO® EC was giving the maximum counts >4.9 X 10^3 CFU/ml when no *E. coli* was inoculated in blank negative test).

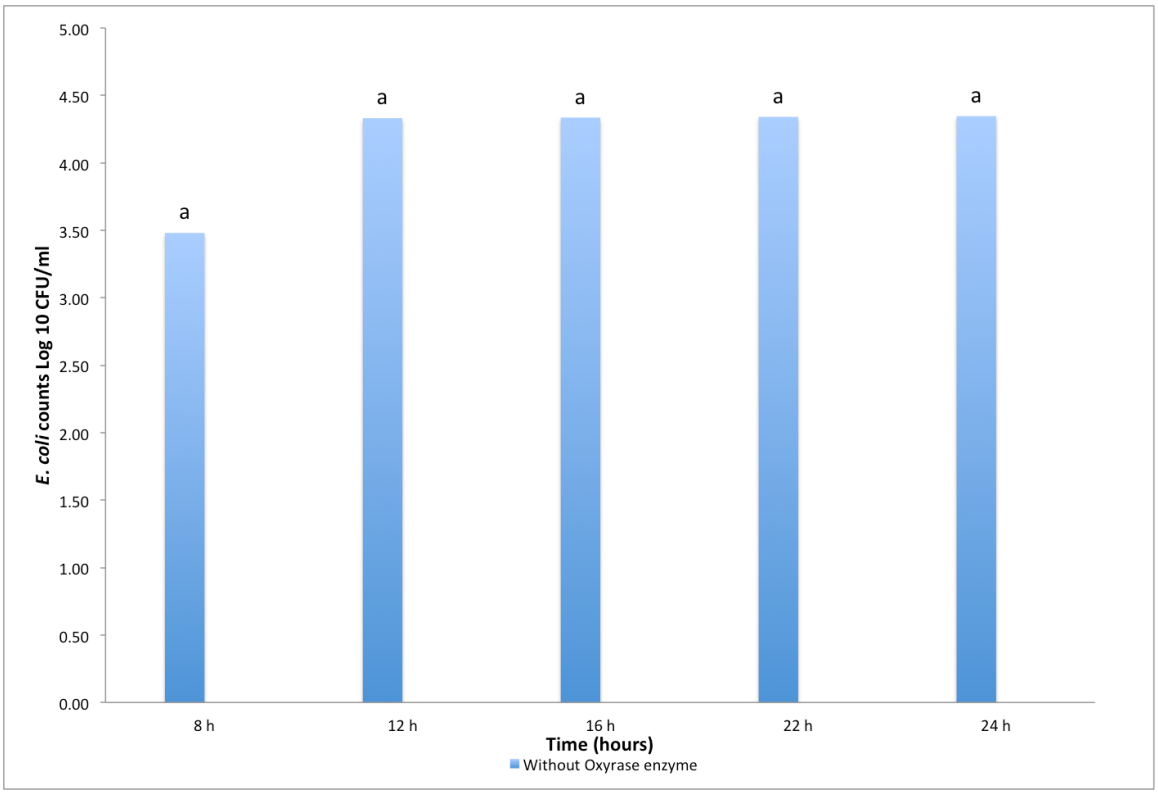
Figure 4.2 Least Square Means of *E. coli* counts (medium concentration) using TEMPO® EC without Oxyrase® enzyme at different times



Note: means with different letters on bars are significantly different (P<0.05). Standard Error= 0.14 results of treatment with Oxyrase® is not shown because of false positive.

Using the medium concentration (2-3 Log $_{10}$ CFU/ml) resulted (Figure 4.2) in no significant difference when reading of the TEMPO® EC card at 12 hours or 22 hours of incubation. That means whenever medium concentration can be read at 12 hours and there is no need to wait for the full incubation period. At 8 hours reading is significantly different from reading at any other times. Results with Oxyrase® enzyme were giving a false positive (i.e., indicates that TEMPO® EC was giving the maximum counts >4.9 X 10^3 CFU/ml when no *E. coli* was inoculated in blank negative test).

Figure 4.3 Least Square Means of *E. coli* counts (high concentration) using TEMPO® EC without Oxyrase® enzyme at different times

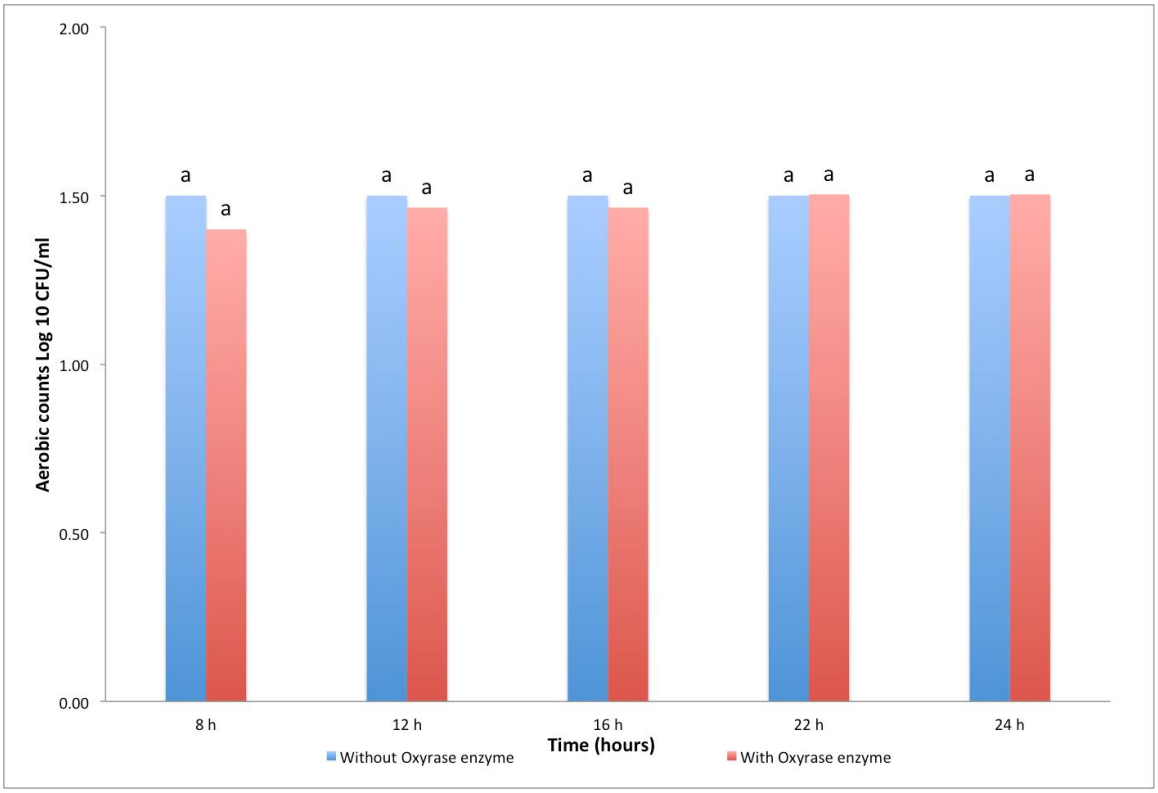


Note: means with different letters on bars are significantly different (P<0.05). Standard Error= 0.29 results of treatment with Oxyrase® is not shown because of false positive.

Using the high concentration (3-5 Log $_{10}$ CFU/ml) resulted (Figure 4.3) in no significant difference when reading of the TEMPO® EC card at 8 hours or 22 hours of incubation. Even though, not significant differences at 8 hours the difference in the mean counts are larger than 0.5 log (0.85 log difference) which considered a difference in microbial count. TEMPO® EC can be read at 12 hours and there is no need to wait for full incubation period. Results with Oxyrase® enzyme were giving a false positive (i.e., indicates that TEMPO® EC was giving the maximum counts >4.9 X 10^3 CFU/ml when no *E. coli* was inoculated in blank negative test).

4.2 Aerobic counts using TEMPO® AC and Oxyrase® enzyme

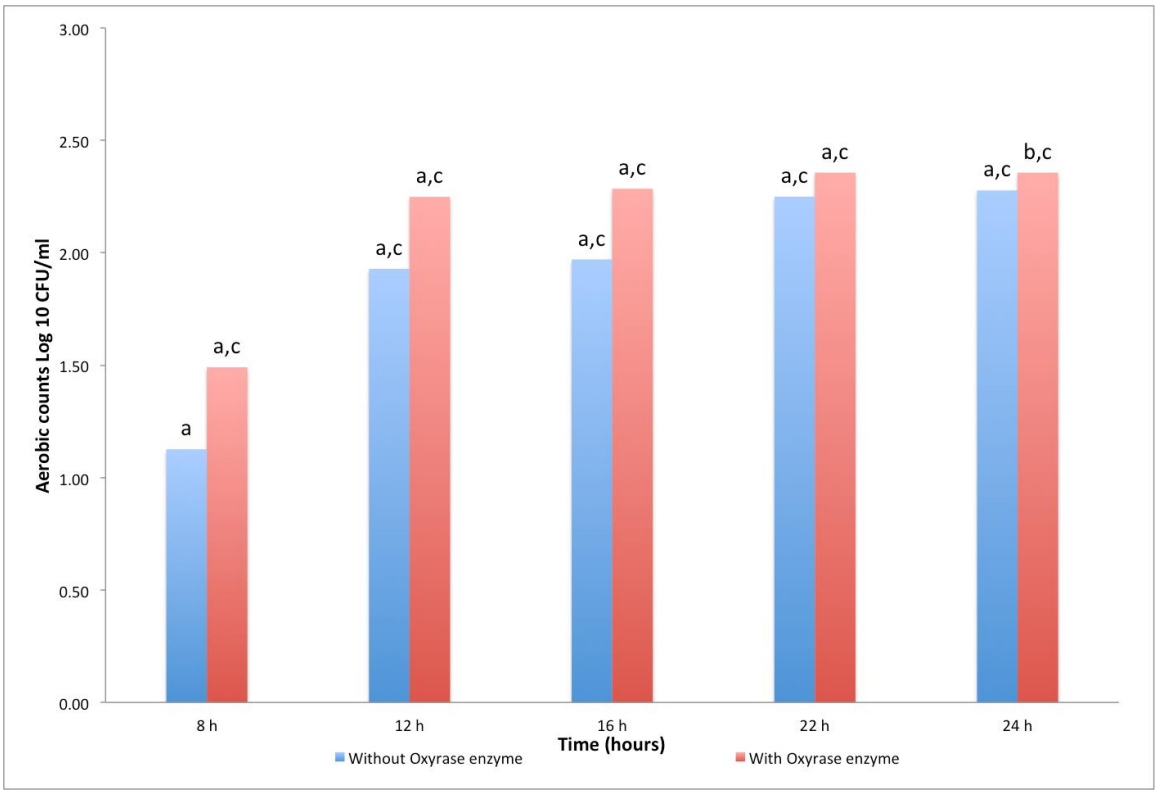
Figure 4.4 Least Square Means of aerobic counts (low concentration) using TEMPO® AC with & without Oxyrase® enzyme at different times



Note: means with different letters on bars are significantly different (P<0.05). Standard Error= 0.24 without Oxyrase® & 0.27 with Oxyrase®.

Using the low concentration (1-2 Log $_{10}$ CFU/ml) resulted (Figure 4.4) in not significant difference when reading of the TEMPO® AC card at 8 hours or 22 hours of incubation. That means whenever low concentration of aerobic counts can be read at 8 hours and there is no need to wait for the full incubation period. Results with Oxyrase® enzyme were not different than without Oxyrase® enzyme at all times tested.

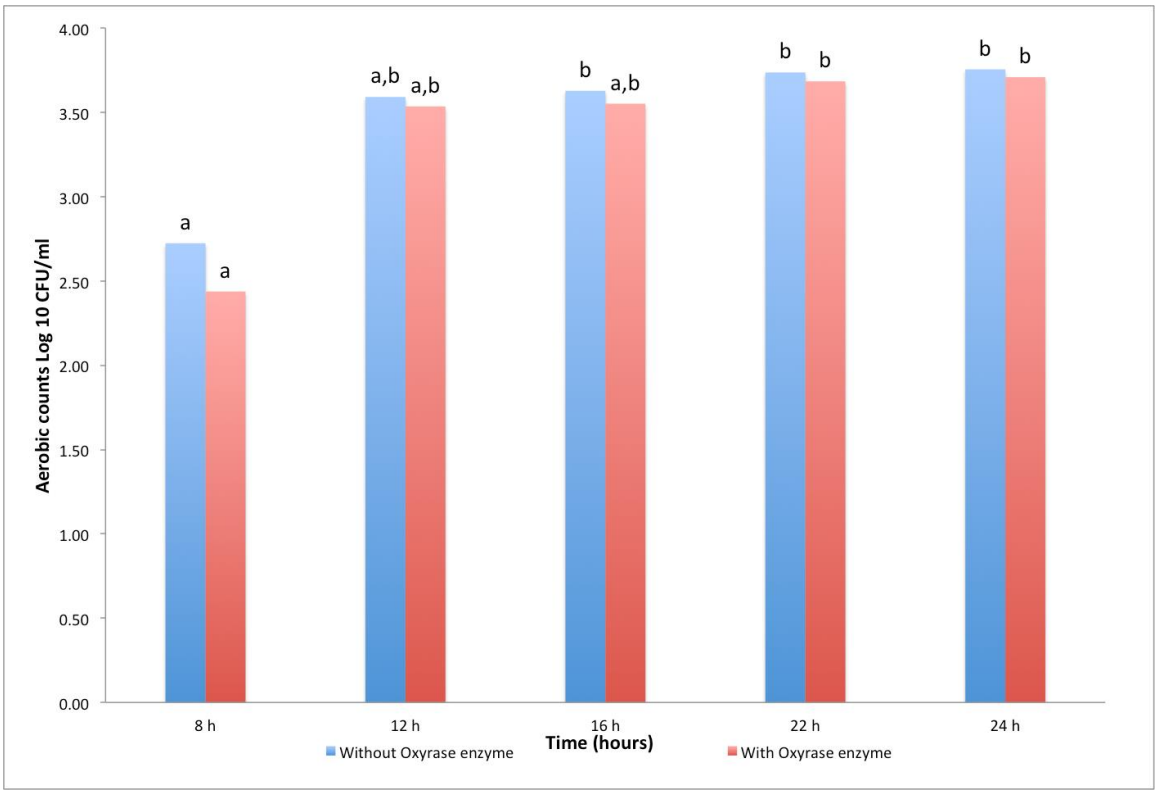
Figure 4.5 Least Square Means of aerobic counts (medium concentration) using TEMPO® AC with & without Oxyrase® enzyme at different times



Note: means with different letters on bars are significantly different (P<0.05). Standard Error= 0.24 without Oxyrase® & 0.24 with Oxyrase®.

Using the medium concentration (2-3 Log $_{10}$ CFU/ml) resulted (Figure 4.5) in significant difference when reading at 8 hours and 24 hours for TEMPO® AC card with Oxyrase®. 12 hours readings were not different from 22 hours. 8 hours readings were differing by more than 0.5 log with other reading times which considered a difference in microbial count. TEMPO® AC can be read at 12 hours and there is no need to wait for the full incubation period, since it is within 0.5 log and not significant difference. Results with Oxyrase® enzyme were not different than without Oxyrase® enzyme at all times tested.

Figure 4.6 Least Square Means of aerobic counts (high concentration) using TEMPO® AC with & without Oxyrase® enzyme at different times

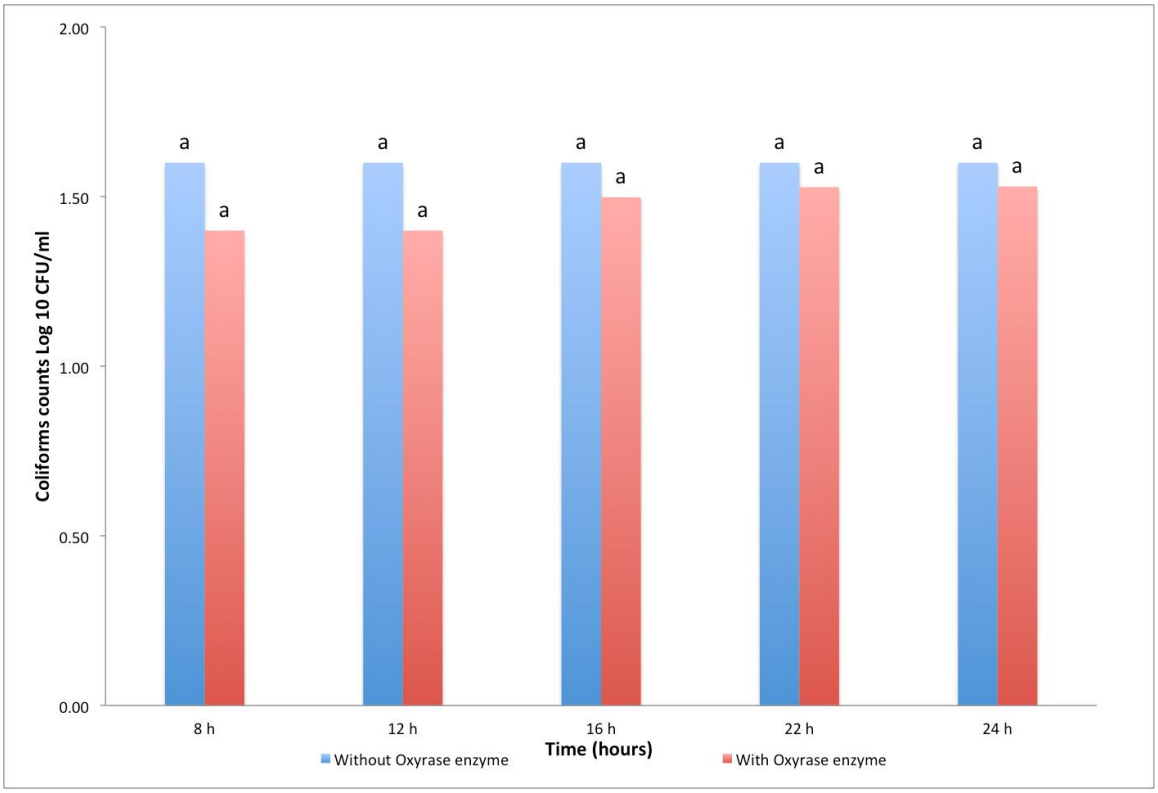


Note: means with different letters on bars are significantly different (P<0.05). Standard Error= 0.24 without Oxyrase® & 0.27 with Oxyrase®.

Using the high concentration (3-5 Log $_{10}$ CFU/ml) resulted (Figure 4.6) in significant difference when reading at 8 hours and at 22 hours for TEMPO® AC card with and without Oxyrase® enzyme. 12 hours readings were not different from 22 hours. TEMPO® AC can be read at 12 hours and there is no need to wait for the full incubation period. Results with Oxyrase® enzyme were not different than without Oxyrase® enzyme at all times tested.

4.3 Coliform counts using TEMPO® CC and Oxyrase® enzyme

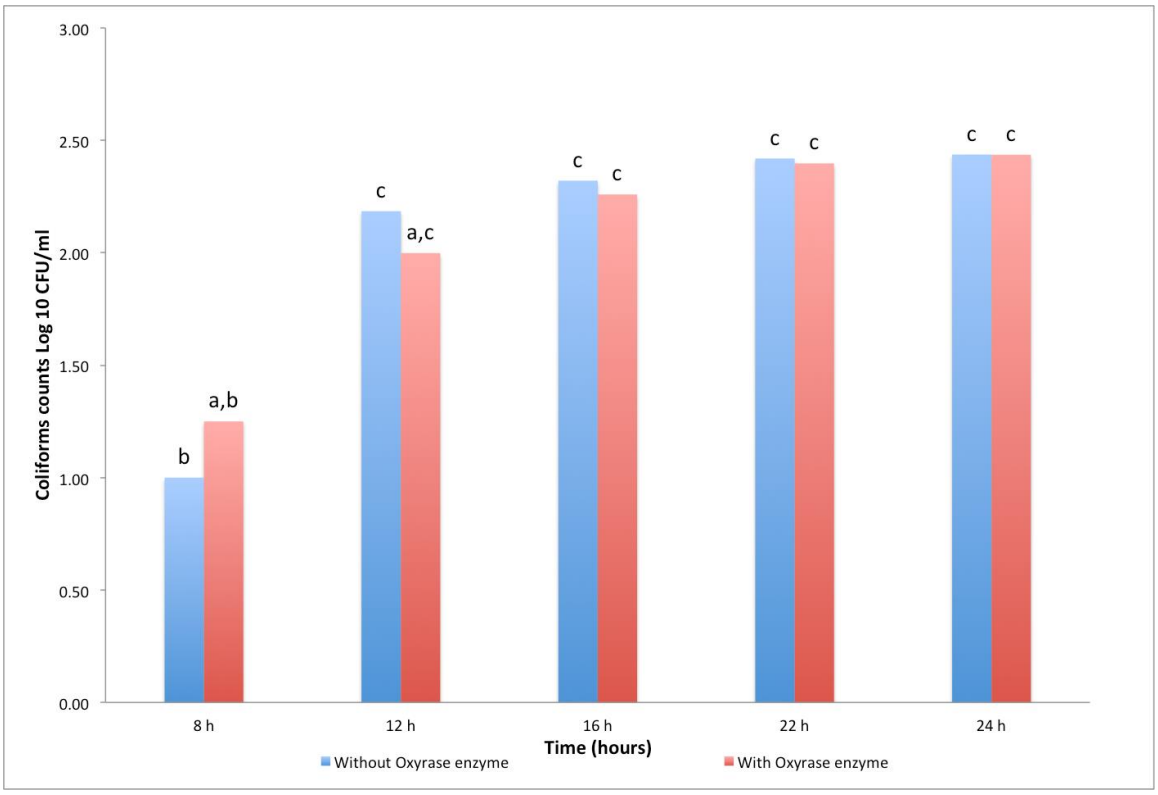
Figure 4.7 Least Square Means of coliforms counts (low concentration) using TEMPO® CC with & without Oxyrase® enzyme at different times



Note: means with different letters on bars are significantly different (P<0.05). Standard Error= 0.24 without Oxyrase & 0.24 with Oxyrase.

Using the low concentration (1-2 Log $_{10}$ CFU/ml) resulted (Figure 4.7) in not significant difference when reading of the TEMPO® CC card at 8 hours or 22 hours of incubation. That means whenever low concentration of coliform counts can be read at 8 hours and there is no need to wait for the full incubation period. Results with Oxyrase® enzyme were not different than without Oxyrase® enzyme at all times tested.

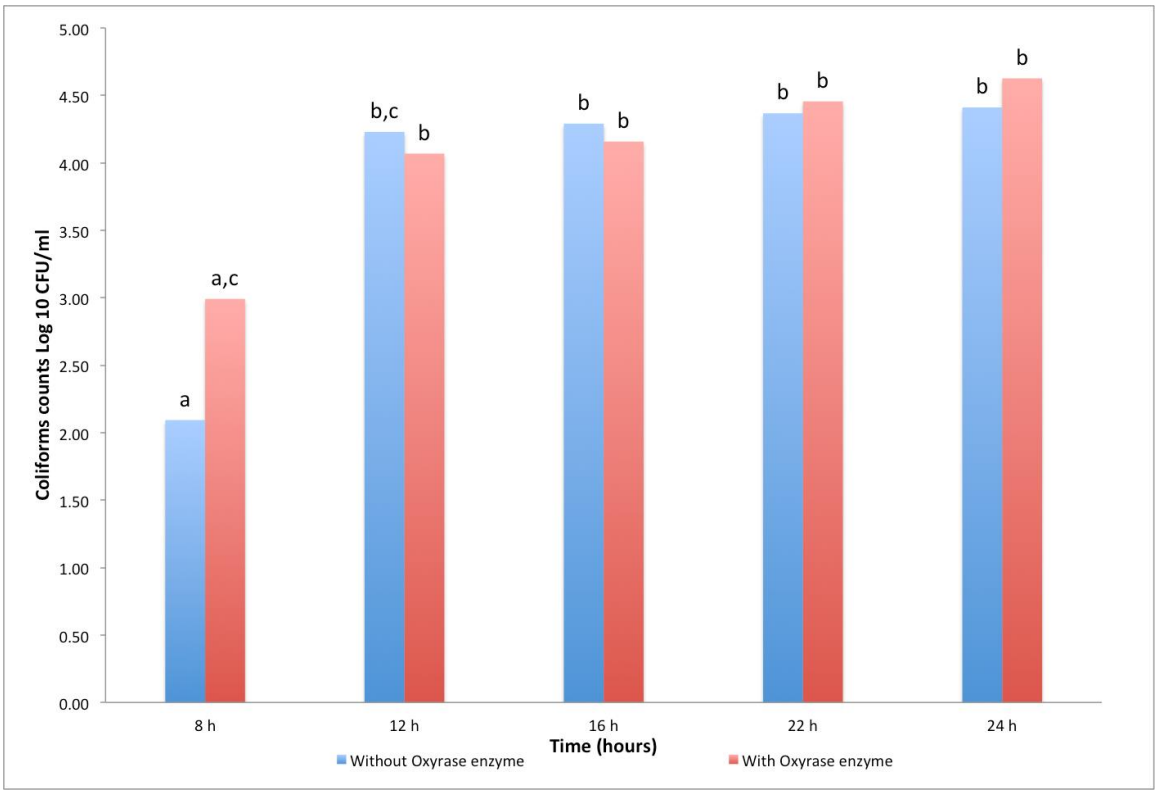
Figure 4.8 Least Square Means of coliforms counts (medium concentration) using TEMPO® CC with & without Oxyrase® enzyme at different times



Note: means with different letters on bars are significantly different (P<0.05). Standard Error= 0.16 without Oxyrase® & 0.16 with Oxyrase®.

Using the medium concentration (2-3 Log $_{10}$ CFU/ml) resulted (Figure 4.8) in significant difference when reading at 8 hours and 24 hours for TEMPO® CC card with Oxyrase®. 12 hours readings were not different from 22 hours. 8 hours readings were differing by more than 0.5 log with other reading times which considered a difference in microbial count. TEMPO® CC can be read at 12 hours and there is no need to wait for the full incubation period, since it is within 0.5 log and not significant difference. Results with Oxyrase® enzyme were not different than without Oxyrase® enzyme at all times tested.

Figure 4.9 Least Square Means of coliforms counts (high concentration) using TEMPO® CC with & without Oxyrase® enzyme at different times

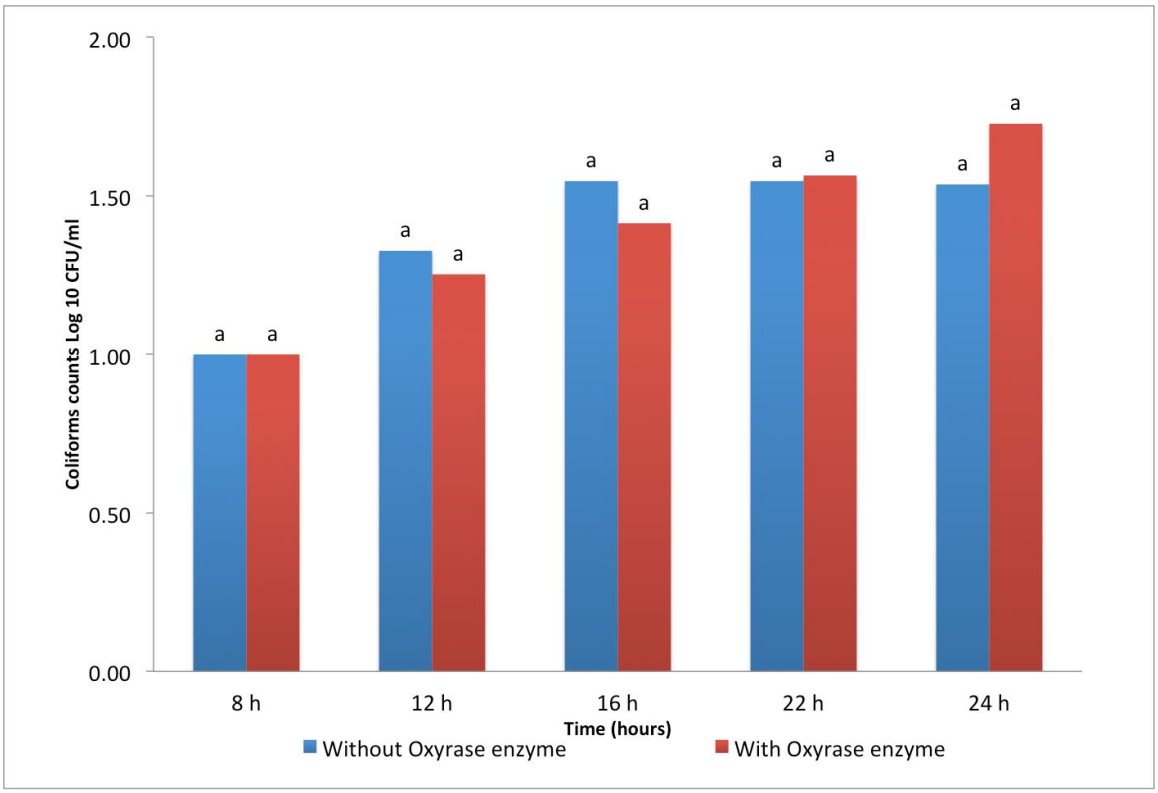


Note: means with different letters on bars are significantly different (P<0.05). Standard Error= 0.27 without Oxyrase® & 0.26 with Oxyrase®.

Using the high concentration (3-5 Log $_{10}$ CFU/ml) resulted (Figure 4.9) in significant difference when reading at 8 hours and at 22 hours for TEMPO® CC card with and without Oxyrase® enzyme. 12 hours readings were not different from 22 hours. 8 hours readings were differing by more than 0.5 log with other reading times which considered a difference in microbial count. TEMPO® CC can be read at 12 hours and there is no need to wait for the full incubation period. Results with Oxyrase® enzyme were not different than without Oxyrase® enzyme at all times tested.

4.4 Coliform counts using TEMPO® CC and Oxyrase® enzyme in ground beef samples

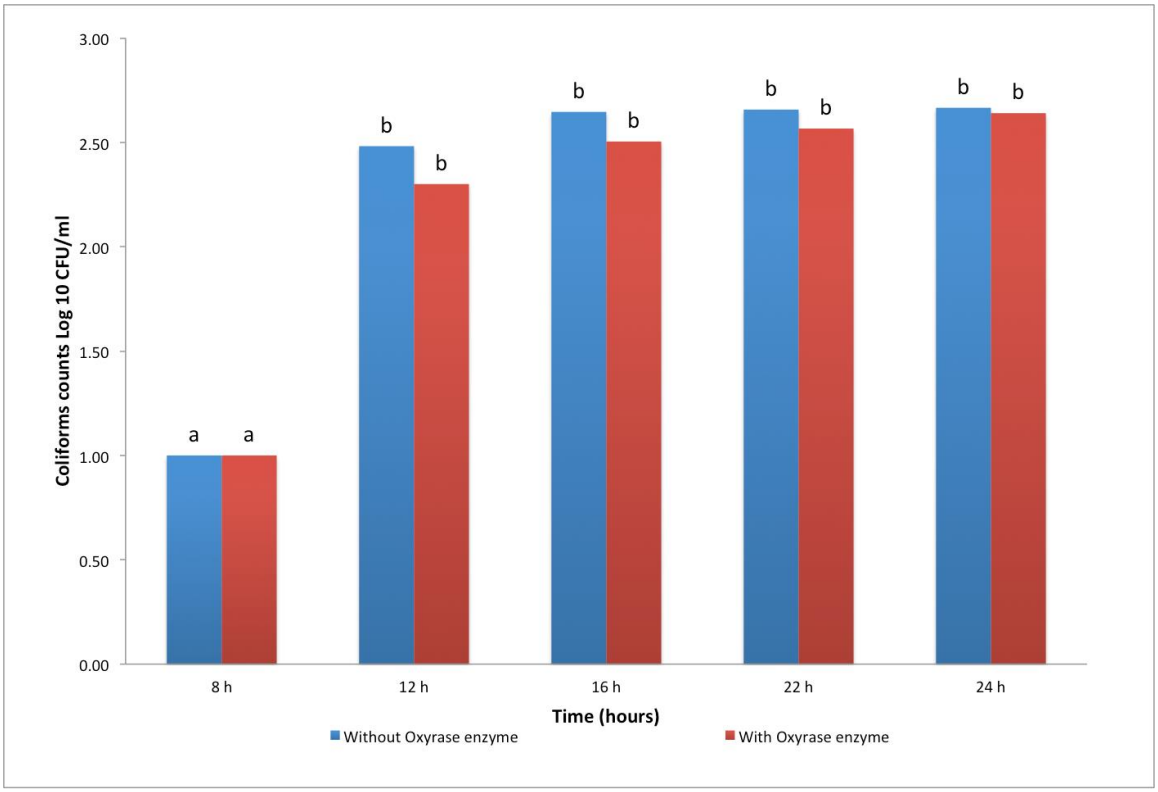
Figure 4.10 Least Square Means of coliforms counts in ground beef (low concentration) using TEMPO® CC with & without Oxyrase® enzyme at different times



Note: means with different letters on bars are significantly different (P<0.05). Standard Error= 0.20 without Oxyrase® & 0.20 with Oxyrase®.

Using the low concentration (1-2 Log $_{10}$ CFU/ml) resulted (Figure 4.10) in not significant difference when reading of the TEMPO® CC card at 8 hours or 22 hours of incubation. That means whenever low concentration of coliform counts can be read at 8 hours and there is no need to wait for the full incubation period. Results with Oxyrase® enzyme were not different than without Oxyrase® enzyme at all times tested.

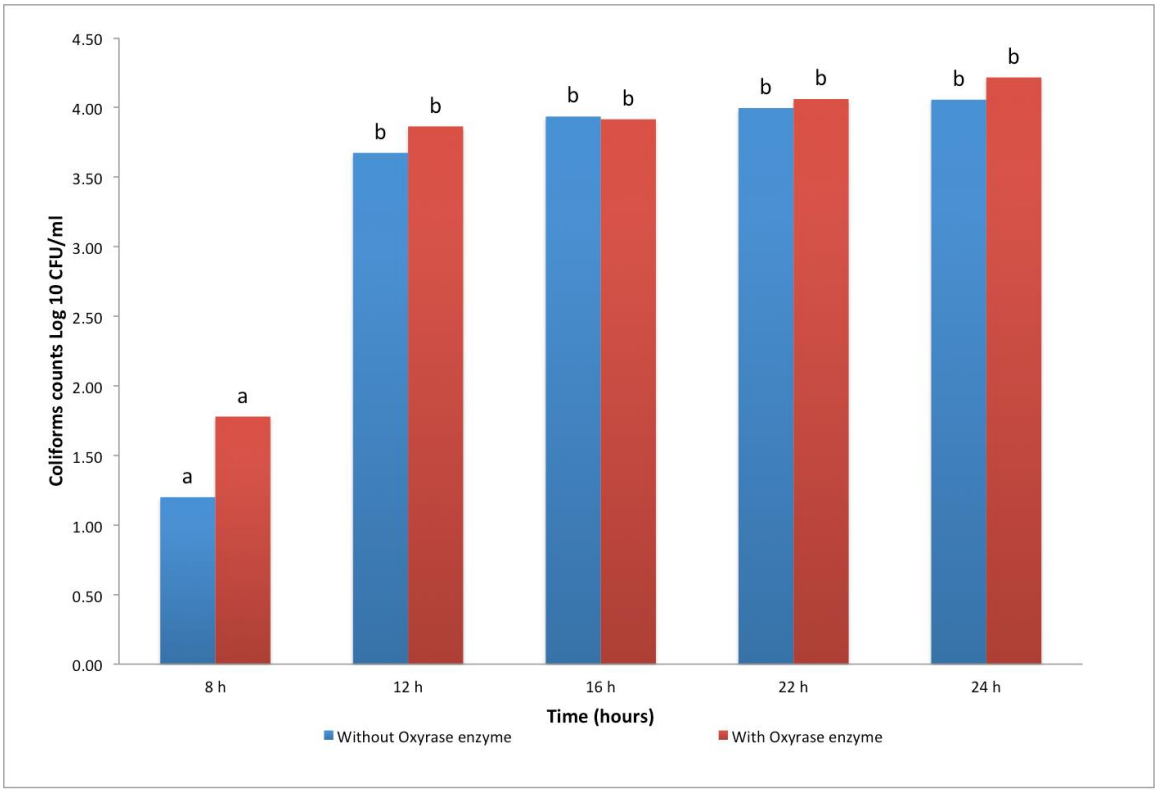
Figure 4.11 Least Square Means of coliforms counts in ground beef (medium concentration) using TEMPO® CC with & without Oxyrase® enzyme at different times



Note: means with different letters on bars are significantly different (P<0.05). Standard Error= 0.11 without Oxyrase® & 0.11 with Oxyrase®.

Using the medium concentration (2-3 Log $_{10}$ CFU/ml) resulted (Figure 4.11) in significant difference when reading at 8 hours and 24 hours for TEMPO® CC card with Oxyrase®. 12 hours readings were not different from 22 hours. 8 hours readings were differing by more than 0.5 log with other reading times which considered a difference in microbial count. TEMPO® CC can be read at 12 hours and there is no need to wait for the full incubation period, since it is within 0.5 log and not significant difference. Results with Oxyrase® enzyme were not different than without Oxyrase® enzyme at all times tested.

Figure 4.12 Least Square Means of coliforms counts in ground beef (high concentration) using TEMPO® CC with & without Oxyrase® enzyme at different times



Note: means with different letters on bars are significantly different (P<0.05). Standard Error= 0.22 without Oxyrase® & 0.24 with Oxyrase®.

Using the high concentration (3-5 Log $_{10}$ CFU/ml) resulted (Figure 4.12) in significant difference when reading at 8 hours and at 22 hours for TEMPO® CC card with and without Oxyrase® enzyme. 12 hours readings were not different from 22 hours. 8 hours readings were differing by more than 0.5 log with other reading times which considered a difference in microbial count. TEMPO® CC can be read at 12 hours and there is no need to wait for the full incubation period. Results with Oxyrase® enzyme were not different than without Oxyrase® enzyme at all times tested.

Table 4.1 Summary of Least Square Means of TEMPO® counts at different times

Test	BC	TRT	8 h	12 h	16 h	22 h	24 h
EC	Low	W/O	1.29[a]	1.58[a]	1.58[a]	1.58[a]	1.58[a]
	Med	W/O	1.55[a]	2.60[b]	2.61[b]	2.62[b]	2.62[b]
	High	W/O	3.48[a]	4.33[a]	4.34[a]	4.34[a]	4.35[a]
AC	Low	W/O	1.50[a]	1.50[a]	1.50[a]	1.50[a]	1.50[a]
	Low	W	1.40[a]	1.46[a]	1.46[a]	1.50[a]	1.50[a]
	Med	W/O	1.13[a]	1.93[a,c]	1.97[a,c]	2.25[a,c]	2.28[a,c]
	Med	W	1.49[a,c]	2.25[a,c]	2.29[a,c]	2.36[a,c]	2.36[a,c]
	High	W/O	2.72[a]	3.59[a,b]	3.63[b]	3.74[b]	3.75[b]
	High	W	2.44[a]	3.54[a,b]	3.55[a,b]	3.68[b]	3.71[b]
CC	Low	W/O	1.00[a]	1.33[a]	1.55[a]	1.55[a]	1.54[a]

	Low	W	1.00[a]	1.25[a]	1.41[a]	1.56[a]	1.73[a]
	Med	W/O	1.00[a]	2.48[b]	2.65[b]	2.66[b]	2.67[b]
	Med	W	1.00[a]	2.30[b]	2.50[b]	2.57[b]	2.64[b]
	High	W/O	1.20[a]	3.67[b]	3.94[b]	4.00[b]	4.06[b]
	High	W	1.78[a]	3.86[b]	3.92[b]	4.06[b]	4.22[b]

Note same super script letter in the row means they are the same statically

Summary of Least Square Means of all TEMPO® counts is shown in Table 4.1. At any given bacterial concentration (BC), 12 hours readings were not different from 22 hours. TEMPO® can be read at 12 hours and there is no need to wait for the full incubation period. Results with Oxyrase® enzyme were not different than without Oxyrase® enzyme at all times tested.

4.5 Detection time of coliform counts using TEMPO® CC

Figure 4.13 Time-to-detection calibration curve for TEMPO® CC

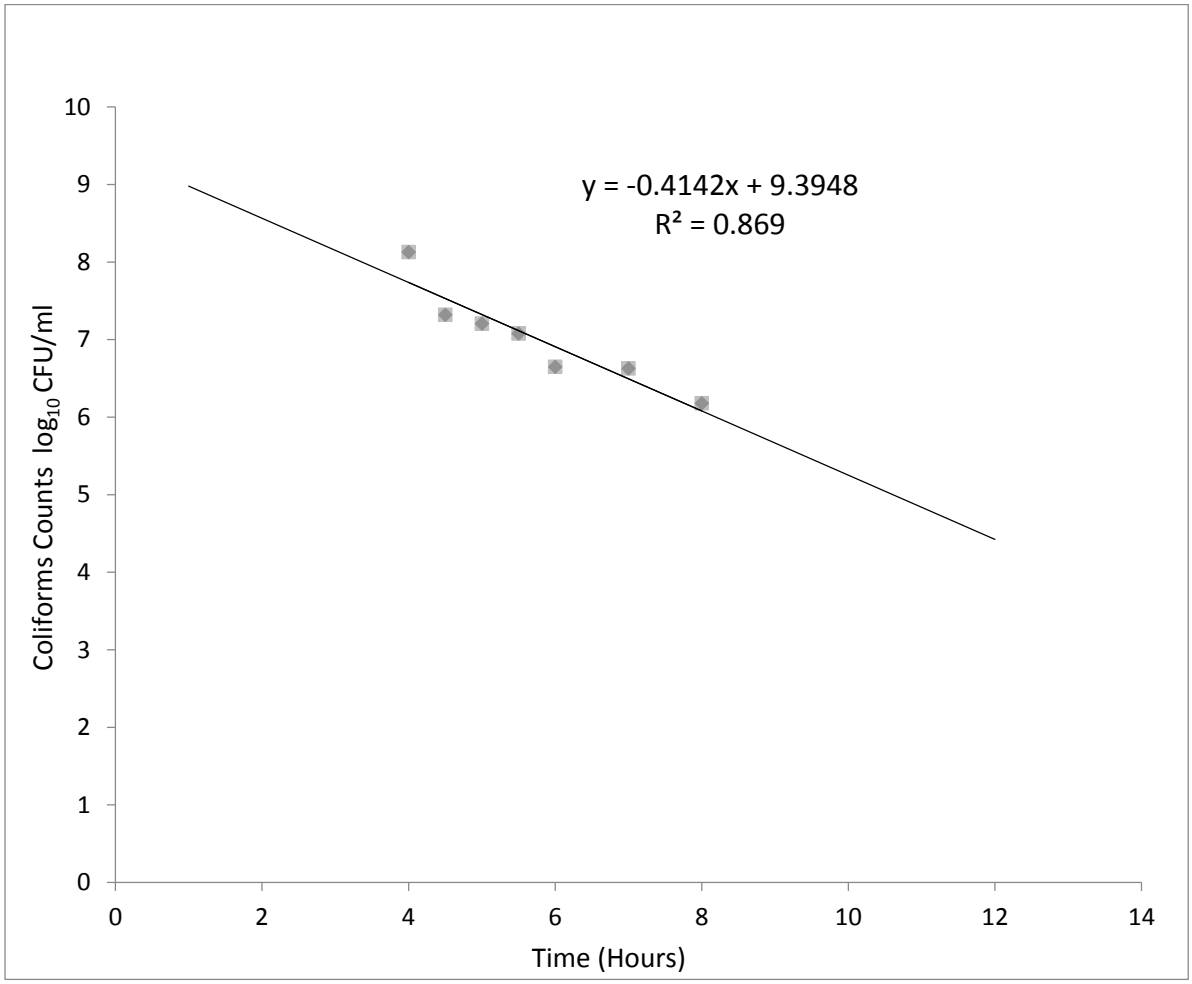
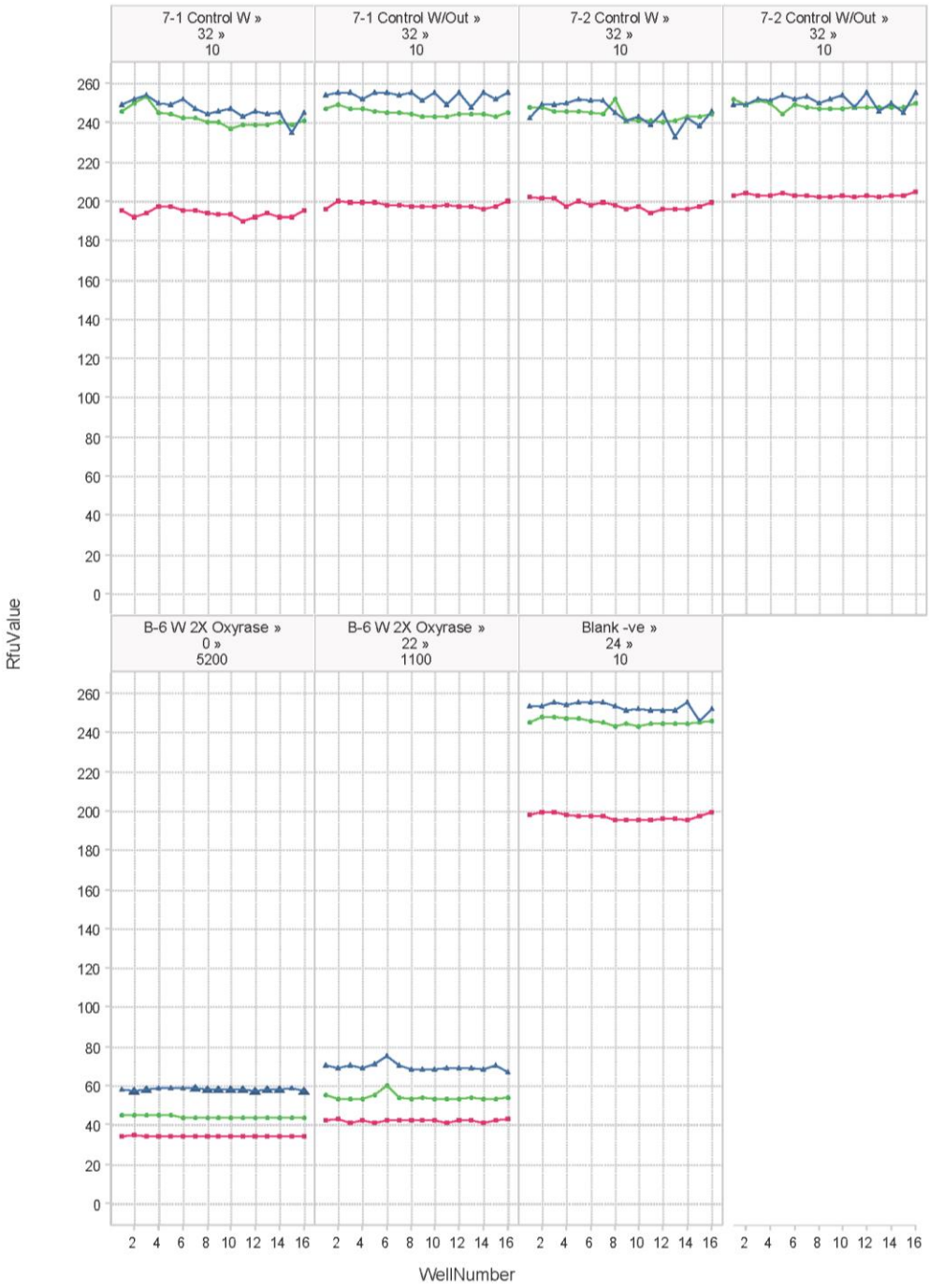


Figure 4.13 shows excellent correlation between a time to detection and very high coliform counts (R^2=0.87) using TEMPO® CC test kits. This modification will allow detection and estimated enumeration between 6-8 log CFU/ml within 4-8 hours range. That is using calibration curve that plot time to detection vs. the coliform counts. It will be more accurate when computed for a large number of samples with same food matrices. That is increasing the enumeration range from 3.7 log to beyond the 8 log range.

4.6 Relative fluorescence units and pH when using TEMPO® CC

Figure 4.14 RFU Values of Blank TEMPO® CC with/without Oxyrase® enzyme



Relative fluorescence units (RFU) are used in measurement of florescence in TEMPO® cards in the TEMPO® reader unit. Figure 4.14 shows that blank TEMPO® CC

without Oxyrase® and with 1X Oxyrase® (which is used in the study 0.1ml Oxyrase® per 1ml of broth) are the same no differences in the florescence graph. When doubling the amount of the enzyme the to 2X or 0.2ml per 1 ml of broth it gives different values for RFU. And gives also a false positive in the system. This graph was obtained from bioMerieux headquarter in France after exporting test results from the TEMPO® system. That related to the changes in pH of the media, which affect the florescence measurement (see Table 4.2).

Table 4.2 pH values in TEMPO® CC reagents with and without Oxyrase®

Oxyrase amount / 1ml of broth	**pH**
0 ml	7.30
0.1 ml	7.23
0.2 ml	7.16

Chapter 5 - Conclusion

Based on the observations of this study the following conclusion were made:

1. Using TEMPO® tests, high counts in food samples (>6 log $_{10}$ CFU/ml) can be read in 6±2 hours of incubation using the time-to-detection calibration curve.
2. TEMPO® system reduces reading time (reading protocol should be changed). There is no need to wait for 22 hours of incubation only 12 hours is required.
3. Oxyrase® enzyme is not needed for the TEMPO® system.

To take the TEMPO® system to next the level of improvement; the following recommendations have been made:

1. Continue reading every 30 min or less. When the 3 consecutive reading are the same or not significantly different the results are final.
2. Reading should start once the cards are entered into the reader. There is no need to log in the computer, results will be automatically saved.
3. Notification of all results should be sent to user mobile devices like smart phones especially when there are high counts and action needs to be taken.
4. There was an excellent correlation (R^2=0.87) between a time-to-detection and very high coliform counts when using TEMPO® CC test kits. This will allow detection and estimated enumeration between 6-8 log CFU/ml within the 4-8 hours range. That is using the calibration curve that plots time to detection vs the coliform counts. It will be more accurate when computed for a large number of samples with the same food matrices. This technique of estimation is used by default in automated microbial enumeration systems like the impedance systems for example:

1. Rapid Automated Bacterial Impedance Technique (RABIT) by Don Whitley Scientific Limited; UK
2. Bactometer by bioMerieux; France
3. Malthus 2000 by Malthus Instruments Limited; UK
4. The Sy-Lab BacTrac 4300 Rapid Microbiology System by SY-LAB; Austria

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Appendix A – Oxyrase® for Broth Product Insert

Oxyrase, Inc.
3000 Park Ave. West
Mansfield, OH 44906
Ph.: 419-589-8800
Fax: 419-589-9919
www.oxyrase.com

Oxyrase® for Broth (OB) Product Insert

Oxyrase® for Broth is a sterile enzyme formulation that uses Nature's Antioxidant® to produce anaerobic conditions in a wide variety of bacteriological broth media.

Precautions:
OB is for In-Vitro Use only. OB is a filter sterilized product and must be handled aseptically to maintain sterility.

Some microorganisms produce significant amounts of gas during growth. To prevent build up of pressure due to gas production, caps can be loosely affixed to allow for the escape of gas and will not compromise the anaerobic conditions maintained in a medium containing OB. A **Material Safety Data Sheet** is available on our website.

Product Performance:
The formulation of OB combines substrates and Oxyrase®.

OB creates an anaerobic environment when added to bacteriological broth medium by keeping oxygen from intruding into the broth; eventually removes oxygen from head space of the sealed container.

When used as recommended within a bacteriological broth medium, OB reduces the oxygen concentration to less than 10 ppB (parts per billion) within 30 minutes and maintains an anaerobic environment within the bacteriological broth medium for at least 16 days at 35°C to 37°C in a sealed vessel incubated without shaking or mixing. OB should only be used with broth media that has an initial pH range of 6.8 to 8.4.
(OB works under temperature and pH conditions beyond those specified. However, you may need to add more product / substrates and / or allow for more time to achieve complete anaerobiosis.)

OB works as specified in many types of broth medium, including the following list of commercially prepared media:

Columbia Broth (CB)	Brain Heart Infusion Broth (BHI)
Schaedler Broth	Brucella Broth
Nutrient Broth (NB)	Trypticase Soy Broth (TSB)
Eugon Broth	Meuller-Hinton Broth

Oxyrase® for Broth is not a substitute for nutrients or gasses required for growth of anaerobic microorganisms. For reduced environments, lower than achieved by complete oxygen removal, a chemical reducing agent is required.

Limitations:
OB contains a penicillin binding protein that may interfere with penicillin and some related antibiotics.

OB is not a substitute for nutrients or gasses required for growth of anaerobic microorganisms. For reduced environments, a chemical reducing agent may be required.

Handling and Storage Instructions:
OB will arrive thawed but cold. The following storage options are listed below:

1. Long Term Storage: Store the product at a constant -20°C or colder to maintain full activity.

   OB can be thawed and re-frozen five times without affecting its activity and performance. In cases where the product will be used infrequently and / or in small amounts, aseptically aliquot the product into smaller, individual, sterile containers (refer to short term storage, if needed). To minimize the amount of freezing and thawing of the product, thaw each container once and discard after use.

2. Short Term Storage: Store the product at 2°C to 8°C for use within 30 days (a precipitant may form at this temperature, but does not affect product performance).

   When stored in this manner, the product will maintain its full activity to the printed expiration date on the label.

Thawing Oxyrase® for Broth:
A convenient way to thaw OB is to place it in the refrigerator overnight.

If necessary, the product can be thawed by warming. *Do **not** exceed a warming temperature of 37°C.* Only apply heat to the outside of the container while ice is still present inside the container. When all ice has melted, keep the product chilled by placing the container in ice until ready for use.

To ensure uniform activity within a thawed sample, *gently* mix the product before use or distribution (*do **not** agitate vigorously).* Vigorous agitation (i.e. shaking) causes foaming and denatures protein in the product, which may result in loss of activity.

In some cases, precipitate may be observed, but will not affect OB performance.

Instructions for Use:
Aseptically, add 0.1 mL of OB to each 1.0 mL of *prepared, sterile* broth medium (if using a disposable transfer pipet, one drop of OB per mL of broth is a sufficient measure). *Do **not** agitate vigorously or aerate the suspension.* Incubate at 35°C to 37°C. Broth will become completely anaerobic in 30 minutes or less. Minimize the area of liquid surface exposed to air when possible.

User Quality Control:
The performance of OB may be verified by inoculating one broth medium tube (BHI or TSB) with OB, and one tube without OB. Using anaerobic control microorganisms (i.e. *B. fragilis* - ATCC 25285 and *F. nucleatum* - ATCC 25586), inoculate mediums with a culture adjusted to a McFarland Turbidity Standard. Incubate both tubes aerobically ***without** agitation* at 35°C to 37°C for 48 to 72 hours.

If the OB is performing as specified, the OB treated broth will show heavy turbidity; whereas, the aerobic control will remain clear.

Guarantee:
OB has a shelf-life of 18 months under recommended storage and use conditions. We guarantee a minimum of 6 months shelf-life from shipment date. If a longer shelf-life is needed, this should be arranged at the time your order is placed.

If OB does not create an anaerobic environment as specified under recommended storage and use conditions, Oxyrase, Inc. will refund your purchase price. To receive a product refund, write or call Oxyrase Inc. with the product lot number which is located on the Oxyrase® for Broth label. Oxyrase, Inc. is available to answer any questions about this product and its applications.

 LAB.0032.v008

Appendix B – TEMPO® CC (coliform count) package insert

REF **80 044**

13534F - en - 2010/12 EN

TEMPO® CC (Coliform Count)

For microbiological control only

TEMPO CC (Coliform Count) is an automated test for use with TEMPO, for the enumeration of coliforms following the BAM definition, in 22-27 hours in food products and environmental samples.

SUMMARY AND EXPLANATION

TEMPO CC is intended for use exclusively with the TEMPO system for the enumeration of coliforms following the Bacteriological Analytical Manual (BAM) (1) definition in 22-27 hours.
This test was developed in order to obtain performance levels similar to the AOAC Official Method 966.24 and the American Public Health Association's Standard Methods for the Examination of Dairy Products (SMEDP) (2).
The enumeration of coliforms is very useful in monitoring the quality of food products. Their presence can also suggest improper food handling or processing conditions.

PRINCIPLE

The TEMPO CC test consists of a vial of culture medium and a card, which are specific to this test.
The culture medium is inoculated with the sample to be tested. The inoculated medium is transferred by the TEMPO Filler into the card containing 48 wells of three different volumes. The card contains 3 sets of 16 wells (small, medium and large wells) with a one log difference in volume for each set of wells. The card is designed to simulate the Most Probable Number (MPN) method (3, 4). The card is then hermetically sealed in order to avoid any risk of contamination during subsequent handling.
The culture medium contains a fluorescent pH indicator which, when its pH is neutral, emits a signal detected by the TEMPO Reader. The coliforms present in the card assimilate the nutrients in the culture medium during incubation, resulting in a decrease in pH and the extinction of the fluorescent signal. Depending on the number and type of the positive wells, the TEMPO system calculates the number of coliforms present in the original sample according to a calculation based on the MPN method.

CONTENT OF THE KIT (48 TESTS) :

TEMPO CC cards 2 x 24	Ready-to-use, disposable cards with a transfer tube.
TEMPO CC culture medium 2 x 24 vials	Each vial contains a single dose of dehydrated culture medium. Dose for 4 ml.
1 package insert provided in the kit or downloadable from **www.biomerieux.com/techlib**	

COMPOSITION OF THE TEMPO CC CULTURE MEDIUM

Theoretical formula in g/l of reconstituted solution.

Lactose (bovine)	15
Nutrients (bovine and porcine)	15
Growth supplements	0.25
Buffer system	5.16
Sodium bile salts (bovine and ovine)	5.75
Fluorescent pH indicator	0.06
Anti-foaming agent	0.4

pH 7.3

MATERIAL AND REAGENTS REQUIRED BUT NOT PROVIDED

Material:
- TEMPO Bags - Bags with lateral filter (bioMérieux Ref. 80 015)
- Stomacher (Model 400 or equivalent)
- Pipettes to dispense exactly 0.10 ml or 1.0 ml of sample
- Vortex-type mixer
- Laboratory incubator (under metrology)

The references below are given as a guide only:

Primary diluents recommended for food samples:
- Butterfield's phosphate-buffered dilution water (1)
- Or any other diluent which has first been validated by the user as compatible for use with the TEMPO system

Primary diluents recommended for environmental testing (swabs – cleaning wipes):
- Difco Neutralizing Buffer (Ref. 236210 Neutralizing Buffer for environmental samples)
- Letheen Broth, Modified (5)
- Or any other diluent which has first been validated by the user as compatible for use with the TEMPO system

Secondary diluents recommended:
- Sterile distilled water or equivalent purified water validated by the user

Material recommended for quality control:
- Densimat (bioMérieux Ref. 99 234)
- Trypcase Soy Agar [TSA] (bioMérieux Ref. 43 011)

WARNINGS AND PRECAUTIONS

- **For microbiological control only.**
- **For professional use only.**
- Comply with Good Laboratory Practice (e.g., standard ISO 7218 (7)).
- This kit contains products of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not totally guarantee the absence of transmissible pathogenic agents. It is therefore recommended that these products be treated as potentially infectious, and handled observing the usual safety precautions (do not ingest or inhale).
- The culture medium should not be used as a manufacturing material or component.
- All samples and inoculated media should be considered infectious and handled appropriately. Aseptic technique and usual precautions for handling the bacterial group studied should be observed throughout this procedure; refer to the Laboratory Biosafety Manual – WHO – Geneva – Latest edition, or the current regulations in the country of use.
- Do not use reagents or disposables after the expiry date indicated on their label.
- Before use, check that the packaging and components are intact.
- Only use culture media which appear to be homogeneous (no agglomerates or moisture).
- Do not use visibly deteriorated cards.
- **Do not allow the sample to come into direct contact with the culture medium (in powder form) before the medium has been reconstituted.**
- Any cards which have not been sealed by the TEMPO Filler must not be used.
- The TEMPO card is not intended for performing subcultures from positive wells.
- Do not write on the card wells or the barcodes.
- Do not stick any labels on the card.
- The TEMPO Reader, TEMPO Filler and racks should be regularly cleaned and decontaminated (see the User's Manuals).
- Any change or modification in the procedure may affect the results and must be validated by the laboratory. bioMérieux will not be held liable for results obtained following any changes or modifications in procedures not validated by bioMérieux. In addition, such changes or modifications may void all warranties.

STORAGE CONDITIONS

- Store the TEMPO CC kit at 2-25°C.
- After opening the kit, and each time cards are removed from the kit, carefully reseal the packaging (pouch or blister pack) containing the remaining cards.
- Do not leave the cards exposed to light (on the workbench or the media stand) for more than 15 days.
- Avoid directly exposing the cards to ultraviolet light.
- If stored according to the recommended conditions, all components are stable until the expiry date indicated on their label.

FOOD SAMPLES

Sample type

The TEMPO system can be used for the analysis of a large variety of food products for consumption by humans and domestic animals.

Preparation

Allow the primary and secondary diluents to come to room temperature (18-25°C) (refer to list of diluents recommended in the paragraph "Material and reagents required but not provided").

Follow the recommendations in the current ISO Standards [or BAM (1) if applicable] for performing sample collection and preparing the stock solution. In particular

- for acidic products, ensure that the pH is restored to neutral when the solution is prepared (EN ISO 6887-4 point 8.2) (6).
- for all aromatic herbs, spices, teas and herbal teas, which may have an inhibitory effect, a minimal dilution of 1/400 should be used (EN ISO 6887-4 point 9.5.4.4) (6).

To prepare the samples, dilute the sample 1/10 (**primary dilution**), using one of the primary diluents recommended. For example, aseptically add 25 g or 25 ml of sample to 225 ml of Butterfield's phosphate-buffered dilution water. Homogenize in the TEMPO bag (see instructions for using the TEMPO bag in the User's Manual for the TEMPO Preparation Station).

The interval between the homogenization of the primary dilution and its transfer into the TEMPO card must not exceed 45 minutes, unless otherwise indicated in the specific international Stanard (7).

INSTRUCTIONS FOR USE

For complete instructions, see the TEMPO system User's Manuals.

Protocol for obtaining performance levels similar to those obtained according to AOAC Official Method 966.24 and the SMEDP (2)

Test procedure for food samples

Example for the preparation of a 1/40 dilution enabling enumeration between 10 and 4.9×10^4 CFU/g. The dilution can be modified according to the expected level of contamination.

1. Remove the required number of vials of culture medium (one vial per test sample) and allow to come to room temperature.
2. Set the dispenser containing the secondary diluent to 3 ml and prime the pump by eliminating the first two volumes dispensed.
3. Log on to the TEMPO preparation station.
4. Following the instructions of the preparation station user interface, identify the sample to be tested, either by manually entering the identifier via the keyboard or using the preparation station barcode reader.
5. Reconstitute the culture medium by dispensing 3 ml of secondary diluent per vial using the dispenser.

6. Using a sterile pipette, take up 1 ml from the filtered compartment of the TEMPO bag and transfer it into the vial containing the reconstituted culture medium. Homogenize for approximately 3 seconds using a vortex-type mixer. The 4 ml of inoculated medium obtained corresponds to a 1/40 dilution of the sample.
7. Remove one card for each vial of inoculated medium, **without touching** the tip of the transfer tube. Check that the codes (colors and abbreviations) on the card and the vial of inoculated medium match.
8. Associate the identifier of the test sample with the barcodes of the corresponding inoculated medium and card using the preparation station barcode reader, following the instructions of the preparation station user interface.
9. Put the vial containing the inoculated medium in the filling rack. Insert the card in the slot opposite the vial, placing the transfer tube of the card inside the vial. The rack can hold up to 6 vials + cards and enables 1-6 TEMPO cards to be filled simultaneously.
10. Place the rack in the TEMPO Filler and start the filling cycle. The inoculated medium is completely aspirated into the card. After the cards have been filled, the TEMPO Filler cuts and seals the transfer tubes. All these operations are performed automatically and take 3 minutes. The filling cycle is the same for all the parameters and enables cards for different parameters to be filled at the same time.
11. Remove the filling rack from the TEMPO Filler and visually check that the vials are empty. Take the cards out of the rack and transfer them into the incubation racks: insert the cards into the slots, with the label on the card facing the user (towards the rack handle). Cards which are to be incubated at the same temperature should be grouped together on the same rack. Each rack can hold up to 20 cards. Do not insert cards in between the slots.
12. Dispose of the used vials and transfer tubes into an appropriate receptacle.
13. Incubate the cards for 22-27 hours at **35 ± 1°C** (**32 ± 1°C** or **35 ± 1°C** for dairy products), in order to obtain performance levels similar to the AOAC Official Method 966.24 and the SMEDP (2).

Foods

Eighteen foods were included in the internal study:
Internal testing included method comparison studies for eighteen of the claimed matrices including raw ground pork, fresh ground beef, heat processed cooked roast beef, fresh ground chicken, frozen cooked chicken, heat processed grilled chicken, frozen catfish, heat processed frozen fish, raw cod, bagged salad, frozen green beans, hashbrown potatoes, vanilla ice cream, pasteurized milk, milk powder, pasteurized eggs, rice and dry pet food.

Four foods were included in the AOAC Research Institute independent lab study:
Independent lab testing included method comparison studies for claimed matrices including fresh ground beef, frozen cooked chicken, raw cod and frozen green beans.

Note 1: The incubation time for the test is managed by the TEMPO Read software which integrates a theoretical interval of 15 minutes between the reading of the card barcode and the start of incubation.
If the real interval is greater than 15 minutes, (without exceeding 2 hours), this extra time must be added to the remaining incubation time displayed by the TEMPO Read software. Reading must always be performed within the 22-27 hour time limit authorized by the software.

Note 2: The ± 1°C tolerance for the incubation temperature must be strictly adhered to.

Reading the cards at the end of incubation

1. Log on to the reading station.
2. Introduce the incubation rack containing the cards to be read into the reader. The reader scans the barcode of each card and interprets the results of fluorescence in the wells. It automatically associates the sample identifier with the type of test, the dilution and the enumeration results.
3. Editing the results : on the reading station screen, the number of colony forming units (CFU) per gram or milliliter of initial product is associated with the sample identifier, the parameter tested and the analysis date.
4. The reading station user interface enables the results to be printed out or transmitted to the laboratory information management system (LIMS). It also enables the records of the results obtained the previous days to be consulted.
5. At the end of the analysis, remove the cards from the rack and dispose of them into an appropriate receptacle.

Note: The TEMPO CC cards may be stored at 2-8°C after inoculation, for a maximum of 48 hours, before incubating for 22-27 hours at 35°C ± 1°C or 32°C ± 1°C. It should be emphasized that the result obtained will include the annotation "The card was read too late". The user can specify in the comment text box that the cards were read after having been refrigerated.

ENVIRONMENTAL SAMPLES

Sample type

The proposed protocol can be used for swabbing equipment, countertops or hands with pre-moistened swabs or for wiping countertops with cleaning wipes or sponges. Given the diversity of environmental samples, users should first validate this protocol or any other protocol.

Preparation

Immediately after swabbing or wiping the countertop, transfer the used swab or wipe/sponge directly into a tube containing a given volume of one of the recommended primary diluents. The dilution obtained is the primary dilution of the sample.

Example of test procedure for environmental swabs

Transfer the swab into a tube containing 10 ml, to obtain a dilution which corresponds to a 1/10 dilution of the sample **(primary dilution)**. Homogenize the suspension carefully by shaking the swab in the diluent. Press out the solution by rotating the swab against the inside edge of the tube. It is recommended to test the samples at a dilution of at least 1 in 40 which will enable enumeration between 10 and 4.9×10^4 CFU/surface swabbed. The dilution can be increased according to the expected level of contamination.

1. Remove the required number of vials of culture medium (one vial per test sample) and allow to come to room temperature.
2. Set the dispenser containing the secondary diluent to 3 ml and prime the pump by eliminating the first two volumes dispensed.
3. Log on to the TEMPO preparation station.
4. Following the instructions of the preparation station user interface, identify the sample to be tested, either by manually entering the identifier via the keyboard or using the preparation station barcode reader.
5. Reconstitute the culture medium by dispensing 3 ml of secondary diluent per vial using the dispenser.
6. Using a sterile pipette, take up 1 ml from the tube containing the suspension obtained after swabbing and transfer it into the vial containing the reconstituted culture medium. Homogenize for approximately 3 seconds using a vortex-type mixer. The 4 ml of inoculated medium obtained corresponds to a 1/40 dilution of the environmental sample collected from the swabbed surface.
7. Follow the TEMPO procedure in the paragraph "Test procedure for food samples" from step 7 onwards.

RESULTS AND INTERPRETATION

Once the reading is completed, the results are automatically analyzed by the computer which determines which wells are positive.
The number of positive wells obtained, in relation to the volume of the wells and the dilution of the sample, gives the enumeration result in CFU per gram or milliliter for the original sample, using the MPN tables.

QUALITY CONTROL

The TEMPO reagents are systematically quality controlled at various stages of their manufacture. For users who wish to perform their own quality control tests to ensure that the TEMPO method has been carried out correctly, the following strains can be used:
Escherichia coli ATCC® 8739
Enterococcus faecalis ATCC 29212
Pseudomonas aeruginosa ATCC 27853

Recommended protocol:

- The different incubation steps should be performed at 35 ± 1°C or at 32 ± 1°C depending on the temperature used by the laboratory.
- Using a 24-hour old culture on Trypcase Soy Agar, prepare a suspension in Peptone water and adjust to 0.5 McFarland, i.e. approximately 10^8 CFU/ml using the Densimat (see " Material and reagents required but not provided"). Perform serial decimal dilutions in Peptone water until a suspension with a theoretical concentration of approximately 10^2 CFU/ml is obtained. For *E. coli*, transfer 1 ml of this suspension into a vial of culture medium which has been reconstituted beforehand with 3 ml of sterile distilled water. Follow the same procedure for *Enterococcus* and *Pseudomonas*, but transfer 1 ml of the 10^5 CFU/ml suspension.
- Modify the default dilution in the TEMPO software by entering "4" in order to obtain a 1/4 dilution.
- Fill one card per vial of medium and incubate.
- At the same time, check the concentration of the suspension which was used to inoculate the TEMPO card by streaking 0.1 ml of the 10^3 CFU/ml suspension on TSA. Incubate.
- After incubation, perform card reading. Count the number of colonies of *E. coli* and check for the presence of *Pseudomonas* and *Enterococcus* on TSA.

Range of expected results:

E. coli strain

Calculate the ratio R:

$$R = \frac{\text{TEMPO result (CFU/g)}}{10 \times \text{no. of colonies on TSA}}$$

R should be between 0.01 and 1.

Pseudomonas and *Enterococcus* strains should be totally inhibited by TEMPO CC (in this case, the TEMPO software indicates: enumeration < 1 CFU/g).

If the enumeration results obtained deviate from the expected values, please contact bioMérieux SA or its local representative.

It is the responsibility of the user to perform Quality Control in accordance with any local applicable regulations.

LIMITATIONS OF THE METHOD

- Invalid results may appear if the card has not been filled correctly (presence of empty wells and/or liquid remaining in the vial after the filling cycle): for example, **use of a filtering bag other than the one recommended** (see paragraph "Material and reagents required but not provided").
- Improper preparation or storage of the samples may lead to incorrect results. In particular, for acidic products or food products known for their inhibitory effects, such as aromatic herbs and spices, teas and infusions, it is advisable to ensure that the recommendations for sample preparation have been followed (see § FOOD SAMPLES, Preparation).

- **Warning:** The TEMPO CC parameter was evaluated using numerous food matrices, excluding soft drinks. However, given the diversity of food matrices and manufacturing processes, users should check that the composition of the matrices tested does not affect result accuracy. In particular, the fluorescent signal may be affected:
  - by certain food products whose the primary dilution is strongly colored (e.g., fruit purées and cocoa) or if it becomes colored during incubation in the card due to an oxidation reaction (e.g., raw mushrooms),
  - or certain dehydrated products such as ready-to-use cake mixes (owing to the turbidity of the primary dilution).

  For the TEMPO CC test, a dilution of these matrices at least equivalent to 1/400 is recommended.

See the TEMPO User's Manuals for more complete information.

The TEMPO CC test, for the enumeration of coliforms in a variety of food products, was validated by the AOAC Research Institute in June 2007 (Certification No. 060702).

PERFORMANCE TESTED AOAC RESEARCH INSTITUTE LICENSE NUMBER 060702

060702 – 06/26/07
PERFORMANCE TESTED METHOD
Certified by AOAC Research Institute
www.aoac.org

WASTE DISPOSAL

Dispose of used or unused reagents as well as any other contaminated disposable materials following procedures for infectious or potentially infectious products.
It is the responsibility of each laboratory to handle waste and effluents produced according to their nature and degree of hazardousness and to treat and dispose of them (or have them treated and disposed of) in accordance with any applicable regulations.

LITERATURE REFERENCES

1. Bacteriological Analytical Manual
   (1998) 8th Edition, Revision A, Chapter 4 “Enumeration of *Escherichia coli* and the Coliform bacteria”.
2. American Public Health Association
   (2004) 17th Edition. Standard Methods for the Examination of Dairy Products, APHA Washington DC.
3. Cochran W.G.
   Estimation of bacterial densities by means of the "Most Probable Number".
   (1950) Biometrics 6, 105-116.
4. Woodward R.L.
   How probable is the most probable number ?
   (1957) J. Am. Water Works Assoc., 49, 1060,1068.
5. Bacteriological Analytical Manual
   (1998) 8th Edition, Revision A, Appendix 3, M.79.
6. International Standard EN ISO 6887-4 – Microbiology of food and animal feeding stuffs - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination. Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products.
7. International Standard EN ISO 7218 – Microbiology of food and animal feeding stuffs – General rules for microbiological examinations.

INDEX OF SYMBOLS

Symbol	Meaning
REF	GB: Catalogue number US: Catalog number
	Manufacturer
	Temperature limitation
	Use by
LOT	Batch code
	Consult Instructions for Use
	Contains sufficient for <n> tests

WARRANTY

bioMérieux disclaims all warranties, express or implied, including any implied warranties of MERCHANTABILITY AND FITNESS FOR A PARTICULAR USE. bioMérieux shall not be liable for any incidental or consequential damages. IN NO EVENT SHALL BIOMERIEUX'S LIABLITY TO CUSTOMER UNDER ANY CLAIM EXCEED A REFUND OF THE AMOUNT PAID TO BIOMERIEUX FOR THE PRODUCT OR SERVICE WHICH IS THE SUBJECT OF THE CLAIM.

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Appendix C – TEMPO® EC (*E. coli*) package insert

REF **80 004**

12597**J** - en - 2013/03 EN

TEMPO® EC (*E. coli*)

For microbiological control only

TEMPO EC (*E. coli*) is an automated test for use with TEMPO, for the enumeration of *Escherichia coli* in 22-27 hours in food products and environmental samples.

SUMMARY AND EXPLANATION

TEMPO EC is intended for use exclusively with the TEMPO system for the enumeration of *Escherichia coli* in 22-27 hours in food products and environmental samples.
This test was developed in order to obtain performance levels similar to the standard EN ISO 16649-2 (1) and AOAC Official Methods 966.23 and 966.24.
Escherichia coli is a commensal host of humans and animals. This species may be responsible for food poisoning if development is profuse. Certain strains are considered pathogenic and can produce one or more toxins.

PRINCIPLE

The TEMPO EC test consists of a vial of culture medium and a card, which are specific to this test.
The culture medium is inoculated with the sample to be tested. The inoculated medium is transferred by the TEMPO Filler into the card containing 48 wells of three different volumes. The card contains 3 sets of 16 wells (small, medium and large wells) with a one log difference in volume for each set of wells. The card is designed to simulate the Most Probable Number (MPN) method (2, 3). The card is then hermetically sealed in order to avoid any risk of contamination during subsequent handling.
Based on ß-glucuronidase activity, *Escherichia coli* present in the card reduce the substrate in the culture medium during incubation and cause a fluorescent signal to appear, which is detected by the TEMPO Reader. Depending on the number and type of the positive wells, the TEMPO system calculates the number of *Escherichia coli* present in the original sample according to a calculation based on the MPN method.

CONTENT OF THE KIT (48 TESTS):

TEMPO EC cards 2 x 24	Ready-to-use, disposable cards with a transfer tube.
TEMPO EC culture medium 2 x 24 vials	Each vial contains a single dose of dehydrated culture medium. Dose for 4 mL.
1 package insert provided in the kit or downloadable from **www.biomerieux.com/techlib**	

COMPOSITION OF THE TEMPO EC CULTURE MEDIUM

Theoretical formula in g/l of reconstituted solution.

Bio-Soyase and nutrients	9
Growth supplement	0.25
MOPS (3-(N-morpholino) propanesulphonic) sodium salt	20.8
MOPS acid (*)	12.6
Sodium deoxycholate (bovine and ovine)	0.7
Substrate and enzyme regulators	0.19
Anti-foaming agent	0.4

pH 7.4

* *Medium Xi: IRRITANT* (28.7% MOPS acid in the dehydrated medium)
- **R36/37/38:** Irritating to eyes, respiratory system and skin.
- **S26:** In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
- **S36/37/39:** Wear suitable protective clothing, gloves and eye/face protection.

For more detailed information consult the material safety data sheet available on request.

MATERIAL AND REAGENTS REQUIRED BUT NOT PROVIDED

Material:
- TEMPO Bags - Bags with lateral filter (bioMérieux Ref. 80 015)
- Paddle blender
- Pipettes to dispense exactly 0.10 mL or 1.0 mL of sample
- Vortex-type mixer
- Laboratory incubator (under metrology)

The references below are given as a guide only:

Primary diluents recommended for food samples:
- Peptone water / Peptone Saline Diluent (90 mL - bioMérieux Ref. 42 021)
- Buffered peptone water (90 mL - bioMérieux Ref. 42 042)
- Sodium citrate solution or dipotassium hydrogen phosphate solution following EN ISO 6887-5: 2010 point 5.3 (4)
- Butterfield's phosphate-buffered dilution water (5)
- Or any other diluent which has first been validated by the user as compatible for use with the TEMPO system

Primary diluents recommended for environmental testing (swabs – cleaning wipes):
- Difco Neutralizing Buffer (Ref. 236210 Neutralizing Buffer for environmental samples)
- Letheen Broth, Modified (6)
- Or any other diluent which has first been validated by the user as compatible for use with the TEMPO system

Secondary diluents recommended:
- Sterile distilled water or equivalent purified water validated by the user
- *Mandatory diluent for milk powder protocol only*:
  sterile MOPS buffer 0.4M, pH 7.5
  MOPS buffer preparation (up to 500 mL):
  - 13.60 g MOPS acid
  - 31.21 g MOPS sodium salt
  Make up to 500 mL with distilled water.
  Sterilize by autoclaving or filtration.

Material recommended for quality control:
- Densimat (bioMérieux Ref. 99 234)
- Trypcase Soy Agar [TSA] (bioMérieux Ref. 43 011)

WARNINGS AND PRECAUTIONS

- **For microbiological control only.**
- **For professional use only.**
- Comply with Good Laboratory Practice (e.g., standard EN ISO 7218 (9)).
- This kit contains products of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not totally guarantee the absence of transmissible pathogenic agents. It is therefore recommended that these products be treated as potentially infectious, and handled observing the usual safety precautions (do not ingest or inhale).
- The dehydrated culture medium contains an irritant agent (28.7% MOPS). Refer to the risk phrases "R" and the safety advice "S" given under the composition of the culture medium.
- The culture medium should not be used as a manufacturing material or component.
- All samples and inoculated media should be considered infectious and handled appropriately. Aseptic technique and usual precautions for handling the bacterial group studied should be observed throughout this procedure ; refer to the Laboratory Biosafety Manual – WHO – Geneva – Latest edition, or the current regulations in the country of use.
- Do not use reagents or disposables after the expiry date indicated on their label.
- Before use, check that the packaging and components are intact.
- Only use culture media which appear to be homogeneous (no agglomerates or moisture).
- Do not use visibly deteriorated cards.
- **Do not allow the sample to come into direct contact with the culture medium (in powder form) before the medium has been reconstituted.**
- Any cards which have not been sealed by the TEMPO Filler must not be used.
- The TEMPO card is not intended for performing subcultures from positive wells.
- Do not write on the card wells or the barcodes.
- Do not stick any labels on the card.
- The TEMPO Reader, TEMPO Filler and racks should be regularly cleaned and decontaminated (see the User's Manuals).
- Any change or modification in the procedure may affect the results and must be validated by the laboratory. bioMérieux will not be held liable for results obtained following any changes or modifications in procedures not validated by bioMérieux. In addition, such changes or modifications may void all warranties.

STORAGE CONDITIONS

- Store the TEMPO EC kit at 2-25°C.
- Do not leave the cards exposed to light (on the workbench or the media stand) for more than 15 days.
- Avoid directly exposing the cards to ultraviolet light.
- If stored according to the recommended conditions, all components are stable until the expiry date indicated on their label.

FOOD SAMPLES

Sample type

The TEMPO system can be used for the analysis of a large variety of food products for consumption by humans and domestic animals.

Preparation

Allow the primary and secondary diluents to come to room temperature (18-25°C) (refer to list of diluents recommended in the paragraph "Material and reagents required but not provided").

Follow the recommendations in the current ISO Standards [or BAM (7) if applicable] for performing sample collection and preparing the stock solution. In particular
- for acidic products, ensure that the pH is restored to neutral when the solution is prepared (EN ISO 6887-4 point 8.2) (8).
- for all aromatic herbs, spices, teas and herbal teas, which may have an inhibitory effect, a minimal dilution of 1/400 should be used (EN ISO 6887-4 point 9.5.4.4) (8).

To prepare the samples, dilute the sample 1/10 (**primary dilution**), using one of the primary diluents recommended. For example, aseptically add 10 g or 10 mL of sample to 90 mL of Peptone water. Homogenize in the TEMPO bag (see instructions for using the TEMPO bag in the User's Manual for the TEMPO Preparation Station).

For the analysis of all milk powder samples, it is mandatory to use sterile MOPS buffer 0.4M, pH 7.5 as secondary diluent in the preparation of the TEMPO 1/40 dilution (the 1/400 dilution does not require the use of MOPS buffer).

The interval between the homogenization of the primary dilution and its transfer into the TEMPO card must not exceed 45 minutes, unless otherwise indicated in the specific international Standard (8).

INSTRUCTIONS FOR USE

For complete instructions, see the TEMPO system User's Manuals.

Protocol certified NF VALIDATION (No. BIO 12/13-02/05) according to the standard EN ISO 16140 (10)

Test procedure for food samples

Preparation of a 1/40 dilution enabling enumeration between 10 and 4.9×10^4 CFU/g. Only the ISO diluents listed on page 1 can be used with this protocol.

1. Remove the required number of vials of culture medium (one vial per test sample) and allow to come to room temperature.
2. Set the dispenser containing the secondary diluent to 3 mL and prime the pump by eliminating the first two volumes dispensed.
3. Log on to the TEMPO preparation station.
4. Following the instructions of the preparation station user interface, identify the sample to be tested, either by manually entering the identifier via the keyboard or using the preparation station barcode reader.
5. Reconstitute the culture medium by dispensing 3 mL of secondary diluent per vial using the dispenser.
6. Using a sterile pipette, take up 1 mL from the filtered compartment of the TEMPO bag and transfer it into the vial containing the reconstituted culture medium. Homogenize for approximately 3 seconds using a vortex-type mixer. The 4 mL of inoculated medium obtained corresponds to a 1/40 dilution of the sample.

7. Remove one card for each vial of inoculated medium, **without touching** the tip of the transfer tube. Check that the codes (colors and abbreviations) on the card and the vial of inoculated medium match.
8. Associate the identifier of the test sample with the barcodes of the corresponding inoculated medium and card using the preparation station barcode reader, following the instructions of the preparation station user interface.
9. Put the vial containing the inoculated medium in the filling rack. Insert the card in the slot opposite the vial, placing the transfer tube of the card inside the vial. The rack can hold up to 6 vials + cards and enables 1-6 TEMPO cards to be filled simultaneously.
10. Place the rack in the TEMPO Filler and start the filling cycle. The inoculated medium is completely aspirated into the card. After the cards have been filled, the TEMPO Filler cuts and seals the transfer tubes. All these operations are performed automatically and take 3 minutes. The filling cycle is the same for all the parameters and enables cards for different parameters to be filled at the same time.
11. Remove the filling rack from the TEMPO Filler and visually check that the vials are empty. Take the cards out of the rack and transfer them into the incubation racks: insert the cards into the slots, with the label on the card facing the user (towards the rack handle). Cards which are to be incubated at the same temperature should be grouped together on the same rack. Each rack can hold up to 20 cards. Do not insert cards in between the slots.
12. Dispose of the used vials and transfer tubes into an appropriate receptacle.
13. Incubate the cards for 24-27 hours at 37 ± 1°C, in order to obtain performance levels similar to the standard EN ISO 16649-2 (1).

Mandatory protocol for analysis of all milk powder samples

Follow steps 1 to 12 of the TEMPO procedure indicated above **mandatorily using sterile MOPS buffer 0.4M, pH 7.5 as secondary diluent.**
Incubate the cards for 24-27 hours at 37 ± 1°C.

Note : As the minimum incubation time authorized by the TEMPO software is 22 hours, the user must pay particular attention when performing the analysis in the context of NF VALIDATION certification to ensure that the 24-hour minimum incubation time is respected.

Protocol for obtaining performance levels similar to those obtained according to AOAC Official Methods 966.23 and 966.24

The AOAC study included 7 different categories of food products:
- meat (fresh ground beef, frozen ground beef, raw ground veal, raw ground pork),
- poultry (raw fresh ground chicken, frozen turkey breast, raw ground turkey, frozen chicken nuggets),
- fish (raw white fish, frozen cooked fish, raw salmon),
- egg products (pasteurized egg),
- vegetables (fresh green beans, lettuce, strawberries),
- dairy (vanilla ice cream, raw milk, mozzarella, yogurt),
- miscellaneous (dry pet food).

Results were obtained using Butterfield's Phosphate as primary diluent.

Follow steps 1 to 12 of the TEMPO procedure indicated above then incubate the cards for 22-27 hours at 35 ± 1°C.

Note: The incubation time for the test is managed by the TEMPO Read software which integrates a theoretical interval of 15 minutes between the reading of the card barcode and the start of incubation.
If the real interval is greater than 15 minutes (without exceeding 2 hours), this extra time must be added to the remaining incubation time displayed by the TEMPO Read software. Reading must always be performed within the 22-27 hour time limit authorized by the software.

Reading the cards at the end of incubation

1. Log on to the reading station.
2. Introduce the incubation rack containing the cards to be read into the reader. The reader scans the barcode of each card and interprets the results of fluorescence in the wells. It automatically associates the sample identifier with the type of test, the dilution and the enumeration results.
   Reading of the TEMPO EC cards may be deferred at the end of incubation by storing them at 2-8°C for a maximum of 48 hours (outside the scope of the NF VALIDATION certification). In this case, allow the cards to come to room temperature (approximately 5-15 minutes) before introducing them into the reader. It should be emphasized that the result obtained will include the annotation "The card was read too late". The user can specify in the comment text box that the cards were read after having been refrigerated.
3. Editing the results: on the reading station screen, the number of colony forming units (CFU) per gram or milliliter of initial product is associated with the sample identifier, the parameter tested and the analysis date.
4. The reading station user interface enables the results to be printed out or transmitted to the laboratory information management system (LIMS). It also enables the records of the results obtained the previous days to be consulted.
5. At the end of the analysis, remove the cards from the rack and dispose of them into an appropriate receptacle.

ENVIRONMENTAL SAMPLES (outside the scope of the NF VALIDATION certification).

Sample type

The proposed protocol can be used for swabbing equipment, countertops or hands with pre-moistened swabs or for wiping countertops with cleaning wipes or sponges. Given the diversity of environmental samples, users should first validate this protocol or any other protocol.

Preparation

Immediately after swabbing or wiping the countertop, transfer the used swab or wipe/sponge directly into a tube containing a given volume of one of the recommended primary diluents. The dilution obtained is the primary dilution of the sample.

Example of test procedure for environmental swabs

Transfer the swab into a tube containing 10 mL, to obtain a dilution which corresponds to a 1/10 dilution of the sample **(primary dilution)**. Homogenize the suspension carefully by shaking the swab in the diluent. Press out the solution by rotating the swab against the inside edge of the tube. It is recommended to test the samples at a dilution of at least 1 in 40 which will enable enumeration between 10 and 4.9×10^4 CFU/surface swabbed. The dilution can be increased according to the expected level of contamination.

1. Remove the required number of vials of culture medium (one vial per test sample) and allow to come to room temperature.
2. Set the dispenser containing the secondary diluent to 3 mL and prime the pump by eliminating the first two volumes dispensed.
3. Log on to the TEMPO preparation station.
4. Following the instructions of the preparation station user interface, identify the sample to be tested, either by manually entering the identifier via the keyboard or using the preparation station barcode reader.
5. Reconstitute the culture medium by dispensing 3 mL of secondary diluent per vial using the dispenser.
6. Using a sterile pipette, take up 1 mL from the tube containing the suspension obtained after swabbing and transfer it into the vial containing the reconstituted culture medium. Homogenize for approximately 3 seconds using a vortex-type mixer. The 4 mL of inoculated medium obtained corresponds to a 1/40 dilution of the environmental sample collected from the swabbed surface.
7. Follow the TEMPO procedure in the paragraph "Test procedure for food samples" from step 7 onwards.

RESULTS AND INTERPRETATION

Once the reading is completed, the results are automatically analyzed by the computer which determines which wells are positive.
The number of positive wells obtained, in relation to the volume of the wells and the dilution of the sample, gives the enumeration result in CFU per gram or milliliter for the original sample, using the MPN tables.

QUALITY CONTROL

The TEMPO reagents are systematically quality controlled at various stages of their manufacture. For users who wish to perform their own quality control tests to ensure that the TEMPO method has been carried out correctly, the following strains can be used:
Escherichia coli ATCC® 25922™
Escherichia coli ATCC® 8739™
Pseudomonas aeruginosa ATCC® 27853™

Recommended protocol:

- The different incubation steps should be performed at 37 ± 1°C or 35 ± 1°C.
- Using a 24-hour old culture on Trypcase Soy Agar, prepare a suspension in Peptone water and adjust to 0.5 McFarland, i.e. approximately 10^8 CFU/mL using the Densimat (see " Material and reagents required but not provided"). Perform serial decimal dilutions in Peptone water until a suspension with a theoretical concentration of approximately 10^2 CFU/mL is obtained. For *E. coli*, transfer 1 mL of this suspension into a vial of culture medium which has been reconstituted beforehand with 3 mL of sterile distilled water. Follow the same procedure for the strains of *Pseudomonas*, but transfer 1 mL of the 10^7 CFU/mL suspension.
- Modify the default dilution in the TEMPO software by entering "4" in order to obtain a 1/4 dilution.
- Fill one card per vial of medium and incubate.
- At the same time, check the concentration of the suspension which was used to inoculate the TEMPO card by streaking 0.1 mL of the 10^3 CFU/mL suspension on TSA. Incubate.
- After incubation, perform card reading. Count the number of colonies of *E. coli* and check for the presence of *Pseudomonas* on TSA.

Range of expected results:

Strains of *E. coli*

Calculate the ratio R:

$$R = \frac{\text{TEMPO result (CFU/g)}}{10 \text{ x no. of colonies on TSA}}$$

R should be between 0.01 and 1.

Pseudomonas aeruginosa should be totally inhibited by TEMPO EC (in this case, the TEMPO software indicates: enumeration < 1 CFU/g).

If the enumeration results obtained deviate from the expected values, please contact bioMérieux SA or its local representative.

It is the responsibility of the user to perform Quality Control in accordance with any local applicable regulations.

LIMITATIONS OF THE METHOD

- Invalid results may appear if the card has not been filled correctly (presence of empty wells and/or liquid remaining in the vial after the filling cycle): for example, **use of a filtering bag other than the one recommended** (see paragraph "Material and reagents required but not provided").
- Improper preparation or storage of the samples may lead to incorrect results.
- **Warning:** The TEMPO EC parameter was evaluated using numerous food matrices, excluding soft drinks. However, given the diversity of food matrices and manufacturing processes, users should check that the composition of the matrices tested does not affect result accuracy. In particular, the fluorescent signal may be affected if the primary dilution is strongly colored (e.g., fruit purées and cocoa): for the TEMPO EC test, a dilution of these matrices at least equivalent to 1/400 is recommended.
- It is not recommended to use TEMPO EC for the microbiological analysis of products with strong enzymatic activity. This restriction applies in particular to raw mollusks and raw red offals.

See the TEMPO User's Manuals for more complete information.

PERFORMANCE

During approval of the TEMPO EC parameter, according to the standard EN ISO 16140 (10), the preliminary study gave the following results:

- Selectivity: out of the 30 *E. coli* strains tested, the 28 strains which produced characteristic colonies with the reference method were detected by TEMPO EC. For the 2 *E. coli* strains (β-glucuronidase negative) which produced non-characteristic colonies on TBX Medium, enumeration was not obtained using TEMPO EC.
  The study of 20 non-*E. coli* strains did not reveal any cross-reactivity.
- Relative accuracy: the analysis was based on 50 samples (including 25 naturally contaminated samples) tested in duplicate and simultaneously using TEMPO EC and the EN ISO 16649-2 (1). The linear regression equation when comparing the 2 methods is:

  log TEMPO EC = 0.99 log ISO + 0.35

  The bias observed between the 2 methods of 0.26 log, in favor of the TEMPO EC method, is due to a better coverage of *E. coli*.

The TEMPO EC parameter was approved according to the standard EN ISO 16140 (10) as an alternative method for the analysis of all food products for consumption by humans and domestic animals, with the exception of drinks and cattle feed. The TEMPO EC method was certified NF VALIDATION by comparison with the reference method described in the international standard EN ISO 16649-2 (1).

The BIO 12/13-02/05 validation certificate can be obtained from our Technical Assistance Dept. or from AFNOR Certification. The date of end of validity for the NF VALIDATION certification is indicated on the certificate.

BIO 12/13 – 02/05
ALTERNATIVE ANALYTICAL METHODS FOR AGRIBUSINESS
Certified by AFNOR Certification
www.afnor-validation.org
www.afnor-validation.com

The TEMPO EC parameter has been validated and certified by the AOAC Official Methods (2009.02) for the enumeration of Escherichia coli. The alternative method was compared in a multi-laboratory collaborative study to AOAC Official Method 966.24. Six food types were artificially contaminated with E. coli: raw ground beef, bagged lettuce, cooked chicken, pasteurized crabmeat, frozen green beans and pasteurized whole milk.

The TEMPO EC test, for the enumeration of Escherichia coli in a variety of food products, was validated by the AOAC Research Institute in August 2006 (Certification No. 080603).

080603 – 08/08/06
PERFORMANCE TESTED METHOD
Certified by AOAC Research Institute
www.aoac.org

WASTE DISPOSAL

Dispose of unused reagents following procedures for hazardous chemical waste.
Dispose of used reagents as well as any other contaminated disposable materials following procedures for infectious or potentially infectious products.
It is the responsibility of each laboratory to handle waste and effluents produced according to their nature and degree of hazardousness and to treat and dispose of them (or have them treated and disposed of) in accordance with any applicable regulations.

LITERATURE REFERENCES

1. International Standard EN ISO 16649-2 (2001) - Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of ß-glucuronidase-positive *Escherichia coli*. Part 2: Colony-count technique at 44°C using 5-bromo-4-chloro-3-indolyl ß-D-glucuronide.
2. Cochran W.G.
   Estimation of bacterial densities by means of the "Most Probable Number".
   (1950) Biometrics 6, 105-116.
3. Woodward R.L.
   How probable is the most probable number ?
   (1957) J. Am. Water Works Assoc., 49, 1060,1068.
4. International Standard EN ISO 6887-5 (2010) – Microbiology of food and animal feeding stuffs - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination. Part 5: Specific rules for the preparation of milk and milk products.
5. Bacteriological Analytical Manual Online
   BAM Reagent R11 (January 2001).
6. Bacteriological Analytical Manual Online
   BAM Media M79 (January 2001).
7. Bacteriological Analytical Manual Online
   Chapter 4 “Enumeration of *Escherichia coli* and the Coliform Bacteria” (September 2002).
8. International Standard EN ISO 6887-4 – Microbiology of food and animal feeding stuffs - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination. Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products.
9. International Standard EN ISO 7218 – Microbiology of food and animal feeding stuffs – General rules for microbiological examinations.
10. International Standard EN ISO 16140 (2003) - Microbiology of food and animal feeding stuffs - Protocol for the validation of alternative methods.

INDEX OF SYMBOLS

Symbol	Meaning
REF	GB: Catalogue number US: Catalog number
	Manufacturer
	Temperature limitation
	Use by
LOT	Batch code
	Consult Instructions for Use
	Contains sufficient for <n> tests

WARRANTY

bioMérieux disclaims all warranties, express or implied, including any implied warranties of MERCHANTABILITY AND FITNESS FOR A PARTICULAR USE. bioMérieux shall not be liable for any incidental or consequential damages. IN NO EVENT SHALL BIOMERIEUX'S LIABLITY TO CUSTOMER UNDER ANY CLAIM EXCEED A REFUND OF THE AMOUNT PAID TO BIOMERIEUX FOR THE PRODUCT OR SERVICE WHICH IS THE SUBJECT OF THE CLAIM.

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Appendix D – TEMPO® AC (aerobic count) package insert

REF **411113**

9301732**B** - en - 2013/01 EN

TEMPO® AC (Aerobic Count)

For microbiological control only

TEMPO AC (Aerobic Count) is an automated test for use with TEMPO, for the enumeration of viable aerobic mesophilic flora in food products and environmental samples.

SUMMARY AND EXPLANATION

TEMPO AC is intended for use exclusively with the TEMPO system for the enumeration of viable aerobic mesophilic flora in food products and environmental samples.
This test was developed in order to obtain performance levels similar to the standard EN ISO 4833 (1), the AOAC Official Method 966.23 and the American Public Health Association's Standard Methods for the Examination of Dairy Products (SMEDP) (2).
The enumeration of this flora is used to determine the sanitary quality of the product and can express its state of freshness or deterioration. For products which are handled or which undergo various technological processes, the total flora count can be used to judge the conditions in which the food was produced, transported and stored.

PRINCIPLE

The TEMPO AC test consists of a vial of culture medium and a card, which are specific to this test.
The culture medium is inoculated with the sample to be tested. The inoculated medium is transferred by the TEMPO Filler into the card containing 48 wells of three different volumes. The card contains 3 sets of 16 wells (small, medium and large wells) with a one log difference in volume for each set of wells. The card is designed to simulate the Most Probable Number (MPN) method (3, 4).
The card is then hermetically sealed in order to avoid any risk of contamination during subsequent handling.
The microorganisms present in the card reduce the substrate in the culture medium during incubation and cause a fluorescent signal to appear, which is detected by the TEMPO Reader. Depending on the number and type of the positive wells, the TEMPO system calculates the number of microorganisms present in the original sample according to a calculation based on the MPN method.

CONTENT OF THE KIT (48 TESTS):

TEMPO AC cards 2 x 24	Ready-to-use, disposable cards with a transfer tube.
TEMPO AC culture medium 2 x 24 vials	Each vial contains a single dose of dehydrated culture medium. Dose for 4 mL.
1 package insert provided in the kit or downloadable from **www.biomerieux.com/techlib**	

COMPOSITION OF THE TEMPO AC CULTURE MEDIUM

Theoretical formula in g/L of reconstituted solution.

Nutrient and growth supplements (bovine/porcine origin)	27
Substrate	0.08
Anti-foaming agent	0.4

pH 7.2

MATERIAL AND REAGENTS REQUIRED BUT NOT PROVIDED

Material:
- TEMPO Bags - Bags with lateral filter (bioMérieux Ref. 80 015)
- Paddle blender
- Pipettes to dispense exactly 0.10 mL or 1.0 mL of sample
- Vortex-type mixer
- Laboratory incubator (under metrology)

The references below are given as a guide only:

Primary diluents recommended for food samples:
- Peptone water / Peptone Saline Diluent (90 mL - bioMérieux Ref. 42 021)
- Buffered peptone water (90 mL - bioMérieux Ref. 42 042)
- Sodium citrate solution or dipotassium hydrogen phosphate solution following EN ISO 6887-5: 2010 point 5.3 (5)
- Butterfield's phosphate-buffered dilution water (7)
- Or any other diluent which has first been validated by the user as compatible for use with the TEMPO system

Primary diluents recommended for environmental testing:
- Difco Neutralizing Buffer (Ref. 236210 Neutralizing Buffer for environmental samples)
- Letheen Broth, Modified (8)
- Or any other diluent which has first been validated by the user as compatible for use with the TEMPO system

Secondary diluents recommended:
- Sterile distilled water or equivalent purified water validated by the user

Material recommended for quality control:
- Densimat (bioMérieux Ref. 99 234)
- Trypcase Soy Agar [TSA] (bioMérieux Ref. 43 011)

WARNINGS AND PRECAUTIONS

- **For microbiological control only.**
- **For professional use only.**
- Comply with Good Laboratory Practice e.g., standard EN ISO 7218 (10)).
- This kit contains products of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not totally guarantee the absence of transmissible pathogenic agents. It is therefore recommended that these products be treated as potentially infectious, and handled observing the usual safety precautions (do not ingest or inhale).
- The culture medium should not be used as a manufacturing material or component.
- All samples and inoculated media should be considered infectious and handled appropriately. Aseptic technique and usual precautions for handling the bacterial group studied should be observed throughout this procedure; refer to the Laboratory Biosafety Manual – WHO – Geneva – Latest edition, or the current regulations in the country of use.
- Do not use reagents or disposables after the expiry date indicated on their label.
- Before use, check that the packaging and components are intact.
- Only use culture media which appear to be homogeneous (no agglomerates or moisture).
- Do not use visibly deteriorated cards.
- **Do not allow the sample to come into direct contact with the culture medium (in powder form) before the medium has been reconstituted.**
- Any cards which have not been sealed by the TEMPO Filler must not be used.
- The TEMPO card is not intended for performing subcultures from positive wells.
- Do not write on the card wells or the barcodes.
- Do not stick any labels on the card.
- The TEMPO Reader, TEMPO Filler and racks should be regularly cleaned and decontaminated (see the User's Manuals).
- Any change or modification in the procedure may affect the results and must be validated by the laboratory. bioMérieux will not be held liable for results obtained following any changes or modifications in procedures not validated by bioMérieux. In addition, such changes or modifications may void all warranties.

STORAGE CONDITIONS

- Store the TEMPO AC kit at 2-25°C.
- Do not leave the cards exposed to light (on the workbench or the media stand) for more than 15 days.
- Avoid directly exposing the cards to ultraviolet light.
- If stored according to the recommended conditions, all components are stable until the expiry date indicated on their label.

FOOD SAMPLES

Sample type

The TEMPO system can be used for the analysis of a large variety of food products for consumption by humans and domestic animals.

Preparation

Allow the primary and secondary diluents to come to room temperature (18-25°C) (refer to list of diluents recommended in the paragraph "Material and reagents required but not provided").

Follow the recommendations in the current ISO Standards [or BAM (6) if applicable] for performing sample collection and preparing the stock solution. In particular

- for acidic products, ensure that the pH is restored to neutral when the solution is prepared (EN ISO 6887-4 point 8.2) (9).
- for all aromatic herbs, spices, teas and herbal teas, which may have an inhibitory effect, a minimal dilution of 1/400 should be used, even after applying the standard EN ISO 6887-4 point 9.5.4.4 (9).

To prepare the samples, dilute the sample 1/10 (**primary dilution**), using one of the primary diluents recommended. For example, aseptically add 10 g or 10 mL of sample to 90 mL of Peptone water. Homogenize in the TEMPO bag (see instructions for using the TEMPO bag in the User's Manual for the TEMPO Preparation Station).

The interval between the homogenization of the primary dilution and its transfer into the TEMPO card must not exceed 45 minutes, unless otherwise indicated in the specific international Standard (10).

PROCEDURE FOR OBTAINING RESULTS EQUIVALENT TO THE ISO 4833 REFERENCE METHOD AT 30°C (1)

For complete instructions, see the TEMPO system user's documentation (available at www.biomerieux.com/techlib).

Sample	Test mode
All food matrices (standard protocol)	40-48h
Raw meat including poultry (specific protocol)	24-28h
Ready-to-eat meal (specific protocol)	24-28h
Production environment including carcasses (specific protocol)	24-28h
Fruit and vegetables (specific protocol)	24-28h

Test procedure for food samples

Example for the preparation of a 1/400 dilution enabling enumeration between 100 and $4.9x10^5$ CFU/g. The dilution can be increased or reduced, according to the expected level of contamination. Only the ISO diluents listed on page 1 can be used with this protocol.

1. Remove the required number of vials of culture medium (one vial per test sample) and allow to come to room temperature.
2. Set the dispenser containing the secondary diluent to 3.9 mL and prime the pump by eliminating the first two volumes dispensed.
3. Log on to the TEMPO preparation station.
4. Following the instructions of the preparation station user interface, identify the sample to be tested, either by manually entering the identifier via the keyboard or using the preparation station barcode reader.
5. Reconstitute the culture medium by dispensing 3.9 mL of secondary diluent per vial using the dispenser.
6. Using a sterile pipette, take up 0.1 mL from the filtered compartment of the TEMPO bag and transfer it into the vial containing the reconstituted culture medium. Homogenize for approximately 5 seconds using a vortex-type mixer. The 4 mL of inoculated medium obtained corresponds to a 1/400 dilution of the sample.
7. Remove one card for each vial of inoculated medium, **without touching** the tip of the transfer tube. Check that the codes (colors and abbreviations) on the card and the vial of inoculated medium match.
8. Associate the identifier of the test sample with the barcodes of the corresponding inoculated medium and card using the preparation station barcode reader, following the instructions of the preparation station user interface.
9. Put the vial containing the inoculated medium in the filling rack. Insert the card in the slot opposite the vial, placing the transfer tube of the card inside the vial. The rack can hold up to 6 vials + cards and enables 1-6 TEMPO cards to be filled simultaneously.
10. Place the rack in the TEMPO Filler and start the filling cycle. The inoculated medium is completely aspirated into the card. After the cards have been filled, the TEMPO Filler cuts and seals the transfer tubes. All these operations are performed automatically and take 3 minutes. The filling cycle is the same for all the parameters and enables cards for different parameters to be filled at the same time.
11. Remove the filling rack from the TEMPO Filler and visually check that the vials are empty. Take the cards out of the rack and transfer them into the incubation racks: insert the cards into the slots, with the label on the card facing the user (towards the rack handle). Cards which are to be incubated at the same temperature should be grouped together on the same rack. Each rack can hold up to 20 cards. Do not insert cards in between the slots.
12. Dispose of the used vials and transfer tubes into an appropriate receptacle.
13. Incubate the cards at 30 ± 1°C for the time indicated for the chosen test mode, in order to obtain performance levels similar to the standard EN ISO 4833 (1).

Note: The incubation time for the test is managed by the TEMPO Read software which integrates a theoretical interval of 15 minutes between the reading of the card barcode and the start of incubation.
If the real interval is greater than 15 minutes (without exceeding 2 hours), this extra time must be added to the remaining incubation time displayed by the TEMPO Read software. Reading must always be performed within the time limit authorized by the software.

Reading the cards at the end of incubation

1. Log on to the reading station.
2. Introduce the incubation rack containing the cards to be read into the reader. The reader scans the barcode of each card and interprets the results of fluorescence in the wells. It automatically associates the sample identifier with the type of test, the dilution and the enumeration results.
   Reading of the TEMPO AC cards may be deferred at the end of incubation by storing them at 2-8°C for a maximum of 48 hours. In this case, allow the cards to come to room temperature (approximately 5-15 minutes) before introducing them into the reader.
   The activation of refrigeration management and type must have been previously configured in ***TEMPO*** Admin.
   This functionality enables the warning « card read too late » obtained for a test result, to be replaced by a message indicating that the card has been refrigerated.
3. Editing the results: on the reading station screen, the number of colony forming units (CFU) per gram or milliliter of initial product is associated with the sample identifier, the parameter tested and the analysis date.
4. The reading station user interface enables the results to be printed out or transmitted to the laboratory information management system (LIMS). It also enables the records of the results obtained the previous days to be consulted.
5. At the end of the analysis, remove the cards from the rack and dispose of them into an appropriate receptacle.

ENVIRONMENTAL SAMPLES

Sample type

The proposed protocol can be used for various types of environmental samples, including swabs, cleaning wipes, process water, dust and carcasses. Given the diversity of environmental samples, users should first validate this protocol or any other protocol.

Preparation

Allow the primary and secondary diluents to come to room temperature (18-25°C) (refer to list of diluents recommended in the paragraph "Material and reagents required but not provided").
If using a collection device which already contains a neutralizing diluent, ensure that the diluent is listed in the paragraph "Material and reagents required but not provided". If it is not listed, users should first validate this collection device.

Example of test procedure for environmental swabs

Transfer the swab into a tube containing 10 mL, to obtain a dilution which corresponds to a 1/10 dilution of the sample **(primary dilution)**. Homogenize the suspension carefully by shaking the swab in the diluent. Press out the solution by rotating the swab against the inside edge of the tube. It is recommended to test the samples at a dilution of at least 1 in 40 which will enable enumeration between 10 and 4.9×10^4 CFU/surface swabbed (CFU/S). The dilution can be adjusted according to the expected level of contamination.

1. Remove the required number of vials of culture medium (one vial per test sample) and allow to come to room temperature.
2. Set the dispenser containing the secondary diluent to 3 mL and prime the pump by eliminating the first two volumes dispensed.
3. Log on to the TEMPO preparation station.
4. Following the instructions of the preparation station user interface, identify the sample to be tested, either by manually entering the identifier via the keyboard or using the preparation station barcode reader.
5. Reconstitute the culture medium by dispensing 3 mL of secondary diluent per vial using the dispenser.
6. Using a sterile pipette, take up 1 mL from the tube containing the suspension obtained after swabbing and transfer it into the vial containing the reconstituted culture medium. Homogenize for approximately 5 seconds using a vortex-type mixer. The 4 mL of inoculated medium obtained corresponds to a 1/40 dilution of the environmental sample collected from the swabbed surface.
7. Modify the default dilution in the TEMPO software by entering "40" in order to obtain a 1/40 dilution.
8. Follow the TEMPO procedure in the paragraph "Test procedure for food samples" from step 7 onwards.

RESULTS AND INTERPRETATION

Once the reading is completed, the results are automatically analyzed by the computer which determines which wells are positive.
The number of positive wells obtained, in relation to the volume of the wells and the dilution of the sample, gives the enumeration result in CFU per gram or milliliter for the original sample, using the MPN tables.

QUALITY CONTROL

The TEMPO reagents are systematically quality controlled at various stages of their manufacture. For users who wish to perform their own quality control tests to ensure that the TEMPO method has been carried out correctly, the following strains can be used:
- *Escherichia coli* ATCC® 25922™,
- *Bacillus subtilis* ATCC® 6633™.

Recommended protocol:

- The different incubation steps should be performed at 30 ± 1°C.
- Using a 24-hour old culture on Trypcase Soy Agar, prepare a suspension in Peptone water and adjust to approximately 10^8 CFU/mL for *E. coli* and 10^7 CFU/mL for *B. subtilis* (which corresponds to a turbidity between 0.4 McFarland and 0.8 McFarland on the Densimat - see "Material and reagents required but not provided"). Perform serial decimal dilutions in Peptone water until a suspension with a theoretical concentration of approximately 10^2 CFU/mL is obtained. For each strain, transfer 1 mL of this suspension into a vial of culture medium which has been reconstituted beforehand with 3 mL of sterile distilled water.
- Modify the default dilution in the TEMPO software by entering "4" in order to obtain a 1/4 dilution.
- Modify the default unit in the TEMPO software by selecting "No unit".
- Fill one card per vial of medium and incubate.
- At the same time, check the concentration of the suspension which was used to inoculate the TEMPO card by streaking 0.1 mL of the 10^3 CFU/mL suspension on TSA. Incubate.
- After incubation, perform card reading.
- At the end of incubation of the plates, count the number of colonies on TSA.

Range of expected results:

For the 2 strains, calculate the ratio R:

$$R = \frac{\text{TEMPO result}}{\text{no. of colonies on TSA}}$$

R should be between 0.1 and 10.

If the enumeration results obtained deviate from the expected values, please contact bioMérieux SA or its local representative.

It is the responsibility of the user to perform Quality Control in accordance with any local applicable regulations.

PROCEDURE FOR OBTAINING RESULTS EQUIVALENT TO THE BAM REFERENCE METHOD AT 35°C (SMEDP REFERENCE METHOD AT 32°C) (2)

For complete instructions, see the TEMPO system user's documentation (available at www.biomerieux.com/techlib).

Sample	Test mode
All food matrices and production environment	22-28h

Test procedure for food samples

Example for the preparation of a 1/40 dilution enabling enumeration between 10 and $4.9x10^4$ CFU/g. The dilution can be adjusted according to the expected level of contamination. Only the diluents listed on page 1 can be used with this protocol.

1. Remove the required number of vials of culture medium (one vial per test sample) and allow to come to room temperature.
2. Set the dispenser containing the secondary diluent to 3 mL and prime the pump by eliminating the first two volumes dispensed.
3. Log on to the TEMPO preparation station.
4. Following the instructions of the preparation station user interface, identify the sample to be tested, either by manually entering the identifier via the keyboard or using the preparation station barcode reader.
5. Reconstitute the culture medium by dispensing 3 mL of secondary diluent per vial using the dispenser.
6. Using a sterile pipette, take up 1 mL from the filtered compartment of the TEMPO bag and transfer it into the vial containing the reconstituted culture medium. Homogenize for approximately 5 seconds using a vortex-type mixer. The 4 mL of inoculated medium obtained corresponds to a 1/40 dilution of the sample.
7. Remove one card for each vial of inoculated medium, **without touching** the tip of the transfer tube. Check that the codes (colors and abbreviations) on the card and the vial of inoculated medium match.
8. Associate the identifier of the test sample with the barcodes of the corresponding inoculated medium and card using the preparation station barcode reader, following the instructions of the preparation station user interface.
9. Put the vial containing the inoculated medium in the filling rack. Insert the card in the slot opposite the vial, placing the transfer tube of the card inside the vial. The rack can hold up to 6 vials + cards and enables 1-6 TEMPO cards to be filled simultaneously.
10. Place the rack in the TEMPO Filler and start the filling cycle. The inoculated medium is completely aspirated into the card. After the cards have been filled, the TEMPO Filler cuts and seals the transfer tubes. All these operations are performed automatically and take 3 minutes. The filling cycle is the same for all the parameters and enables cards for different parameters to be filled at the same time.
11. Remove the filling rack from the TEMPO Filler and visually check that the vials are empty. Take the cards out of the rack and transfer them into the incubation racks: insert the cards into the slots, with the label on the card facing the user (towards the rack handle). Cards which are to be incubated at the same temperature should be grouped together on the same rack. Each rack can hold up to 20 cards. Do not insert cards in between the slots.
12. Dispose of the used vials and transfer tubes into an appropriate receptacle.
13. Incubate the cards for 22-28 hours
    - at 35 ± 1°C, in order to obtain performance levels similar to those obtained according to AOAC Official Method 966.23.
    - at 32 ± 1°C, in order to obtain performance levels similar to the Standard Methods for the Examination of Dairy Products (SMEDP) (2).

The TEMPO method was compared to AOAC Official Method 966.23 and SMEDP (2). The AOAC study included the following categories of food products:
- meat (fresh raw ground beef, cooked deli roast beef),
- poultry (fresh ground chicken breast, chicken carcass rinse),
- seafood-based products (raw fresh cod, heat processed frozen white fish),
- fruit and vegetables (fresh fruit salad, fresh tomatoes),
- dairy (pasteurized milk, vanilla ice cream),
- miscellaneous (almonds, pasteurized liquid eggs, dry onions),
- feed (dry pet food),
- environmental (stainless steel surfaces).

Note: The incubation time for the test is managed by the TEMPO Read software which integrates a theoretical interval of 15 minutes between the reading of the card barcode and the start of incubation.
If the real interval is greater than 15 minutes (without exceeding 2 hours), this extra time must be added to the remaining incubation time displayed by the TEMPO Read software. Reading must always be performed within the time limit authorized by the software.

Reading the cards at the end of incubation

1. Log on to the reading station.
2. Introduce the incubation rack containing the cards to be read into the reader. The reader scans the barcode of each card and interprets the results of fluorescence in the wells. It automatically associates the sample identifier with the type of test, the dilution and the enumeration results.
   Reading of the TEMPO AC cards may be deferred at the end of incubation by storing them at 2-8°C for a maximum of 48 hours. In this case, allow the cards to come to room temperature (approximately 5-15 minutes) before introducing them into the reader.
   The activation of refrigeration management and type must have been previously configured in ***TEMPO*** Admin.
   This functionality enables the warning « card read too late » obtained for a test result, to be replaced by a message indicating that the card has been refrigerated.

3. Editing the results: on the reading station screen, the number of colony forming units (CFU) per gram or milliliter of initial product is associated with the sample identifier, the parameter tested and the analysis date.
4. The reading station user interface enables the results to be printed out or transmitted to the laboratory information management system (LIMS). It also enables the records of the results obtained the previous days to be consulted.
5. At the end of the analysis, remove the cards from the rack and dispose of them into an appropriate receptacle.

ENVIRONMENTAL SAMPLES

Sample type

The proposed protocol can be used for swabbing equipment, countertops or hands with pre-moistened swabs or for wiping countertops with cleaning wipes or sponges. Given the diversity of environmental samples, users should first validate this protocol or any other protocol.

Preparation

Immediately after swabbing or wiping the countertop, transfer the used swab or wipe/sponge directly into a tube containing a given volume of one of the recommended primary diluents. The dilution obtained is the primary dilution of the sample.

Example of test procedure for environmental swabs

Transfer the swab into a tube containing 10 mL, to obtain a dilution which corresponds to a 1/10 dilution of the sample **(primary dilution)**. Homogenize the suspension carefully by shaking the swab in the diluent. Press out the solution by rotating the swab against the inside edge of the tube. It is recommended to test the samples at a dilution of at least 1 in 40 which will enable enumeration between 10 and 4.9×10^4 CFU/surface swabbed (CFU/S). The dilution can be increased according to the expected level of contamination.

1. Remove the required number of vials of culture medium (one vial per test sample) and allow to come to room temperature.
2. Set the dispenser containing the secondary diluent to 3 mL and prime the pump by eliminating the first two volumes dispensed.
3. Log on to the TEMPO preparation station.
4. Following the instructions of the preparation station user interface, identify the sample to be tested, either by manually entering the identifier via the keyboard or using the preparation station barcode reader.
5. Reconstitute the culture medium by dispensing 3 mL of secondary diluent per vial using the dispenser.
6. Using a sterile pipette, take up 1 mL from the tube containing the suspension obtained after swabbing and transfer it into the vial containing the reconstituted culture medium. Homogenize for approximately 5 seconds using a vortex-type mixer. The 4 mL of inoculated medium obtained corresponds to a 1/40 dilution of the environmental sample collected from the swabbed surface.
7. Follow the TEMPO procedure in the paragraph "Test procedure for food samples" from step 7 onwards.

RESULTS AND INTERPRETATION

Once the reading is completed, the results are automatically analyzed by the computer which determines which wells are positive.
The number of positive wells obtained, in relation to the volume of the wells and the dilution of the sample, gives the enumeration result in CFU per gram or milliliter for the original sample, using the MPN tables.

QUALITY CONTROL

The TEMPO reagents are systematically quality controlled at various stages of their manufacture. For users who wish to perform their own quality control tests to ensure that the TEMPO method has been carried out correctly, the following strains can be used:
- *Escherichia coli* ATCC® 25922™,
- *Bacillus subtilis* ATCC® 6633™.

Recommended protocol:

- The different incubation steps should be performed at 35 ± 1°C.
- Using a 24-hour old culture on Trypcase Soy Agar, prepare a suspension in Peptone water and adjust to approximately 10^8 CFU/mL for *E. coli* and 10^7 CFU/mL for *B. subtilis* (which corresponds to a turbidity between 0.4 McFarland and 0.8 McFarland on the Densimat - see "Material and reagents required but not provided"). Perform serial decimal dilutions in Peptone water until a suspension with a theoretical concentration of approximately 10^2 CFU/mL is obtained. For each strain, transfer 1 mL of this suspension into a vial of culture medium which has been reconstituted beforehand with 3 mL of sterile distilled water.
- Modify the default dilution in the TEMPO software by entering "4" in order to obtain a 1/4 dilution.
- Modify the default unit in the TEMPO software by selecting "No unit".
- Fill one card per vial of medium and incubate.
- At the same time, check the concentration of the suspension which was used to inoculate the TEMPO card by streaking 0.1 mL of the 10^3 CFU/mL suspension on TSA. Incubate.
- After incubation, perform card reading.
- At the end of incubation of the plates, count the number of colonies on TSA.

Range of expected results:

For the 2 strains, calculate the ratio R:

$$R = \frac{\text{TEMPO result}}{\text{no. of colonies on TSA}}$$

R should be between 0.1 and 10.

If the enumeration results obtained deviate from the expected values, please contact bioMérieux SA or its local representative.

It is the responsibility of the user to perform Quality Control in accordance with any local applicable regulations.

LIMITATIONS OF THE METHOD

- Invalid results may appear if the card has not been filled correctly (presence of empty wells and/or liquid remaining in the vial after the filling cycle) : for example, **use of a filtering bag other than the one recommended** (see paragraph "Material and reagents required but not provided").
- Improper preparation or storage of the samples may lead to incorrect results.
- **Warning:** The TEMPO AC parameter was evaluated using numerous food matrices. However, given the diversity of food matrices and manufacturing processes, users should check that the composition of the matrices tested does not affect result accuracy. In particular, the fluorescent signal may be affected if the primary dilution is strongly colored (e.g., fruit purées and cocoa): for the TEMPO AC test, a dilution of these matrices at least equivalent to 1/400 is recommended.
- For food products with strong reducing activity, e.g., foods rich in vitamin C and raw mussels, a dilution greater than 1/40 is recommended.
- The TEMPO AC reagent enables the enumeration of total aerobic mesophilic flora as described in the standardized protocols. Incubation conditions may not be optimum for slow-growing bacteria, such as certain lactic bacteria. Laboratories testing products which may contain this type of microorganism, e.g., fermented products, are therefore advised to perform an internal verification.

See the TEMPO User's Manuals for more complete information.

The TEMPO AC test, for the enumeration of total aerobic mesophilic flora in a variety of food products and environmental samples, was validated by the AOAC Research Institute in December 2012 (Certification No. 121204).

PERFORMANCE TESTED AOAC RESEARCH INSTITUTE LICENSE NUMBER 121204

121204 – 12/19/12
PERFORMANCE TESTED METHOD
Certified by AOAC Research Institute
www.aoac.org

WASTE DISPOSAL

Unused cards may be considered as non hazardous waste and disposed of accordingly.
Dispose of used reagents or unused vials of TEMPO AC culture medium as well as any other contaminated disposable materials following procedures for infectious or potentially infectious products.
It is the responsibility of each laboratory to handle waste and effluents produced according to their nature and degree of hazardousness and to treat and dispose of them (or have them treated and disposed of) in accordance with any applicable regulations.

LITERATURE REFERENCES

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(2004) 17th Edition. Standard Methods for the Examination of Dairy Products, APHA Washington DC.
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Estimation of bacterial densities by means of the "Most Probable Number".
(1950) Biometrics 6, 105-116.
4. Woodward R.L.
How probable is the most probable number ?
(1957) J. Am. Water Works Assoc., 49, 1060,1068.
5. International Standard EN ISO 6887-5 (2010) – Microbiology of food and animal feeding stuffs - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination. Part 5: Specific rules for the preparation of milk and milk products.
6. Bacteriological Analytical Manual Online,
BAM Chapter 3 “Aerobic Plate Count”
(January 2001).
7. Bacteriological Analytical Manual Online
BAM Reagent R11 (January 2001).
8. Bacteriological Analytical Manual Online
BAM Media M79 (January 2001).
9. International Standard EN ISO 6887-4 – Microbiology of food and animal feeding stuffs - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination. Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products.
10. International Standard EN ISO 7218 – Microbiology of food and animal feeding stuffs – General rules for microbiological examinations.

INDEX OF SYMBOLS

Symbol	Meaning
REF	GB: Catalogue number US: Catalog number
	Manufacturer
	Temperature limitation
	Use by
LOT	Batch code
	Consult Instructions for Use
	Contains sufficient for <n> tests

WARRANTY

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Appendix E – Mcfarland equivalence turbidity standards package insert

remel

McFARLAND EQUIVALENCE TURBIDITY STANDARDS

INTENDED USE
Remel McFarland Equivalence Turbidity Standards are used as standards in adjusting densities of bacterial suspensions.

SUMMARY AND EXPLANATION
Original McFarland standards were prepared by adding $BaCl_2$ to H_2SO_4, resulting in $BaSO_4$ precipitation.[1] The McFarland Equivalence Turbidity Standards are prepared from suspensions of uniform polystyrene microparticles with absorbance values similar to the original $BaSO_4$ standards. Stability of suspensions, shelf life, and ease of comparison have been improved with the McFarland Equivalence Turbidity Standards.

PRINCIPLE
Polystyrene microparticles are suspended in a special buffer and adjusted to an acceptable absorbance range using a spectrophotometer with a 1 cm light path set at 600 nm or 625 nm, depending on the standard used.[2,3] Adjusting a bacterial suspension turbidity to the McFarland Equivalence Turbidity Standard produces bacterial counts in an expected range.

REAGENTS*
Electrically charged polystyrene microparticles suspended in a special buffer.
*Adjusted as required to meet performance standards.

PRECAUTIONS
This product is for Laboratory Use only and should be used by properly trained individuals. Directions should be read and followed carefully.

STORAGE
This product is ready for use and no further preparation is necessary. Store product in its original container at room temperature (20-25°C). Do not freeze or overheat.

PRODUCT DETERIORATION
This product should not be used if (1) there is evidence of dehydration, (2) the product is contaminated, (3) the color has changed, (4) the expiration date has passed, or (5) there are other signs of deterioration.

MATERIALS REQUIRED BUT NOT SUPPLIED
(1) Loop sterilization device, (2) Inoculating loops, swabs, or transfer pipettes, (3) Sterile tube, (4) Saline or broth, (5) Light source.

PROCEDURE
1. Invert the McFarland Equivalence Turbidity Standard gently to fully suspend the polystyrene microparticles.
2. Visually compare the turbidity of an actively growing broth culture or a bacterial suspension prepared from an 18-24 hour culture to the appropriate McFarland Standard. (**Note:** The bacterial suspension tubes should be of similar diameter as the McFarland Equivalence Turbidity Standard).
3. For visual comparison, use adequate light and read the tubes against the white card with contrasting black lines.
4. Equal obliteration or distortion of black lines indicates a turbidity match.

INTERPRETATION
Bacterial suspensions are standardized when distortion of black lines is equal to that of the corresponding McFarland Equivalence Turbidity Standard.

QUALITY CONTROL
All lot numbers of McFarland Equivalence Turbidity Standards have been tested spectrophotometrically and found to be acceptable.

LIMITATIONS

1. The use of broth media which is dark yellow, orange, or brown in color may result in bacterial suspensions of incorrect densities. Trial comparisons should be performed. Use adequate light to read the Standard and test broth against a white card with contrasting black lines.[2]
2. Visually comparing McFarland Equivalence Turbidity Standards and bacterial suspensions by use of backlight illumination could result in bacterial suspensions of incorrect densities.
3. Bacterial densities may be too heavy when colonies of *Haemophilus influenzae* ≤ 24 hours old are used to prepare suspensions.[3]
4. McFarland Equivalence Turbidity Standards are recommended when performing visual comparisons or when using a spectrophotometer adjusted to the proper setting.[4] Use with instruments which use alternative light sources, such as scattered light, has not been validated.

EXPECTED VALUES

Standard No.	0.5	1.0	2.0	3.0	4.0	5.0
Approximate Cell Density (x 10^8/ml)	1.5	3.0	6.0	9.0	12.0	15.0

PERFORMANCE CHARACTERISTICS

A study comparing McFarland Equivalence Turbidity Standards to barium sulfate standards resulted in agreement between the two methods.

BIBLIOGRAPHY

1. McFarland, J. 1907. JAMA. 14:1176-1178.
2. Clinical and Laboratory Standards Institute (CLSI). 2009. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard, 10th ed. M2-A10. CLSI, Wayne, PA.
3. Doern, G.V. and R.N. Jones. 1988. Antimicrob. Agents Chemother. 32:1747-1753.
4. Lorian, V. 1986. Antibiotics in Laboratory Medicine, 2nd ed. Williams & Wilkins, Baltimore, MD.

PACKAGING

Each standard is packaged in a plastic case with a visual comparison card. Tube size is 15 x 103 mm and fits most spectrophotometers.

REF R20410, McFarland Equivalence Turbidity Standard 0.5.....Each
REF R20411, McFarland Equivalence Turbidity Standard 1.0.....Each
REF R20412, McFarland Equivalence Turbidity Standard 2.0.....Each
REF R20413, McFarland Equivalence Turbidity Standard 3.0.....Each
REF R20414, McFarland Equivalence Turbidity Standard 4.0.....Each
REF R20415, McFarland Equivalence Turbidity Standard 5.0.....Each
REF R20421, McFarland Equivalence Turbidity Standard Set .....Set*

*Contains 1 each of 0.5, 1.0, 2.0, 3.0, and 4.0 standards

Symbol Legend

REF	Catalog Number
IVD	In Vitro Diagnostic Medical Device
LAB	For Laboratory Use
	Consult Instructions for Use (IFU)
	Temperature Limitation (Storage Temp.)
LOT	Batch Code (Lot Number)
	Use By (Expiration Date)

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