Red One™
Rapid and Quantitative Detection of Microorganisms in Food Matrices
A Feasibility Study on Semi-Skimmed UHT Milk

Abstract

Redberry has developed a method for the rapid detection of microorganisms in semi-skimmed UHT milk based on the Red One™ system. This application note compares the effectiveness of this new method with the traditional compendial approach for the aerobic mesophilic count. The study involved introducing various microorganisms, both spore-forming and non-spore-forming, into milk samples to simulate different contamination scenarios. The Limit of Detection (LOD) and Time to Result (TTR) for the Red One™ system were key focus areas. The system achieved an LOD of approximately 100 cells/mL, which is in compliance with the standards set by the European Directive 92/146/EEC-1992-CHII-B3. Additionally, the Red One™ system can detect a low level of microbial contamination in a 1-liter milk sample within 24 hours, underscoring its capability for the quick detection of microorganisms in semi-skimmed UHT milk.

Introduction

UHT milk is the most widely consumed type of milk in Europe. For UHT milk to be sold, it must meet the criteria for commercial sterility, which means it should be free from microorganisms capable of growing in the milk under normal, ambient storage conditions. Traditionally, commercial sterility has been verified using the conventional plate count method, the standard technique for such testing. However, this traditional method, often requiring up to 15 days for results, is labor-intensive and unsuitable for immediate, on-site testing. To overcome these challenges and decrease the time to results (TTR), Redberry has introduced a rapid and sensitive detection method capable of delivering results in less than 24 hours.

This study aims to assess the capability of the Red One™ system to detect low levels of microorganisms in semi-skimmed UHT milk, comparing its performance with the conventional plate count method, regarded as the benchmark. Specifically, the study focuses on establishing the limit of detection (LOD) and the TTR for the Red One™ system.

Red One™: A Solid-Phase Cytometry (SPC) Platform

Red One™ is a state-of-the-art, fully automated rapid microbiology system leveraging Solid-Phase Cytometry (SPC) technology for the detection of single-cell microorganisms. It offers immediate and quantitative microbiological results, with outcomes available in 10 minutes.
The system simplifies use into a two-step process:

- Placement of the sample onto a cap.
- Filtration of the sample through a membrane.

Following these steps, a fluorophore (staining agent) is automatically injected to stain live microorganisms. The Redberry dye, through fluorescence, indicates cellular metabolism. The system examines staining kinetics, the change in fluorescence over time, to distinguish between microorganisms and inert particles. Every phase, from sample preparation to the final analysis, including filtration, staining, image capture, and cell counting, is automated, ensuring a seamless workflow.

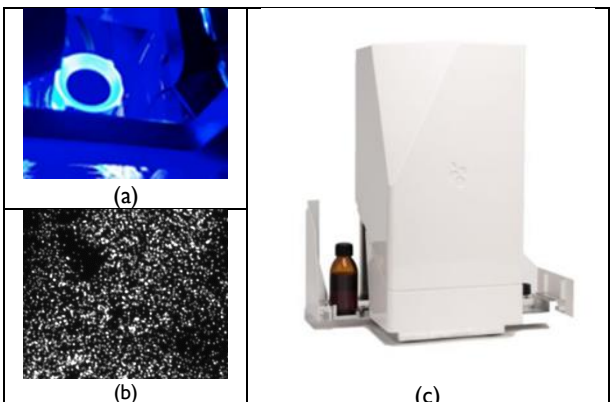


Figure 1 – (a) Red Caps under 480 nm excitation, (b) High Resolution picture of *Saccharomyces cerevisiae*, (c) Red One™ System

The staining agent, Red_One™_DVC, is an esterase-sensitive fluorophore chosen for its minimal toxicity to living cells. This characteristic ensures accurate and reliable identification of microorganisms.

Protocol

Tested products

- Semi skimmed UHT milk
- Semi skimmed UHT milk vitamins supplemented

Tested microorganisms

A panel of relevant spoilage microorganisms was selected, including both spore-forming and non-spore-forming types. The tested strains are summarized in ***Table 1***.

Table 1 | Tested microorganisms

Microorganisms	Forms
Bacillus subtilis ATCC 6633	Both vegetative and sporulated
Escherichia coli ATCC 8739	Vegetative
Pseudomonas aeruginosa ATCC 9027	Vegetative
Pseudomonas fluorescens ATCC 13525	Vegetative

An overnight pre-culture was prepared by inoculating 1 colony from a TS agar plate into 5 mL of TS broth and incubating at 37°C.

LOD (Limit of Detection) Determination

From the overnight pre-culture, pure suspensions of *P. fluorescens* and *B. subtilis* (vegetative form) were diluted in milk to obtain concentrations of 100, 1,000, and 10,000 CFU/mL. To verify the inoculum, plate counts were performed on PCA plates in accordance with ISO 4833-1, and enumeration was conducted after 72 hours of incubation at 30°C.

TTR (Time to Results) Determination

Starting with different pure pre-cultures, serial dilutions were made to achieve low CFU suspensions of *P. aeruginosa*, *B. subtilis*, and *E. coli*. The B. subtilis suspension was initially heated for 10 minutes at 80°C to select for spores. These microorganisms were then inoculated into 1 L milk bottles using a syringe, and the bottles were resealed with beforehand disinfected Parafilm M. The milk bottles were incubated at 30°C for either 24 or 48 hours. The same process was repeated with non-inoculated product to serve as a negative control. The concentration of spiked microorganisms was determined using plate counts on PCA plates, with enumeration after 72 hours of incubation at 30°C, as specified in ISO 4833-2 [4].

Testing workflow

Before analysis on the Red One™ device, all samples underwent a pre-treatment protocol (***Figure 2***), which includes a 1:10 dilution of milk in physiological saline followed by a pre-treatment step involving heating the milk solution with a mix of enzymes and surfactant.

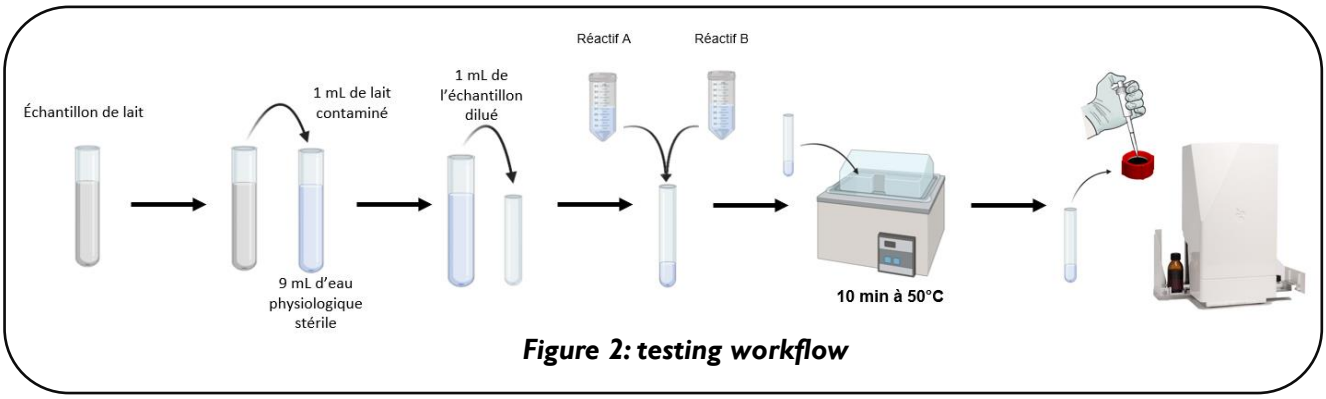


Figure 2: testing workflow

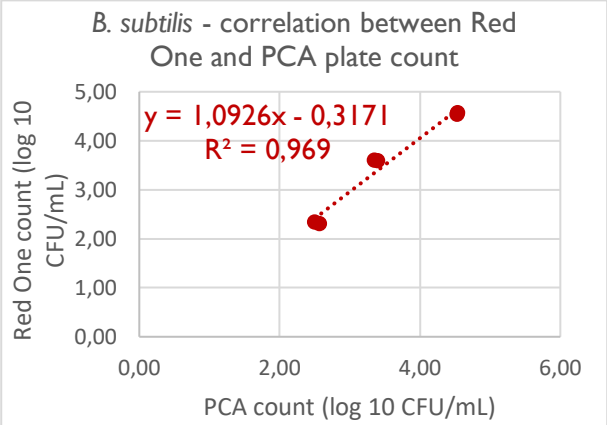
Results

Background Noise Analysis

Background noise was assessed by analyzing six different milk samples, either immediately at T=0 hours or after 48 hours of incubation at 30°C. For each milk sample tested, a very low level of background noise was observed, which is compliant with the requirements for analysis using the Red One™ system. Further details are provided in the appendix.

Enumeration results – LOD and correlation

The linearity, accuracy, and Limit of Detection (LOD) of the Red One™ system were determined by comparing its performance against the plate count method, which serves as the reference standard. This comparison was based on 12 separate counts for each strain, conducted using both the Red One™ system and the plate count method in accordance with ISO 4833-1 standards [3].



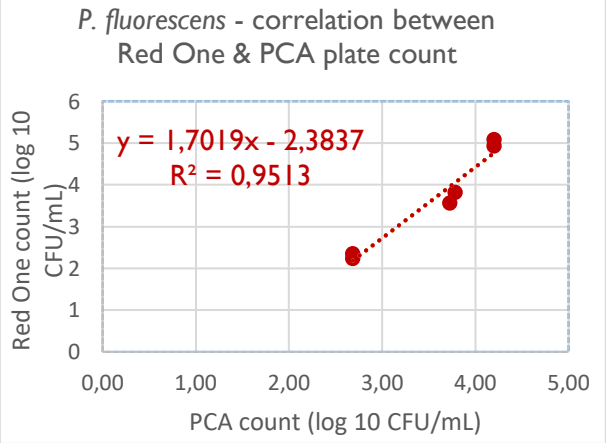


Figure 3: Correlation between Red One™ and PCA plate count 72h

The Red One™ system successfully detected concentrations as low as 100 CFU/mL in milk for all tested microorganisms. The system's lower limit of detection is approximately 100 cells per cap, while its upper limit reaches around 5 × 10^5 cells per cap. These detection limits align with the specifications outlined in the European Directive 92/146/EEC-1992-CHII-B3 [2].

The enhanced sensitivity of the Red One™ system, particularly for measurements ranging from 10^2 to 10^4 cells/mL, showcases its superior performance over other rapid detection techniques applied to dairy products. This increased sensitivity can be attributed to the system's pre-treatment process, notably the heating step. In contrast, ATP detection methods, which can deliver results within 24 hours, do not match the sensitivity of the Red One™ system. Typically, ATP detection methods have a lower limit ranging from 3 to 4 log10 CFU/mL of milk. Moreover, ATP detection yields indirect results that vary with the specific microbial flora present, leading to potential issues with repeatability. [1,5].

TTR

The Red One™ system successfully detected as low as 10 CFU per 1L of milk within 24 hours of incubation at 30°C, with detections exceeding 10^5 cells after this period. Table 2 presents the results.

Analysis demonstrated a strong correlation between the Red One™ system and the plate count method, with correlation coefficients (R^2) of 0.95 for P. aeruginosa and 0.97 for *B. subtilis* across all tested concentrations. A difference of 0.3 log10 is typically considered acceptable in food microbiology; the study found that B. subtilis measurements stayed within this limit across all concentration levels, whereas P. aeruginosa differed by more than 0.3 log10 at low and high concentrations. Detailed findings are available in the appendix.

Microorganisms	Spike count	+24h Red One count
B. subtilis (spores)	15 CFU/L	>10^6 CFU/mL
	24 CFU/L	>10^6 CFU/mL
	89 CFU/L	>10^7 CFU/mL
E. coli	11 CFU/L	>10^7 CFU/mL
	8 CFU/L	>10^7 CFU/mL
	4 CFU/L	>10^7 CFU/mL
P. aeruginosa	32 CFU/L	>10^6 CFU/mL
	8 CFU/L	>10^6 CFU/mL
	5 CFU/L	>10^6 CFU/mL

Table 2 | 24h time to result

Conclusion

The evaluation of the Red One™ system for detecting viable cells in semi-skimmed UHT milk indicates a consistent correlation with established plate count methods for detecting microbial counts in the range of 10^2 to 10^4 CFU/mL. This suggests a level of sensitivity that compares favorably with existing rapid detection technologies, such as ATP detection, within the specific scope of this study.

With its detection limit positioned around 100 CFU/mL, the system meets the standards outlined by the European directive 92/146-1992-CHII-B3. The ability of the Red One™ to provide results in under 24 hours offers a potential reduction in the waiting period associated with traditional methods, which may, in turn, support more timely responses to quality and safety concerns in milk production.

The system's design facilitates ease of use, which could lead to improvements in the efficiency of sample storage and analysis. Moreover, the capability for further detailed analysis of the samples for microbial identification is an additional feature that may prove useful for more in-depth sterility assessments.

It appears that the Red One™ system could provide certain advantages in the context of semi-skimmed UHT milk testing. However, the practicality of implementing this system within established quality assurance procedures and its comparative effectiveness in broader applications warrant cautious further investigation.

More information on this workflow available on:
Red One - Rapid Microbiology System: https://youtu.be/9MvN_QatZ0k

Partnership: Aerial, Technological Resource Center and Technical Institute for Food Industry (France)
Find out more at www.redberry.net and www.aerial-crt.com

Bibliography

[1] Diep, B. *et al.* (2019). Validation protocol for commercial sterility testing methods. *Food Control* **103**, pp 1-8.

[2] Council Directive 92/46/EEC of 16 June 1992 laying down the health rules for production and placing on the market of raw milk, heat-treated milk and milk-based products.

[3] ISO 4833 – 1. Microbiology of the food chain — Horizontal method for the enumeration of microorganisms — Part 1: Colony count at 30 °C by the surface pour plate technique.

[4] ISO 4833 – 2. Microbiology of the food chain — Horizontal method for the enumeration of microorganisms — Part 2: Colony count at 30 °C by the surface plating plate technique.

[5] Ziyaina, M., Rasco, B. & Sablani, S.S. (2020). Rapid methods of microbial detection in dairy products. *Food Control* **110**, art 107008.

Appendix

Supplementary table 1: Background noise on negative controls

		Sample	Red One™ count CFU/milk mL	PCA count CFU/milk mL
J0	Negative controls	Milk 1, sample 1	0,00E+00	<1
			0,00E+00	<1
		Milk 1, sample 2	0,00E+00	<1
			0,00E+00	<1
		Milk 2, sample 1	1,00E+01	<1
			0,00E+00	<1
		Milk 2, sample 1	0,00E+00	<1
			0,00E+00	<1
+48h of incubation	Negative controls	Milk 3 sample 1	0,00E+00	<1
		Milk 3 sample 2	0,00E+00	<1
		Milk 3 sample 3	0,00E+00	<1
		Milk 4, sample 1	0,00E+00	<1
		Milk 4, sample 2	0,00E+00	<1
		Milk 4, sample 3	0,00E+00	<1
		Milk 5, sample 1	0,00E+00	<1
		Milk 5, sample 2	0,00E+00	<1
		Milk 5, sample 3	2,00E+01	<1
		Milk 6, sample 1	0,00E+00	<1
		Milk 6, sample 2	0,00E+00	<1
		Milk 6, sample 3	0,00E+00	<1
		Mean	**1,50E+00**	**<1**
		Standard deviation	**4,77E+00**	**/**

Supplementary table 2: Red One vs plate count on **P. fluorescens** *samples*

Target concentration	Milk sample	Red One count (log10)	PCA count (log10)	ΔPCA count - Red One™ count (log 10)
1,00E+02	Milk 1, sample 1	2,20	2,69	0,46
1,00E+02	Milk 1, sample 2	2,26	2,68	
1,00E+02	Milk 2, sample 1	2,15	2,67	0,35
1,00E+02	Milk 2, sample 2	2,53	2,70	
1,00E+03	Milk 1, sample 1	3,48	3,72	0,17
1,00E+03	Milk 1, sample 2	3,63	3,72	
1,00E+03	Milk 2, sample 1	3,81	3,81	-0.03
1,00E+03	Milk 2, sample 2	3,81	3,76	
1,00E+04	Milk 1, sample 1	4,89	4,23	-0.72
1,00E+04	Milk 1, sample 2	4,95	4,18	
1,00E+04	Milk 2, sample 1	5,07	4,23	-0.87
1,00E+04	Milk 2, sample 2	5,08	4,18	

Supplementary table 3: Red One™ vs plate count on **B.subtilis** *samples*

Target concen-tration	Milk sample	Red One™ count (log10)	PCA count (log10)	Δ PCA count (log 10) - Red One™ count
1,00E+02	Milk 1, sample 1	2,23	2,56	0,26
1,00E+02	Milk 1, sample 2	2,38	2,58	
1,00E+02	Milk 2, sample 1	2,40	2,52	0,16
1,00E+02	Milk 2, sample 2	2,28	2,48	
1,00E+03	Milk 1, sample 1	3,58	3,38	-0,19
1,00E+03	Milk 1, sample 2	3,59	3,41	
1,00E+03	Milk 2, sample 1	3,56	3,36	-0,24
1,00E+03	Milk 2, sample 2	3,63	3,34	
1,00E+04	Milk 1, sample 1	4,56	4,54	-0,02
1,00E+04	Milk 1, sample 2	4,56	4,53	
1,00E+04	Milk 2, sample 1	4,56	4,48	0,00
1,00E+04	Milk 2, sample 2	4,49	4,58	