Application Note
F13002

GreenLight® System for
Determination of Microbial Load

An assessment of Bacterial Load in Raw Milk using GreenLight® Technology

Abstract

The GreenLight® system was designed for applications across the food industry with further uses in environmental measurements. The core technology is a novel oxygen-depletion sensor that can detect very small changes in oxygen content making the system ideal for use in enumerating aerobic microbes. Here we describe a small comparative study conducted by Luxcel Biosciences, assessing correlation between the GreenLight system and standard plate count (SPC) for local raw milk. GreenLight demonstrated strong correlation to SPC with improvements in time-to-result, limit of detection and preparation costs over SPC. GreenLight has been AOAC and Microval certified for raw meats and poultry and further certifications in dairy products is ongoing.

Introduction

GreenLight Model 930 is designed to address the increasing demand for faster, simpler methods of determining bacterial load in food samples. The industry standard for TVC determination (ISO 4833:2003), also known as Standard Plate Count (SPC), is widely used but presents users with some very significant drawbacks. The method is both material and labor intensive, requiring the preparation and analysis of multiple agar plates per sample. More importantly, the method is slow, with 48-72 hours typically required for a definitive result [1-3] and determinations are inherently subjective.

Figure 1: GreenLight Model 930

The GreenLight series of instruments address these limitations, with GreenLight Model 930 providing a rapid high-throughput method for the assessment of bacterial load through analysis of microbial oxygen consumption [4,5]. The test sample is prepared in the usual manner and then simply added to a APCheck™ test vial. An oxygen sensor at the base of each vial is then interrogated kinetically by the GreenLight 930 instrument, producing oxygen profiles which reflect microbial growth (Fig. 2). As bacteria replicate, oxygen consumption rate increases. At a critical point, oxygen consumption exceeds back-diffusion and the sensor signal increases significantly. It then passes a pre-set threshold (Fig. 2A). The higher the initial load, the earlier this threshold level is reached, with instrument software automatically determining the time- to-result

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(Onset time) for each sample and subsequent bacterial load values using a pre-determined calibration (Fig. 2B).

This simple procedure allows the rapid determination microbial contamination levels with results available in 1-12 hours depending on microbial load. GreenLight provides the capacity to handle high sample volumes and facilitates rapid turn-around times in critical limited shelf-life applications.

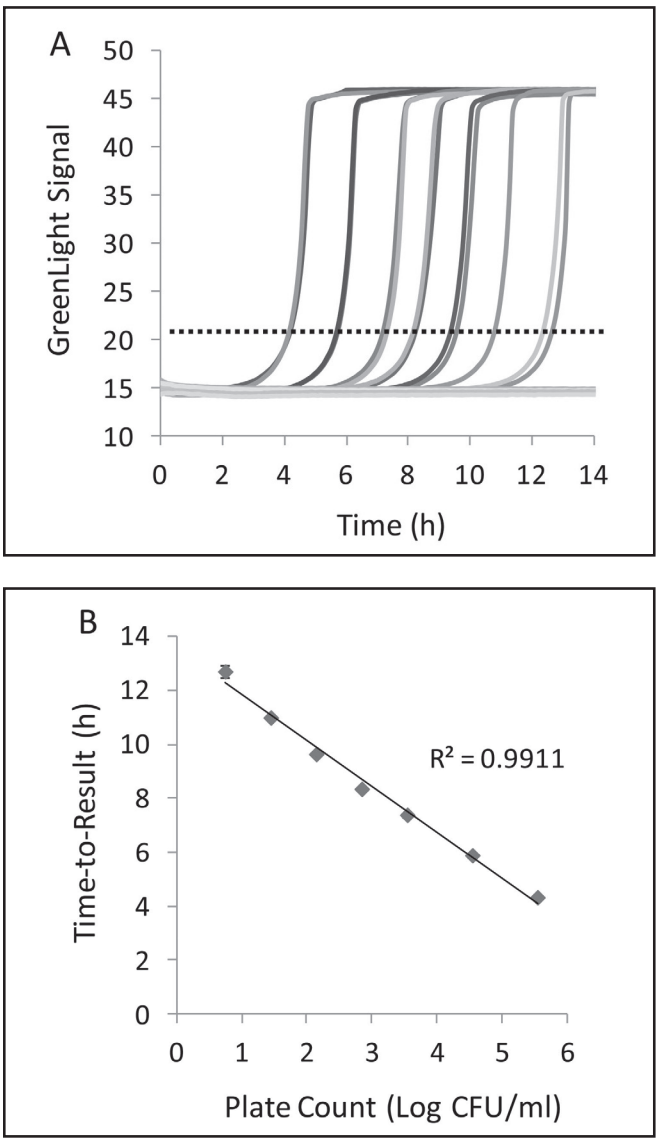


Figure 2: A) Oxygen-based growth curves from a serial dilution of E.coli across a range of contamination levels. B) The correlation between measured GreenLight time to result and bacterial load as determined by agar plate counting (ISO 4833:2003).

To assess the feasibility of determining bacterial load in raw milk samples, a study was performed examining discrete samples over multiple days. A calibration curve was generated relating GreenLight time-to-result to SPC. Discrete samples were then measured using GreenLight 930 and the SPC reference method in parallel in order to assess assay performance and method correlation.

Materials & Methods

Standard Plate Count

Prior to sampling, the raw milk samples were mixed thoroughly by inverting. A serial 10-fold dilution of each raw milk sample was prepared in PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 KH_2PO_4 g/L, pH 7.4) with 5 dilution steps per sample. 1mL of each dilution was added to a sterile agar plate in duplicate. 15mL of plate count agar, pre-cooled to 45°C, was poured onto each plate and swirled gently to mix. After agar solidification the plates were inverted and incubated for 72h at 30°C. Plates containing less than 300CFU/mL were counted and used to calculate the concentration of cells in the corresponding GreenLight 930 sample.

GreenLight 930 Measurement

Prior to sampling, the raw milk samples were mixed thoroughly by inverting. For standard curve generation, 1:10 and 1:100 dilutions were then prepared in a sterile nutrient diluent (10g/L granulated yeast extract, 3.5 g/L Na_2HPO_4, 1.5 g/L KH_2PO_4, 5g/L NaCl). For enumeration, only 1:10 dilutions were performed.

2mL of each dilution was added to an APCheck vial in duplicate. The samples were loaded as per manufacturer's instructions and measured kinetically at 5 minute intervals on the GreenLight 930 instrument at 30°C. Maximum run time was set for 24 hours in order to accommodate negative control readings.

Results & Discussions

Preliminary Calibration Generation

Samples were measured as outlined above at multiple dilutions to generate data points at both high and low levels of bacterial contamination. Samples were diluted in a nutrient broth to ensure optimum and uniform growth conditions, thereby circumventing any potential difficulties associated with milk sample variability and also facilitating the enumeration of samples with a bacterial load of greater than ~$2x10^7$CFU/mL. This approach generated a dataset in excess of 70 data points between 10^2 and 10^6 CFU/mL which provided a calibration function to convert measured time-to-result into microbial load values in units of LogCFU/mL.

Method Evaluation & Comparison

Using the calibration function generated as outlined above, a series of discrete samples were measured at 1:10 dilution and the predetermined calibration used to convert the measured time-to-result into a calculated microbial load in units of Log CFU/mL. These values

were then compared to plate count data to provide an indication of correlation to SPC and a Pearson correlation coefficient of 0.854 was observed, illustrating strong correlation (Fig.3).

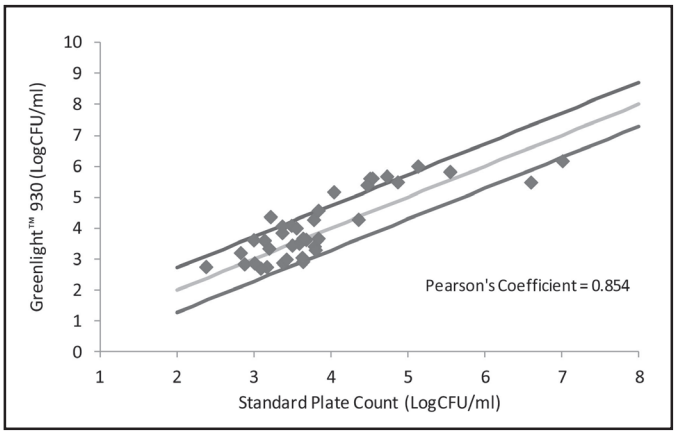


Figure 3: Correlation between GreenLight 930 and plate count measurements of microbial load in raw milk, including tramlines illustrating +/- 2 standard deviations as derived from an independent analysis of SPC repeatability [7].

The error associated with measurement is another important consideration. To examine this, the percentage coefficient of variance was calculated for both GreenLight and ISO reference methods across each sample tested. These data are summarised in figure 4 and illustrate comparable levels of deviation, with the GreenLight system showing slightly better reproducibility. This is not a comprehensive error assessment of the two methods involved but it does illustrate that both methods contribute to any deviation observed in figure 3. This observation correlates with previous observations where GreenLight 960 displayed repeatability equal to or greater than SPC as well as very similar reproducibility [7] (Appendix1).

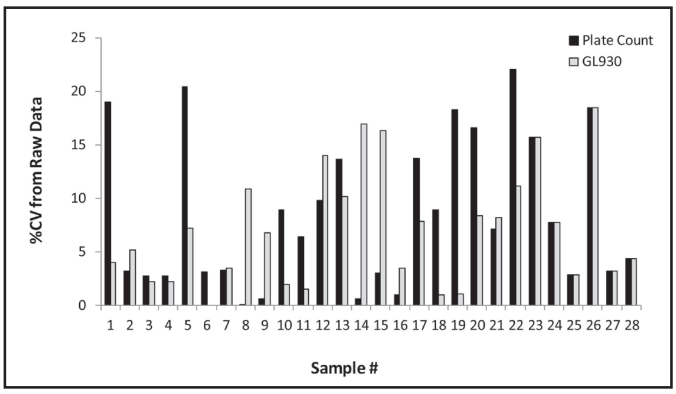


Figure 4: %CV values for each method across the samples tested (n=2).

The use of a 1:10 dilution in a nutrient broth ensures that the impact of factors such as regional or seasonal variability in milk content on bacterial enumeration are minimised resulting in a much more robust assay protocol. These variations are believed to derive from the relative ability of 'neat' milk to support bacterial growth. Dilution also facilitates enumeration of very high levels of microbial load. Repeatability values equal or superior to those previously published should be obtainable through analysis of a larger sample set [7] (Appendix1).

Conclusions

The data presented suggests that GreenLight Model 930 facilitates a simple, high throughput procedure for the evaluation of microbial load in raw milk whereby the sample is added directly to the APCheck vial and microbial load values quantified using an appropriate calibration. The data generated shows a strong correlation with plate count and in this study, showed comparable reproducibility to the reference method.

Using pre-determined criteria, the GreenLight Traffic Light System [RED = Fail, YELLOW is Caution, and GREEN is Pass] critically evaluates the results as required by the laboratory. The GreenLight approach is therefore much less labor intensive and less subjective than the standard plate count method.

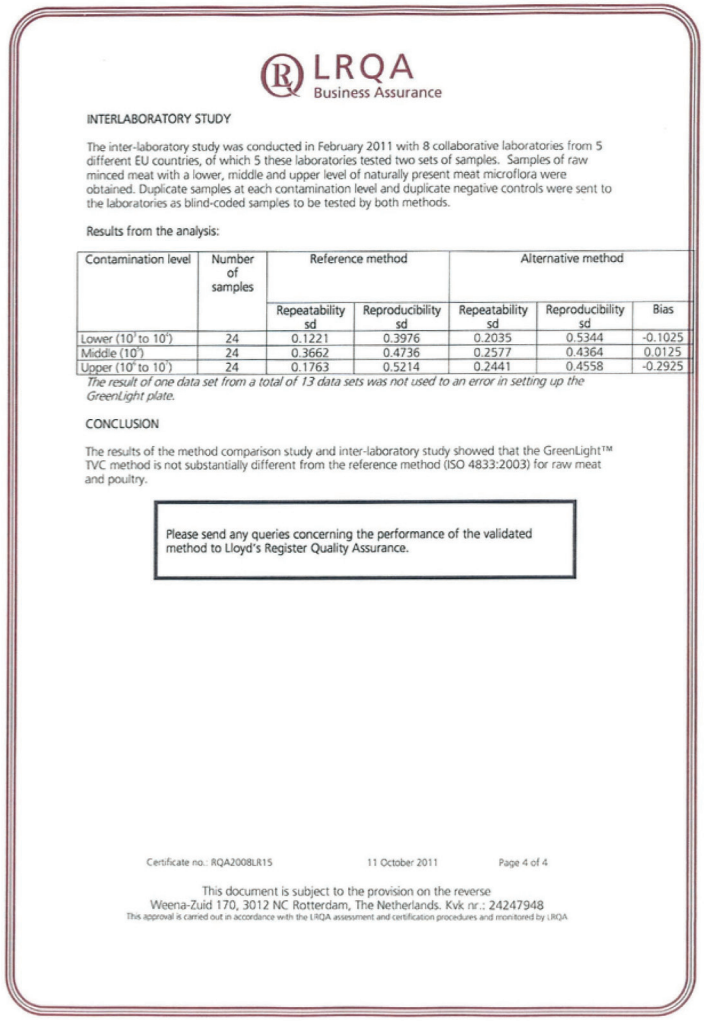
In comparison to the conventional approach, GreenLight 930 provides a much improved 'time-to-result' with data available in a few hours in comparison to the 72 hours required by the standard method. Also, contamination levels are calculated automatically using the standard 930 instrument software yielding results in Log CFU/mL or other commonly used units of measure. This contrasts with the subjective nature of standard agar plate counting.

Potential users of GreenLight are encouraged to conduct their own comparative analyses against SPC data in order to account for local variability in milking environment and microflora and to produce an adequate correlation for use in production testing.

References

1. ISO:4833:2003. (2003). Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of microorganisms. Colony-count technique at 30°C.
2. Baylis, C. L. (2003). Manual microbiological methods for food and drinks industry (fourth ed). CCFRA.
3. Collins, C. H. & Lyne, P. M. (1995) Microbiological methods (seventh ed.). Butterworth & Co. Ltd.
4. O'Mahony, F. C &Papkovsky, D. B. (2006). Rapid high throughput assessment of aerobic bacteria in complex samples by fluorescence based oxygen respirometry. Appl. Environ. Microbiol., 72,1279-1287.
5. O'Mahony, et al (2009). Analysis of total aerobic viable counts in samples of raw meat using fluorescence-based probe and oxygen consumption assay.
6. Commission Regulation (EC) No 2073/2005 on the microbiological criteria for food stuffs.
7. Model 960 PTM Report (12-0318.) Journal of AOAC International. Submitted.

APPPENDIX 1: Excerpt from Microval Certificate: Reproducibility data table



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INTERLABORATORY STUDY

The inter-laboratory study was conducted in February 2011 with 8 collaborative laboratories from 5 different EU countries, of which 5 these laboratories tested two sets of samples. Samples of raw minced meat with a lower, middle and upper level of naturally present meat microflora were obtained. Duplicate samples at each contamination level and duplicate negative controls were sent to the laboratories as blind-coded samples to be tested by both methods.

Results from the analysis:

Contamination level	Number of samples	Reference method		Alternative method		
		Repeatability sd	Reproducibility sd	Repeatability sd	Reproducibility sd	Bias
Lower (10^1 to 10^2)	24	0.1221	0.3976	0.2035	0.5344	-0.1025
Middle (10^5)	24	0.3662	0.4736	0.2577	0.4364	0.0125
Upper (10^6 to 10^7)	24	0.1763	0.5214	0.2441	0.4558	-0.2925

The result of one data set from a total of 13 data sets was not used to an error in setting up the GreenLight plate.

CONCLUSION

The results of the method comparison study and inter-laboratory study showed that the GreenLight™ TVC method is not substantially different from the reference method (ISO 4833:2003) for raw meat and poultry.

Please send any queries concerning the performance of the validated method to Lloyd's Register Quality Assurance.

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