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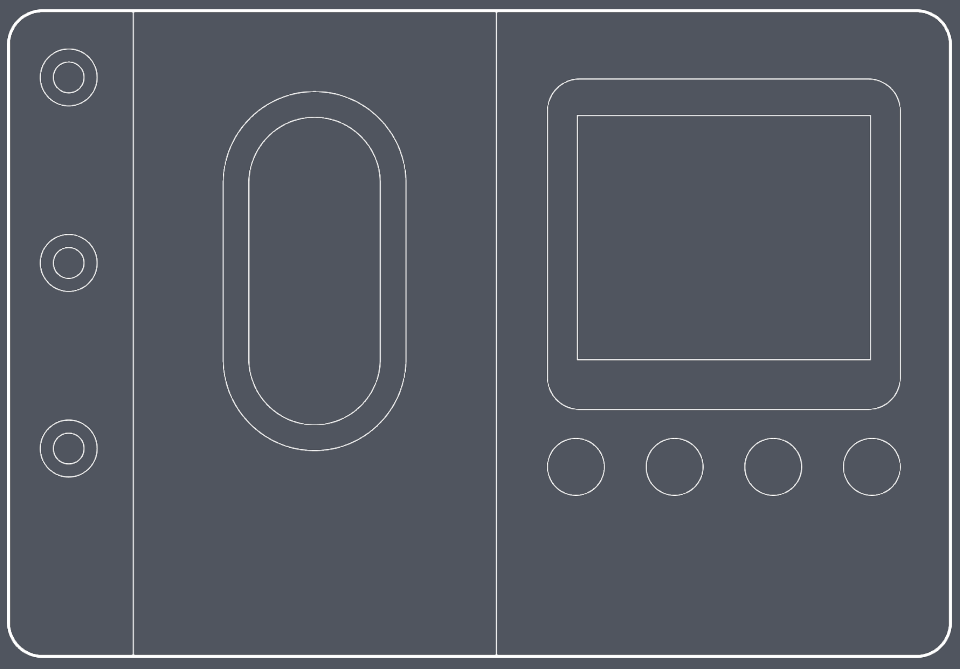
Application Note



CFUs vs BactoBox

Abstract

This application note presents a comprehensive comparison between the traditional method of microbial enumeration, Colony Forming Units (CFUs), and BactoBox. A comparative analysis was conducted using five distinct bacterial species, demonstrating a strong correlation between BactoBox's intact cell concentrations (ICC) and CFUs for actively growing cultures. Despite variations in morphology and cell wall composition among the studied bacteria, BactoBox results paralleled those of the plate count method.

The precision of BactoBox was also evaluated, revealing a significantly lower Coefficient of Variation (CV) compared to CFUs. BactoBox's ability to operate effectively with higher bacterial concentrations contributed to this lower CV, reducing the variance introduced by multiple dilution steps required in the CFU method.

The BactoBox system also offers cost and labor efficiency, with each measurement costing roughly half that of a typical dilution series and aerobic plate count. Furthermore, BactoBox's rapid results facilitate immediate adjustment of sample concentration, reducing the risk of overgrowth or insufficient colonies for statistically valid results.

In conclusion, BactoBox provides a rapid, precise, and cost-effective alternative to traditional CFU methods, offering immediate, actionable results for microbial enumeration.

Keywords

Total viable count (TVC), standard methods agar (SMA), plate count agar (PCA), Drigalsky, microorganism, rapid microbiology methods, serial dilution, agar plate, aerobic plate count (APC), good manufacturing processes (GMPs), growth, bacterial concentration, in-process samples, pour plate, spread plate, spiral plating, rapid microbiology methods

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Plate counting is the most frequently used method for microbial enumeration

Plate counts or colony forming units, CFUs, are considered the golden standard for enumeration of microorganisms [1]. The method has a very low detection limit, and it is inexpensive to establish because it only needs modest equipment and reagents. An additional benefit is that selective and/or chromogenic media can be exploited to quantify a specific microorganism.

Drawbacks with the plate count method

Everyone knows the time-to-result for plate counts is measured in days or even weeks. in extreme cases like the 10-16 hour generation times of *Mycobacterium tuberculosis*, time-to-results can be more than 2 weeks [2]. Even though millions of plate spreads are done every day, the plate count method is far from a perfect method, and it has other obvious shortcomings that are not discussed as frequently:

- Bacteria can be “viable but non-cultureable, VBNC”: The VBNC state is considered a long-term survival mechanism to environmental stressors like temperature and chemicals [4], [5]. VBNC bacteria do not form colonies on plates and therefore the plate count may underestimate the true cell count.
- One-size-fits-all growth conditions do not exist: Bacteria are extremely diverse. Some require highly specific growth factors such as certain combinations of acid/base, temperature, and anaerobic/aerobic conditions. Another challenge is that some bacteria grow fast on standard media and can completely overshadow slow-growing bacteria. These two general problems explains the often extreme underestimation of bacteria also referred to as “the great plate count anomaly” [3].
- Plate counts are associated with analytical imprecision: There is high technical variance associated with the plate count method. For concentrated bacterial suspensions, it is normal to have coefficient of analytical variation, CV, of 15-35% [6].
- Plate counts are expensive and laborious: Even though sophisticated equipment and reagents are not required, it takes a long time to prepare reagents, dilute samples and perform plating. When adding up reagents (excluding working hours), the price is often around 15 EUR per sample. In addition, plate counts are low throughput, so it typically requires the analyst an entire day to prepare, dilute and plate e.g. 100 samples.
- Plate count results are influenced by the analyst: It takes a great deal of practice and skill to get consistent plate spreads. Contamination can be a serious issue.

Advantages of BactoBox measurements over plate counts

The BactoBox has been innovatively designed to resolve the long-standing challenges tied to the century-old plate count method. Offering rapid results in as little as two minutes, it provides the complete bacterial count, empowering you with expedient, actionable insights for faster batch releases and hastened learning cycles.

Operating independently of cultivation, the BactoBox relies on impedance flow cytometry, thereby overcoming bias that arises from specific growth condition requirements. It also ensures that Viable but Non-Culturable (VBNC) bacteria are unable to elude detection.

Finally, the BactoBox addresses the crucial issue of contamination during sample handling. Its rapid measurement capabilities considerably reduce the window for bacterial growth, significantly minimizing the risk of contamination and enhancing overall accuracy.

Correlation between BactoBox and CFUs for actively growing cultures

There's often a question of how results from the BactoBox align with those acquired from the traditional plate count method. To examine this in detail, a comparative analysis was carried out using the five distinct bacterial species listed in Table 1. These species were intentionally chosen to encompass a range of characteristics, including both Gram-positive and Gram-negative bacterial envelopes, and different physical structures, namely cocci (spherical) and rods. All measurements were performed in technical triplicates with repeated dilution series from the homogenized primary sample.

Table 1: The correlation between BactoBox and plate counts was investigated using five different bacterial species.

Species	**Envelope type**	**Shape**
Escherichia coli	Gram-neg.	Rod
Listeria innocua	Gram-pos.	Rod
Klebsiella aerogenes	Gram-neg.	Rod
Staphylococcus epidermidis	Gram-pos.	Round (coccoid)
Acinetobacter baumannii	Gram-neg.	Rod (exp. stage). Round (stationary phase)

This study used actively growing cultures, ensuring that all bacteria would be capable of forming colonies on a plate. As depicted in Figure 1, both the Colony Forming Units (CFUs, represented by dashed lines) and the concurrent BactoBox intact cell concentrations (ICC, represented by solid lines) were evaluated for five different bacterial species.

Each of these species was cultivated in Erlenmeyer shake flasks. Samples were then homogenized to create single-cell suspensions, which were subsequently diluted to fall within the measurement ranges required for both the BactoBox method (10,000 – 5,000,000 total particles/mL) and the plate count method (30-300 colonies per plate).

Despite the variations in morphology and cell wall composition among the studied bacteria, the BactoBox results paralleled those of the plate count method. This underscores the feasibility of obtaining rapid, CFU-like measurements with minimal sample handling using BactoBox.

For the plate count method, most colonies were countable within a span of 1-2 days. However, there were several instances where additional colonies were observed after a period of 3-4 days, demonstrating the varying growth rates among different bacterial species.

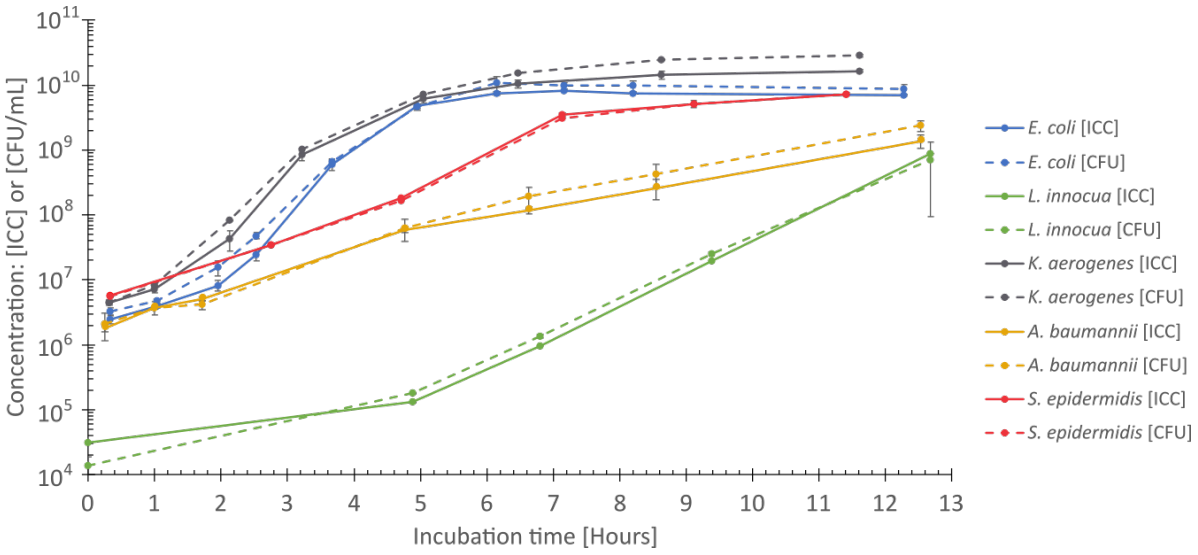


Figure 1: Demonstrating a strong correlation between BactoBox's intact cell concentrations (ICC) and Colony Forming Units (CFU) for actively growing cultures. The growth curves for five bacterial species are presented; plate counts (CFU/mL) are represented by dashed lines, whereas the intact cells/mL, or intact cell concentrations (ICC), reported by BactoBox are represented by solid lines.

Precision of BactoBox and CFUs

To meet the 30-300 colony measurement range for the plate count method, a series of 1:10 dilutions are required. However, each pipetting and vortexing step inevitably contributes to variation, which when multiplied with the dilution factor, results in considerable variance, particularly evident during the growth's latter stages.

One case in point is the E. coli growth curve sampled at the 6.1-hour point. Practically, the number of colonies per plate were relatively consistent across the triplicate dilution series (105, 81 and 136), but with the implementation of eight serial dilutions, the plate count is multiplied by a factor of 10^8, leading to a Coefficient of Variation (CV) of 26%.

In contrast, BactoBox operates effectively with relatively higher bacterial concentrations. For the equivalent time point (7.3 hours), a simpler two-step 1:100 dilution procedure was carried out, culminating in a final 1:10,000 dilution of the primary sample and resulting in a significantly lower CV of 6%.

An interesting case where both the CFU method and BactoBox exhibit relatively high variation is A. baumannii. Known for its propensity to adhere strongly to polymer surfaces and form biofilms [7], the observed variation is likely due to the bacterial adhesion to pipette tips and vials during the dilution series preparation.

The table entries highlighted in green indicate measurements where the CV was below 10%. For the plate count method, this criterion was met in 36% of the sampling points, while for BactoBox, this figure stands significantly higher at 69%. Maintaining low variation is crucial for accurate decision-making. With lower "noise" in the measurements, it's easier to determine whether a culture's bacterial concentration is increasing or decreasing, thereby making reliable decisions. Furthermore, fewer replicates are needed to achieve statistically significant results when evaluating experimental parameters. In conclusion, BactoBox provides immediate results, reduces tedious work, and offers more reliable data.

K. aerogenes (ATCC 13048)

Time [H]	CFU CV%	ICC CV%
0.3	5%	10%
1.0	10%	3%
2.1	18%	4%
3.2	17%	5%
5.1	13%	0%
6.5	10%	6%
8.6	7%	4%
11.6	3%	7%

A. baumannii (ATCC 12457)

Time [H]	CFU CV%	ICC CV%
0.2	45%	12%
1.0	22%	2%
1.7	16%	6%
4.8	37%	9%
6.6	37%	13%
8.5	39%	34%
12.5	18%	24%

E. coli (ATCC 8739)

Time [H]	CFU CV%	ICC CV%
0.3	13%	15%
1.0	9%	7%
1.9	26%	19%
2.5	11%	19%
3.7	10%	18%
4.9	10%	8%
6.1	26%	6%
7.2	10%	5%
8.2	19%	2%
12.3	17%	2%

S. epidermidis (ATCC 12228)

Time [H]	CFU CV%	ICC CV%
0.3	4%	3%
2.7	19%	4%
4.7	7%	3%
7.1	8%	7%
9.1	12%	11%
11.4	11%	5%

L. innocua (ATCC 33090)

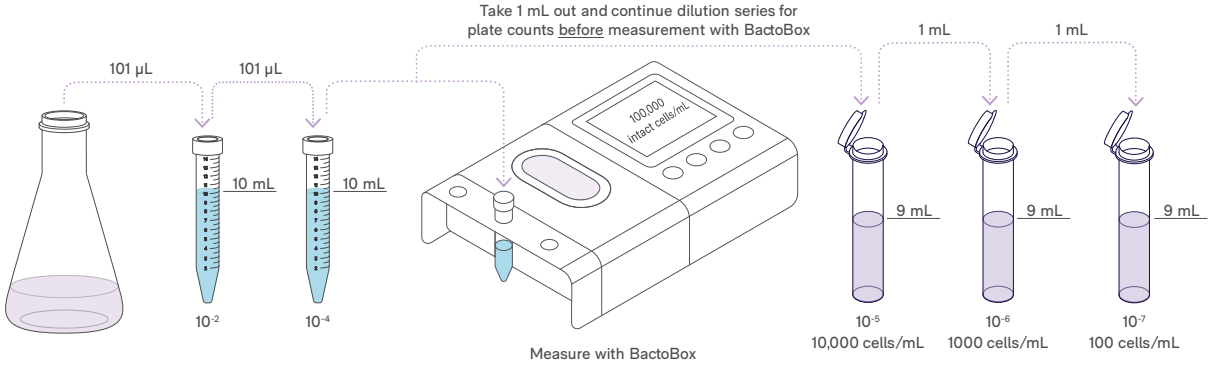
Time [H]	CFU CV%	ICC CV%
0.0	37%	20%
4.9	2%	36%
6.8	6%	2%
9.4	7%	3%
12.7	87%	2%

Figure 2: Precision of BactoBox vs. precision of plate counts. Summary of coefficients of variation of the mean (CV%) for both BactoBox and CFUs. CVs below 10% are green. 36% of plate counts are green while the same number for BactoBox is 69%.

A complimentary case study: Use BactoBox to avoid overkill plating

Any analytical method has its strengths and weaknesses. In the present context, the strengths of BactoBox are speed, ease-of-use, and precision. Nonetheless, plate counting is more suitable if time is not an issue, and you need low detection limit as well as species-specific results. In many standards, plate counts are also a legislative norm that can't easily be substituted by an alternative method without validation efforts. Admittedly, plate counts are indispensable in some cases, but one major issue is that plate counts are often performed "in the dark", because the exact concentration of the primary bacterial sample is unknown. The colonies pr plate should typically be 30-300 and if the proper dilution is missing, the plates could be useless either because they're i) completely overgrown and impossible to count or ii) devoid of colonies.

Fear of missing out of the right dilution leads to extensive dilution series and an unnecessarily high number of plated samples, i.e. more workload and higher costs on reagent and personnel hours. A solution to the excessive workload is to use BactoBox to determine the concentration in the culture prior to plating. As shown in the illustration below, by knowing the bacterial concentration, it is easy to determine the dilution factor necessary to get 30-300 colonies per plate. With this BactoBox-guided plating workflow, it will often be sufficient to plate a single dilution series instead of the typical plating of 2-8 dilutions and plate spreads.



BactoBox-guided plating workflow saves time, costs, and frustrations. Initially a dilution series is performed in BactoBox diluent and 1 mL of the final dilution is used to extend the dilution series to the plate count concentration range. Knowing the concentration of bacteria means that unnecessary dilution and plating is avoided.

In short – if plate counts are indispensable – you can use BactoBox to avoid groping in the dark. The BactoBox-guided dilution workflow will cut costs, save time, and make the plate counting method much less frustrating.

BactoBox saves time, money, and frustrations

The data gathered from the five growth curves affirm that BactoBox maintains a close correlation with plate counts throughout the lag, exponential, and stationary phases. The primary benefit of BactoBox is its capacity to deliver immediate, actionable results, allowing for proactive interventions if bacterial growth is found deviating from the expected patterns. The advantages BactoBox holds over the traditional plate count method are outlined as follows:

- Immediate, Actionable Results: BactoBox delivers results rapidly, with sample preparation and measurement each completed in less than two minutes.

- Enhanced Precision: While the plate count method usually exhibits Coefficients of Variation (CVs) between 15-35% for high bacterial concentrations, BactoBox thrives on concentrated samples and typically records CVs below 10%. Additionally, BactoBox results are uninfluenced by the analyst's manual handling as measurements are automatically initiated with the push of a button.

- Avoidance of Cultivation Biases: BactoBox quantifies bacterial count in a sample without the need for pre-cultivation, circumventing issues associated with colony formation like specific medium composition, growth conditions, and domination by fast-growing organisms. It's also capable of detecting viable but non-culturable (VBNC) bacteria, which might not form colonies on a plate.

- Cost and Labor Efficiency: Whereas a typical dilution series and aerobic plate count costs approximately 10 EUR per sample, a BactoBox measurement costs roughly 5 EUR per sample. BactoBox's ability to immediately reveal bacterial concentration aids in refining the dilution workflow, complementing the plate count method and avoiding unnecessary plating.

- Reduction of Overgrowth Risk: Plate counts can occasionally yield plates that are uncountable due to overgrowth or an insufficient number of colonies for statistically valid results. With BactoBox, results are available within two minutes, facilitating the immediate adjustment of sample concentration if it is either too high or falls below the limit of quantification.

Summarizing these advantages succinctly, we borrow a favorite customer statement shared on LinkedIn: "After trying the BactoBox, plating seems like a waste of time".

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