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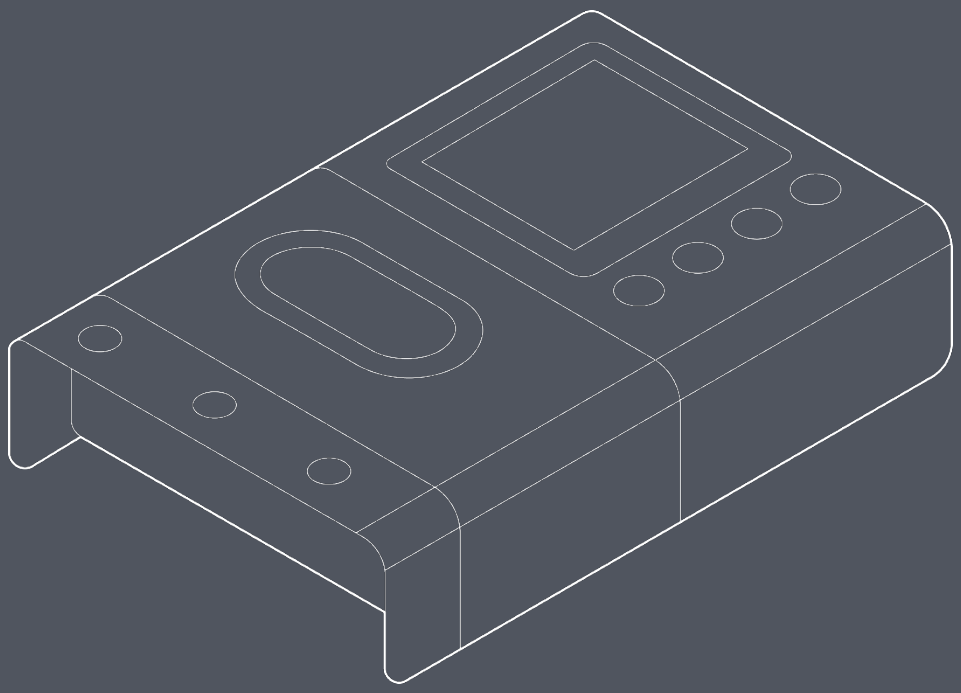
Application Note



# OD600 vs BactoBox

## Abstract

The plate count method is by far the most typical method for determining the number of viable bacteria in a sample and therefore often stated as the gold standard method for this purpose[1]. Unfortunately - even for some of the fastest growing bacteria - it typically takes more than 10 hours for colonies to appear to the naked eye. Plate counts do therefore not provide actionable here-and-now knowledge for fermentation processes. Optical density at 600 nm (OD600) is one of the most frequently used proxy methods for plate counts, but it is well-known that there are pitfalls with this technique. BactoBox represents a novel rapid microbiology alternative to track in-process fermentation samples and bacterial test suspensions. The data provided here for an *E. coli* growth curve show that BactoBox detects growth sooner than OD600 and clearly provides more consistent correlations with plate counts.

## Keywords

Spectrophotometry, OD600, OD660, dry weight estimates, biomass estimates, particles, light scattering, impedance flow cytometry, impedance spectroscopy, rapid microbiology methods, turbidity, wavelength, absorbance.

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## Pitfalls and disadvantages with OD600

Within spectrophotometry, OD600 is often used as a time-saving proxy measurement for biomass concentration2. The measurement principle is based on light-scattering as shown in the illustration below: Light is passed through the liquid medium and collides with individual bacteria and other particles in the suspension. Higher bacterial concentrations will result in less light reaching the detector.

The wavelength 600 nm is typically chosen because it offers an acceptable tradeoff between signal strength and specificity where most of the light "loss" is caused by light scattering and not by pigment absorption. While OD600 is possible to multiplex in 96-well plates, extremely cheap, simple and can be performed on-line, there are obvious pitfalls and drawbacks for quantification of microorganisms:

- OD600 provides a number with an arbitrary unit. It does not provide true bacterial concentrations unless laborious OD600/CFU calibration curves are carried out.
- Spectrophotometer configurations vary and therefore the OD600 measurements from one device can't be compared directly with another device unless a calibration with measurement standards are performed[3].
- OD600 does not differentiate between bacteria and other particles. If the medium contains a background of non-bacterial particles or a high background of dead cells, this could result in misleading results.
- Multiple scattering (Figure 1A, red arrow) can lead to non-linear effects where the incident light reaches the detector because it "bounces off" several particles in solution.
- Many bacteria produce pigments that absorb in the OD600 range. The pigment production can vary over time and therefore the OD600/CFU ratio will be inconsistent.
- Bacterial cell size typically shifts during a growth curve and because larger cells spread light more than smaller bacteria, this will also give a variable OD600/CFU ratio during the growth curve.
- Some bacteria – especially photosynthetic – transmit light very effectively through the cells and are therefore sometimes referred to as "OD-transparent" because even very high concentration of cells (>$10^9$ CFU/mL) result in very little light scattering[2]. This means that they are difficult to detect by OD600 measurements.

## Advantages with BactoBox

While OD600 is based on simultaneous scattering by several bacteria/particles in solution, BactoBox measures **individual** cells as illustrated below. BactoBox exploits a novel, label-free, impedance flow cytometry technology. Objects larger than bacteria (>5 µm) are diverted away from the measuring using microfluidic principles, so they do not lead to clogging or interference with measurements. Bacteria enter the measuring channel, where sets of electrodes detect the individual bacteria on a single-cell basis. Because the detection is based on perturbance of electrical fields, pigmentation or other optical phenomena do not obscure the results and even OD-transparent cells can't hide from the BactoBox. The electrical signatures for bacteria differ substantially from that of other small micrometer-sized objects and therefore the BactoBox has high specificity for bacteria with intact membranes. These are referred to as intact cells and the BactoBox measurement will report the intact cell concentration (ICC) as a subset of the total particle concentration (Total).

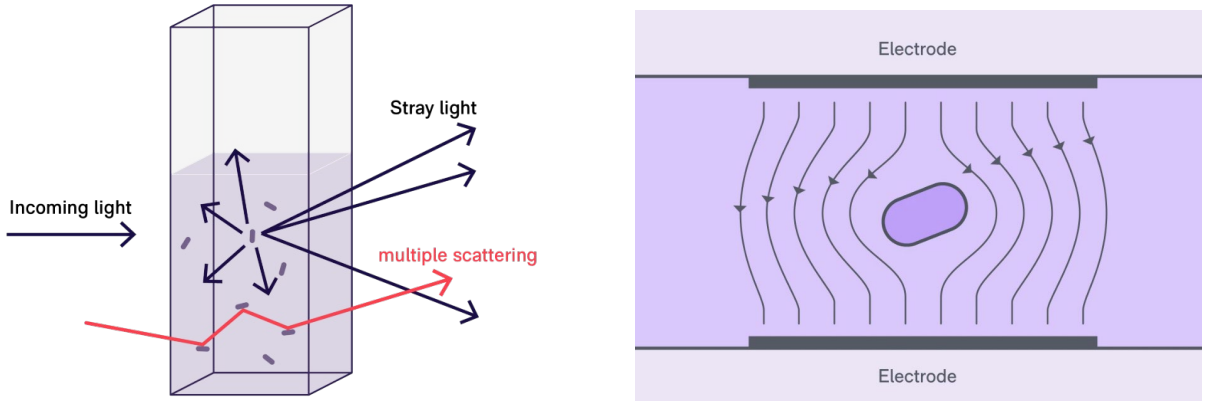


Figure 1: Illustration of differences in the measurement principles. OD600 is based on light scattering by multiple bacteria and other particles. BactoBox measures the fingerprint of the individual intact bacteria in a flow.

## Head-to-head comparison of BactoBox and OD600 methods

In theory, these properties should make BactoBox a more reliable proxy method for plate counts for actively growing bacteria. But the proof is in the pudding, and we therefore set out to investigate this in practice with simultaneous head-to-head measurements on a classical *E. coli* growth curve:

- Which technique provides the best correlation with plate count during the growth curve?
- Which technique reveals active growth earliest?

## BactoBox returns reliable bacterial concentrations in minutes

All measurements were performed in technical triplicates from the primary sample, i.e. the replicates were freshly diluted from the homogenized shake flask sample at the given incubation time.

At first glimpse on the normal axis representation below, both ICC and OD600 tracks growth relatively well. However, if the duration from 3-4 hours is inspected, OD600 somewhat exaggerates the growth relative to CFU and BactoBox measurements. BactoBox ICC is very consistent with the CFU concentration except for the 6.4-hour time point where the large standard deviation on the CFU/mL is the most likely explanation for the offset. On the log10 axis representation, there is excellent correlation between ICC and CFU. On the other hand, at one of the most critical time points (6.4 hours), the high standard deviation on the OD600 curve means that it is not possible to determine if the cell counts are increasing or growth has halted.

Based on these data, BactoBox provides better guidance for the operator at the most critical time points. On the contrary, OD600 has relatively high uncertainty. In addition, BactoBox provides an actual cell concentration and not just an arbitrary unit that needs calibration factors.

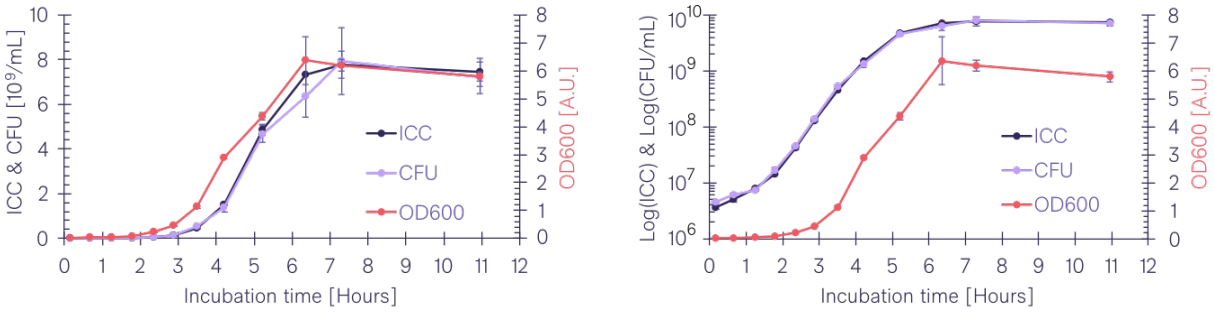


Figure 2: BactoBox ICC provides values close to CFU concentrations. Left graph shows the measurements depicted on a normal axis, while the right graph depicts ICC and CFU concentrations on a log axis. All data points are average values of triplicate measurements, where a new dilution series was started for each replicate.

## BactoBox detects growth earlier than the OD600 method

One of the most frequent questions when starting a fermentation reactor is “are my cells growing or not”? Several things can go wrong (medium, acid/base control, inoculum, growth-inhibiting substances etc.), and if the cells are not growing you want an answer as quick as possible to avoid wasting further time. In fact, “quick” and sensitive is not enough – you also want robust measurements with a stable baseline as otherwise an increase in the signal could simply be due to “noise”.

In the figure below, the earliest sampling points of the *E. coli* growth curve are inspected in greater detail in the bar charts below. Both the plate count method and BactoBox measurements show a highly significant ($p < 0.01$) increase in bacterial concentration for the 40min. sample. On the other hand, with the OD600 measurements, the variation (error bars) is so high that it is not possible to conclude if the cells are growing or not before the 75 min. sampling point. In addition, the numerical increase in OD600 at 75 min. is very small relative to the 10 min. measurement - as an operator, this could likely be interpreted as “no growth yet”.

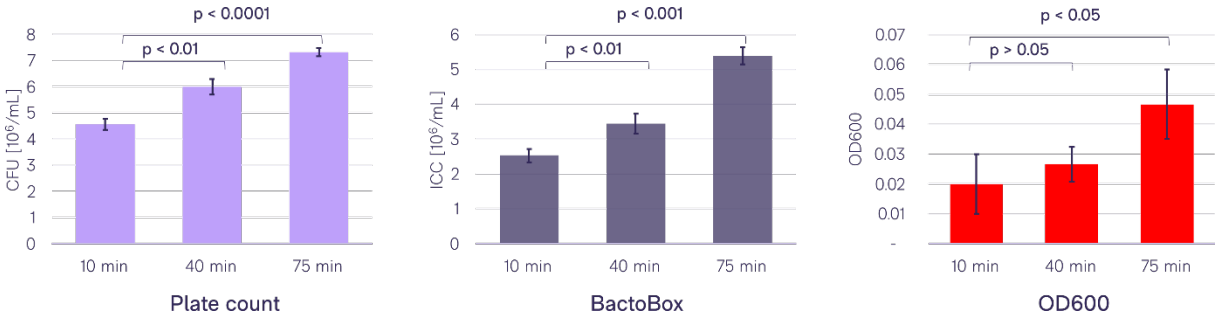


Figure 3: BactoBox reflects increase in growth earlier than OD600. Comparison of measurement principles for the earliest sampling points of the E. coli growth curve. Statistical analysis shows a highly significant increase already at the 40 min point for ICC and CFU concentrations (Student’s T-test, $p < 0.01$). For OD600 the cell density has not increased significantly before the 75 min. sampling point ($p < 0.05$).

Another classical pitfall with OD600 is that the (supposedly sterile) medium used to “zero” the spectrophotometer can be contaminated by growth. This could make initial growth seem to have even lower OD600 values than the blank.

In brief, BactoBox is significantly more sensitive at detecting early growth at low bacterial concentrations than the OD600 method.

## BactoBox correlation with CFU is consistent over time

Because BactoBox detects single events as they pass by the electrodes in the measurement channel, it is not affected by the shortcomings of many optical cuvette-based light-scattering methods. This is indeed reflected in the figure below where the BactoBox ICC is very consistent over time with almost a perfect 1:1 correlation with plate counts. On the other hand, OD600 to CFU ratio is not consistent over time which is clearly showed with the red curve where the calibration factor changes from $0.8*10^9$ to $6.4*10^9$ over time, i.e., a factor 8 difference during the bacterial growth curve.

The offsets in the OD600/CFU ratio over time can be a problem because OD600 is very often used in conjunction with CFUs to create a calibration curve for test suspensions in e.g. antimicrobial studies. Once the calibration curve has been established the analyst will use this correlation as a proxy to determine the here-and-now concentration of CFU/mL before performing experiments. The data in the present investigation show that there are serious pitfalls with this approach. For example, a calibration curve for an 11-hour (overnight) culture is not valid for a 4-hour culture since the CFU/mL concentration would be overestimated by 200%.

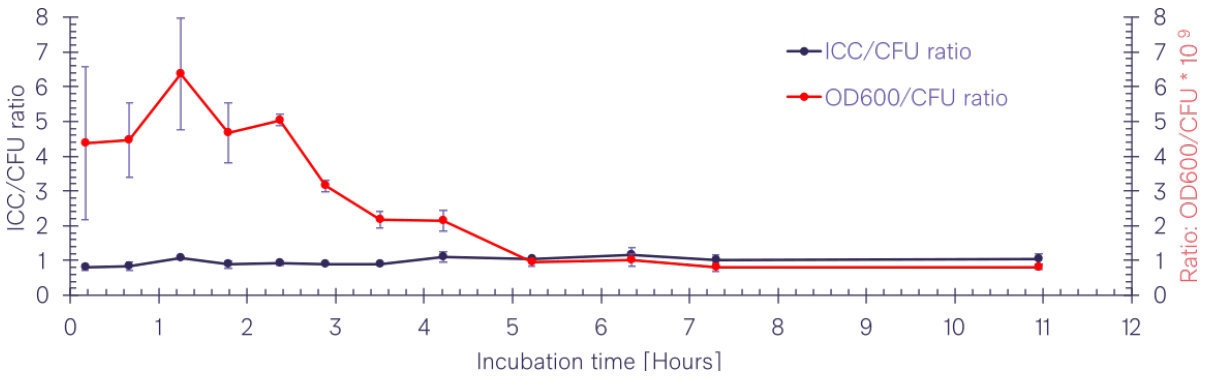


Figure 4: BactoBox has an almost perfect 1:1 correlation to plate counts. The intact cell concentration correlates closely with CFU concentrations. This is not the case for OD600.

## BactoBox as a superior method for in-process bacterial analyses

Here we provided a head-to-head comparison of BactoBox intact cell concentrations and OD600 for tracking the growth of a shake flask culture of *E. coli*. This comparison clearly showed that BactoBox is a fast and reliable growth tracker for in-process samples of actively growing bacteria.

- BactoBox is more sensitive than the OD600 method. In practice this means that BactoBox can detect growth much sooner than OD600.
- The accuracy (similarity to the plate counts) of the BactoBox method is better than that of the OD600.
- The correlation of BactoBox intact cell concentration relative to plate counts is consistent over the entire incubation period.
- BactoBox provides absolute concentrations for bacteria, not arbitrary units that must be converted via calibration curves.

## References

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3. Stevenson, K., McVey, A. F., Clark, I. B. N., Swain, P. S. & Pilizota, T. General calibration of microbial growth in microplate readers. *Sci. Reports 2016 61* **6**, 1–7 (2016).