2025 PDA Pharmaceutical Microbiology Conference | Oct 27 - Oct 29, 2025 | Washington, DC

COMPARISON OF THREE METHODS TO DETERMINE THE "SAFETY MARGIN" USED TO ESTABLISH FINAL RELEASE TIME OF SHORT-LIVED PRODUCTS PER USP<72>

Stéphanie Hempstead[1], Jean Claude Raymond[2], Caroline Kassim Houssenaly[2]
[1] R&D Microbiology, bioMérieux Inc, Durham, North Carolina, United States • [2] R&D Pharma Quality Control, bioMérieux SA, Craponne, Rhône-Alpes, France

INTRODUCTION

Automated growth-based methods are often used for rapid microbial contamination detection in short shelf-life pharmaceutical products such as Cell and Gene Therapies (CGT). For such products, every hour gained on release testing is vital to patients in need.

The BACT/ ALERT® 3D (BTA) Solution is an automated, non-destructive, growth-based, rapid microbial method, capable of detecting a variety of aerobic and anaerobic microorganisms including fungi, utilizing a combination of culture bottles and incubation temperatures. Although most relevant microorganisms are detected in less than 72 hours, the most common practice is the release of product after 7 days incubation (1).

The introduction of USP <72> now gives manufacturers the opportunity to release products in less than 7 days, by providing guidance on the determination of the required incubation time. This determination considers the slowest-growing microorganism plus a safety margin (2).

Using the most common microorganisms encountered in CGT, we compare different methods assessing applicability, repeatability, and robustness of: traditional plate enumeration, optical density, and a novel approach using the kinetics of the BACT/ALERT® system (TTD method).

The purpose of this work is to demonstrate principles and present data for and alternate generation time calculation method using BACT/ALERT (r) 3D (BTA) Solution.

DOUBLING TIME & TTD METHOD

The Doubling time or Generation time (***G***), is the time required for a bacterial population (N_0) to double its number under optimal growth conditions during the exponential growth phase(3).

This value is characteristic of each bacterial species and varies significantly among organisms, from a few minutes to several hours(1). Although generation time has previously been established using the traditional enumeration method, other methodologies can be considered such as a kinetic (TTD method) utilizing BTA technology.

During the exponential growth phase, the time (***TTD***) needed for a culture to reach a detectable concentration (N_{det}) logarithmically depends on the initial concentration (N_0) (Equation A). When considering the exponential phase and after mathematical rearrangement Equation B is obtained. Plotting of such an equation allows the direct determination of ***G*** where t_0 is the lag time in hours, and ***N(t)*** the total bacterial population at time ***t*** in hours (4).

Per USP<72>, the incubation time for microbial detection in the product to be examined can be calculated by using Equation C and the generation time is then calculated by using Equation D.

$$N(t) = N_0 \cdot 2^{\left(\frac{t - t_0}{G}\right)} \quad \text{(A)}$$

$$TTD = t_0 + \frac{G}{\log_{10}(2)} \log_{10}\left(\frac{N_{det}}{N_0}\right) \quad \text{(B)}$$

$$T = t_{TTD} + \left(\log_2(10) \times G\right) \quad \text{(C)}$$

$$G = \frac{t}{3.3 \times \log_{10}(N/N_0)} \quad \text{(D)}$$

MATERIALS AND METHODS

BACT/ALERT® 3D Dual-T system is a growth/respiration-based detection method for aerobic and anaerobic bacteria and fungi using a combination of BACT/ALERT® Culture Media Bottles as required by regulatory bodies. Presence of microorganisms is determined based on dissolved CO_2 production monitored by pairing of a colorimetric sensor and reflected light measurement, providing continuous, automated, and objective reading for early detection alerts.

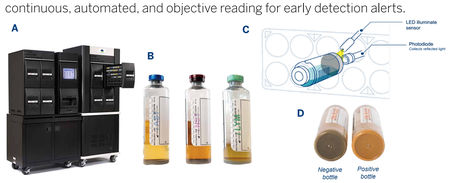

Figure 1. *A/ BACT/ALERT® 3D DUAL-T system; B/ Industry culture media bottle; C/ Detection principle; D/ Colorimetric sensor indicative of positive result.*

Study Design

Microorganisms and their growth conditions evaluated in this study are reported in Table 1. ***These were chosen based on the work from Improved time to detection of slow-growers microorganisms with an automated growth-based method*** (5).

To evaluate the generation time, three distinct methods were evaluated: traditional plate enumeration, optical density and a novel approach using the kinetics of the BACT/ALERT® system (TTD Method).

For enumeration and optical density methods, replicate bottles were spiked with the same starting inoculum and incubated in the BACT/ALERT® system. Throughout microbial growth, regular sampling from separate BACT/ALERT® bottles was carried out. Sample was either diluted and plated in triplicate for enumeration or transferred to cuvette in triplicate for optical density measurement. Plotting either $\log_{10}(N)$ or $\log_{10}(OD_{600nm})$ and applying a regression curve allows determination of *G* which is the slope multiplied by $\log_{10}(2)$(B).

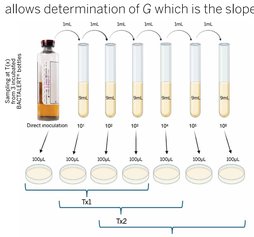

Figure 2. Enumeration Methodology for Generation Time Calculation. *At each sampling time point, a series of tenfold dilutions is performed from the incubated BACT/ALERT bottles. From each dilution, 3 agar plates are inoculated, incubated, and colonies counted.*

For the TTD method, a microbial stock solution at 10^8 CFU/mL is prepared and further diluted to obtain inocula (N_0) ranging from 10^6 to 10 CFU/mL. For each concentration, 3 bottles are inoculated and incubated on the system until declared positive. TTD is plotted against $\log_{10}(N_0)$ which allows for the determination of the generation time.

For each condition reported in ***Table 1,*** at least two repetitions of the kinetic method were run with at least one being paired with enumeration method for comparison. The optical density method was only run once for comparison.

RESULTS

For each organism where enumeration and Kinetic TTD Method could be directly compared, calculated generation time consistently had differences of less than 75 minutes. The examples shown in ***Figures 3 & 4*** show only a 6.6 minute difference between methods for *C. acnes* 6919 in iNST at 36°C. Organisms that demonstrate aggregate growth such as *A. brasiliensis,* or those that swarm on plates such as *P. vulgatus* present significant challenges for the enumeration method regarding sampling reliability and consistent colony counts on plates, respectively. As a result, enumeration experiments regarding these two organisms were determined to be inconclusive in this study. However, the Kinetic TTD Method demonstrated high levels of success and consistency in these organisms, shown in ***Table 1.***

The optical density method was inconclusive and determined to be not viable as readings needed to be done at very specific stages at high frequencies to obtain adequate signal for measurements (data not shown). Additionally, this method could not be applied to organisms with certain properties such as aggregation (clump-forming). For each experiment, all regression coefficients were above 0.95 and therefore considered to be acceptable for further analysis.

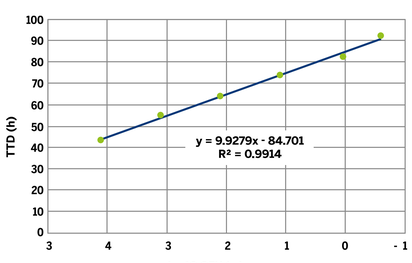

Figure 3. *Kinetic (TTD) Method Example data and Calculation Example for C. acnes 6919 in iNST at 36°C*

When plotting TTD against $\log_{10}$ (CFU), slope = $\frac{G}{\log_{10}(2)} = 3.3 \cdot G$, hence G = 3.01h

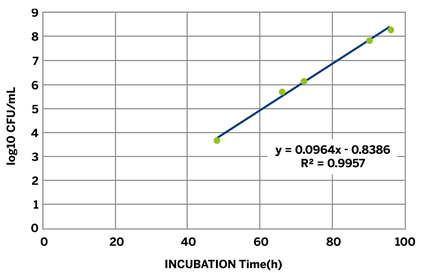

Figure 4. *Kinetic (TTD) Method Example data and Calculation Example for C. acnes 6919 in iNST at 36°C*

When plotting $\log_{10}$ (CFU) against TTD, slope = $\frac{G}{\log 10(2)} = \frac{G}{3.3}$. With a slope of 0.0964, G = 3.12h

STRAIN NAME	STRAIN ID	Experimental CONDITIONS	Enumeration Generation TIME (R^2)	TTDm Generation TIME (R^2)	TTDm REPEAT Generation TIME (R^2)
Aspergillus brasiliensis	NCPF 2275 / ATCC 16404	iAST 22.5°C	N/A	3.37 (0.9929)	3.42 (0.9724)
		iAST 32.5°C	N/A	1.67 (0.9818)	1.83 (0.977)
		iLYM 22.5°C	N/A	2.44 (0.9804)	2.50 (0.9968)
Cutibacterium acnes	DSM 1897 / ATCC 6919	iNST 32.5°C	4.01 (0.9576)	4.42 (0.9731)	4.1 (0.9898)
			3.19 (0.9747)	3.55 (0.993)	4.40 (0.9901)
			3.39 (0.9869)	3.77 (0.9976)	3.71 (0.9973)
		iNST 36°C	2.43 (0.9913)	3.43 (0.9869)	3.53 (0.9936)
			3.12 (0.9957)	3.01 (0.9914)	3.19 (0.9977)
	ATCC11827	iNST 32.5°C	5.18 (0.9605)	4.33 (0.9951)	4.33 (0.9971)
		iNST 36°C	4.23 (0.9549)	3.34 (0.9981)	3.52 (0.9953)
Micrococcus spp.	API82-06-57 / ATCC 700405	iAST 22.5°C	2.22 (0.9926)	2.67 (0.9959)	2.36 (0.991)
Phocaeicola vulgatus	ATCC 8482	iNST 36°C	N/A	N/A	0.97 (0.977)
		iNST 32.5°C	N/A	N/A	1.17 (0.9824)

Table 1: *Generation Time Summary Table*

DISCUSSION

Although labor-intensive, the enumeration method remains the standard reference in microbiology to determine the Generation Time which directly measures the number of viable and cultivable cells by counting. In comparison, optical density, is easier to implement but remains far less precise and is not adaptable for all organisms.

Both the enumeration and optical density methods require significant amounts of time, effort, expertise, and resource organization not always available in the lab. Additionally, while *C. acnes* can be considered as one of the slowest-growing microorganisms, others could be considered based on manufacturer risk assessment and environmental monitoring findings. This can include molds for which, our results confirm that enumeration method is not a viable option. Microorganisms forming clumps or prone to swarming are also a limitation to this method.

Our data set demonstrates that the generation time determination using the kinetic (TTD) method was not substantially different from the traditional enumeration method and demonstrated superior repeatability across multiple organisms and test conditions. Offering an alternative to enumeration method, the kinetic (TTD) method can be launched in a single day, by a single person, with far less supplies and hands-on time needed.

Upon assessing several microorganisms, including aerobic and anaerobic bacteria as well as molds, we confirm that the kinetic method is not only relevant, but a universal and reproducible method to determine generation time as defined by USP <72>.

CONCLUSION

- Detection time-based methods offer an effective approach for estimating bacterial growth parameters, particularly in large-scale studies.
- From these parameters, it is possible to determine the generation time based on CO_2 production measured by the BACT/ALERT® system.
- Kinetic generation time determination offers an alternative to the traditional enumeration method which is time-consuming and requires significant technical expertise, time, and resources.
- **The results herein demonstrate that BACT/ALERT®-based TTD analysis enables precise and efficient safety margin determination, supporting confident, faster release decisions in line with USP <72>.**

REFERENCES:

1. European Pharmacopoeia. 2024. Chapter 2.6.27. Microbiological examination of cell-based preparations
2. U.S. Pharmacopoeia. 2025. General Chapter <72> Respiration-Based Microbiological Methods for the Detection of Contamination in Short-Life Products.
3. Baranyi, J., & Pin, C. (1999). Estimating bacterial growth parameters by means of detection times. Applied and Environmental Microbiology, 65(2), 732-736.
4. Delignette-Muller, M.L. (1998). Relation between the generation time and the lag time of bacterial growth kinetics. International Journal of Food Microbiology, 43(1-2), 97-104.
5. Raymond JC & al. Improved time to detection of slow-growers microorganisms with an automated growth-based method. PDA Microbiology 2024.

ACKNOWLEDGEMENTS:

The authors would like to warmly thank Edgard Minassian, Doriane Piazza, Charlotte Bollinger, and Taylor McDonald.