



Understanding the Variability in Bioburden Test Results in Biomanufacturing

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Variability in bioburden testing presents a significant challenge in ensuring accurate microbial enumeration during biopharmaceutical manufacturing. This study provides a quantitative framework for understanding and minimizing errors in colony-forming unit (CFU) counts, integrating regulatory guidance with statistical modeling. Through detailed analysis, the author demonstrates how factors such as microbial distribution, dilution steps, and test volume selection contribute to variability, particularly at low CFU levels where Poisson-distributed behavior dominates. Using models grounded in relative error and confidence-based metrics, the author calculates the percentage error associated with replicate counts and test conditions. A root sum of squares approach is further proposed to estimate total combined error from independent sources that include CFU/plate variability, number of replicates, serial dilution, and sample volume. This work offers a statistically rigorous method to enhance microbial detection reliability, thereby supporting consistent quality control and regulatory compliance in biopharmaceutical environments.

B ioburden testing plays a crucial role in ensuring the microbiological quality of biopharmaceutical products by quantifying microbial contamination at various stages of production. Accurate microbial enumeration is essential for compliance with regulatory standards, product safety, and process control. However, numerous factors introduce variability in colony-forming unit (CFU) counts, including microbial distribution, test volume selection, dilution errors, and plating techniques. Understanding the statistical principles that govern these factors is critical to minimizing error and improving the reliability of bioburden testing.

One of the key challenges in microbial enumeration is establishing an acceptable range for CFU counts per plate to ensure accurate results while avoiding errors caused by overcrowding or underrepresentation. Regulatory guidelines such as *United States Pharmacopeia (USP) <1227>*, *Validation of Microbial Recovery from Pharmaceutical Articles*, and American Society for Testing and Materials (ASTM) standards provide specific CFU ranges to maintain consistency in bioburden assessments. Additionally, sources of variability such as pipetting errors and microbial recovery rates can significantly impact CFU enumeration. Addressing these challenges requires the application of statistical models to quantify errors and establish robust bioburden testing methodologies that enhance data integrity.

This article explores the impact of test volume, microbial distribution, and dilution errors on bioburden testing variability. It presents statistical approaches to estimate percentage error and discusses strategies to optimize microbial enumeration techniques in biopharmaceutical quality control.

Acceptable CFU counts per plate

Establishing an appropriate range for CFU counts per plate is fundamental to ensuring accurate microbial enumeration while mitigating errors arising from overcrowding or underrepresentation. Historical investigations, such as those by Breed and Dotterer (1), identified key factors affecting CFU enumeration accuracy, including colony crowding, nutrient competition, and interactions between adjacent colonies. Their findings established that plates with more than 400 CFU were unreliable due to overlapping colonies, while counts below 30 CFU lacked statistical significance. Based on these observations, they proposed an optimal range of 50–200 CFU per plate (1). Later refinements by Tomasiewicz *et al.*, through triplicate plating experiments with raw milk samples, recommended a broader range of 25–250 CFU per plate (2).

Regulatory guidelines reinforce the necessity of defined CFU ranges for different testing methodologies. *USP <1227>* specifies an acceptable

range of 25–250 CFU per plate for most bacterial species and *Candida albicans*, whereas *Aspergillus niger* enumeration requires a narrower range of 8–80 CFU per plate (3). Harmonized microbial enumeration guidelines define limits for total aerobic microbial count (TAMC) at ≤ 250 CFU per plate and total yeast and mold count (TYMC) at ≤ 50 CFU per plate. Additionally, ASTM standards specify CFU limits based on enumeration techniques, including 20–80 CFU per membrane for membrane filtration, 20–200 CFU per plate for spread plate methods, and 30–300 CFU per plate for pour plate methods (4). FDA's *Bacteriological Analytical Manual (BAM)* aligns with these recommendations, endorsing the 25–250 CFU range for most microbial enumeration applications (5). While these standardization efforts ensure consistency, variability in microbial enumeration remains a challenge, influenced by a range of technical and biological factors.

Statistical criteria for accepting variability

To ensure the reliability of duplicate plate counts, statistical criteria must be established to define acceptable variability. While universally accepted guidelines for plate count ratios are not explicitly defined, maintaining consistency between duplicate plates is critical for microbial enumeration accuracy. FDA's *Pharmaceutical Microbiology Manual* mandates that all plate counts be performed in duplicate to enhance reliability. Furthermore, the manual states that for a control count of 80 CFU, acceptable duplicate plate counts should be no lower than 40 CFU, implying that a twofold difference in CFU counts (i.e., a ratio of the higher CFU count to the lower count of up to 2.0) is generally permissible. If the observed ratio exceeds this threshold, further investigation may be warranted to identify potential sources of variability, such as technical errors, inconsistencies in sample handling, or filtration inefficiencies (6). Implementing statistical control measures in bioburden enumeration ensures data consistency and enhances the robustness of microbial monitoring programs in pharmaceutical quality control.

Common sources of variability

Despite strict guidelines, several factors contribute to variability in bioburden enumeration. Microbial distribution within a sample is often uneven, leading to inconsistent CFU counts across plates. Pipetting and handling errors, even minor ones, can further affect accuracy. In membrane filtration methods, factors like retention efficiency, flow rate, and microbial adherence impact microbial recovery. The performance of 0.45 μm membranes is particularly critical, as it influences retention and passage of microorganisms. Additionally, manual colony counting can introduce subjectivity, especially at high CFU densities where colonies may merge or overlap. These challenges highlight the importance of rigorous validation to ensure reproducible and reliable bioburden results.

Error in bioburden results as a function of CFU/plate and number of replicates. The accuracy and reliability of bioburden enumera-

tion are directly influenced by the inherent variability in CFU counts and the number of replicates used in plating. Given that microbiological methods naturally introduce variability, particularly at low CFU levels, it is essential to quantify the percentage error (% error) associated with reported bioburden results to ensure method precision and data reliability.

A key challenge in bioburden enumeration is that CFU counts exhibit a Poisson-like distribution, where the variance is proportional to the mean (3). The US Forum on Environmental Measurements (FEM) Microbiology Action Team demonstrates that the margin of error in bioburden enumeration for n replicates, at a specified confidence level $(1-\alpha)$, can be statistically estimated based on the CFU count per plate (C) and the number of replicates (n), as described in **Equation 1** (7):

$$E_{CFU,n} = \frac{100 \times Z_{(1-\alpha)} \times RE}{C^{0.5} \times n \times (p)} \quad [\text{Eq. 1}]$$

where $Z_{(1-\alpha)}$ is the inverse of the normal cumulative distribution function at confidence level $(1-\alpha)$, typically set at 95%, RE is the relative error and p represents the Poisson-scaled error factor, which adjusts sensitivity estimates based on microbial enumeration uncertainty.

Variability in microbial plate counts is a well-documented phenomenon, particularly at low CFU levels, where stochastic effects contribute to significant fluctuations in enumeration results. This variability is recognized in pharmacopeial guidelines such as *USP <1227>*, which highlights the increased percentage error associated with lower CFU counts following a Poisson distribution. Given that, for a Poisson distribution, the standard deviation is equal to the square root of the mean, the relative error (RE) is calculated as shown in **Equation 2** (3):

$$RE = (SD/\mu) = (\sqrt{C}/C) \quad [\text{Eq. 2}]$$

Given this inherent variability, the accuracy of microbial detection is influenced by the relationship between the mean CFU count (C) and the RE , particularly at lower CFU levels where stochastic effects dominate. The Poisson-scaled error factor (p) can be estimated using **Equation 3**:

$$p = (RE) \times C^{(-RE)} \quad [\text{Eq. 3}]$$

where $C^{(-RE)}$ represents the probability of microbial detection; this term is derived from the Poisson distribution, where higher CFU counts increase the probability of detecting at least one colony. It reflects how likely it is to observe microbial presence based on the CFU count. RE represents

the variability in counting; this accounts for the uncertainty introduced by random fluctuations in CFU enumeration, especially at lower CFU levels. It shows that error decreases as CFU count increases.

Equation 3 reflects the inverse dependence of error on CFU count, where larger microbial loads result in reduced RE, while lower CFU levels amplify variability. The exponent $(-RE)$ functions as a confidence-weighted correction factor, modifying the influence of error percentage on the probability of detection.

Traditional Poisson-based sensitivity estimation models, such as $P1 = 1 - e^{-c}$, estimate the chance of detecting at least one microbe. But they assume a fixed relationship between the number of microbes and detection. They don't consider counting errors, which can cause inaccuracies, especially when CFU levels are low, and results fluctuate more.

In contrast, the improved model introduces (p) , which includes both the probability of microbial detection and the variability in counting. This makes bioburden assessments more accurate and reliable, particularly when CFU counts are low, ensuring better microbiological quality control.

Together, the $C^{(-RE)}$ and RE terms ensure that p adjusts for both the likelihood of detection and the uncertainty in microbial enumeration, making it a more refined metric for bioburden sensitivity estimation. As a result, (p) serves as a more comprehensive and practical metric for assessing bioburden sensitivity in microbiological quality control, offering improved statistical reliability over traditional sensitivity estimations.

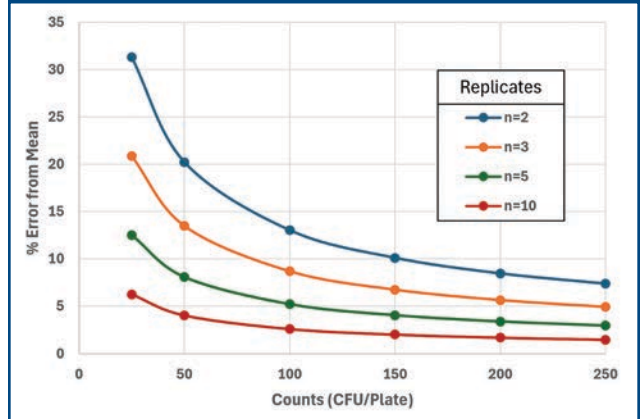
Using **Equation 1**, the percentage error from the mean at a 95% confidence level is calculated as a function of CFU per plate across different replicate numbers (n), as illustrated in **Figure 1**. The observed trend and calculated values align with those reported by Scott (8). Additionally, the trend is consistent with the recommendation by Breed and Dotterer (1), indicating that triplicate plates can provide CFU plate count estimates within 20% of the mean at an estimate of 25 CFU/plate.

Error in serial dilution and its contribution to variability. Serial dilution is a fundamental technique in microbiological testing used to achieve countable CFU levels; however, each dilution step introduces systematic and random errors that contribute to variability in the final reported result. One major source of error arises from pipetting inconsistencies, where inaccuracies in transferring small liquid volumes can introduce errors of approximately $\pm 5.5\%$ per dilution (8). If each dilution step contributes an error percentage ($DE\%$) due to pipetting, mixing, and handling inaccuracies, then the cumulative error ($E_D\%$) over (n) dilution steps can be given by **Equation 4**:

$$E_D = n \times DE \quad [\text{Eq. 4}]$$

Error in bioburden testing due to test volume and microbial distribution. Microbial counts in bioburden testing follow

Figure 1: Percentage error from the mean at 95% confidence level as a function of colony-forming unit (CFU) per plate across different replicate numbers.



a Poisson distribution, where individual microorganisms are randomly dispersed within a test sample. Therefore, test volume plays a crucial role in the reliability of bioburden enumeration. Smaller test volumes increase variability due to uneven microbial distribution, whereas larger volumes enhance statistical confidence in microbial quantification. The relative error (EV) due to test volume can be estimated using **Equation 5** (9):

$$E_V = \frac{100\%}{\sqrt{N}} = \frac{100\%}{\sqrt{V_{test} \times C_{actual}}} \quad [\text{Eq. 5}]$$

where V_{test} represents the test volume in mL; N is the total CFU in tested volume; and C_{actual} denotes the actual microbial concentration in sample CFU/mL. As test volume decreases, the probability of underestimating microbial presence increases, leading to significant errors in bioburden quantification. This analysis underscores the necessity of optimizing test volumes and employing robust statistical models to ensure the accuracy and reproducibility of microbial enumeration in quality control applications.

Calculation of combined error. Because errors due to actual CFU/plate, dilution, and sample volume originate from independent sources, their total combined percentage error (E_{Total}) can be estimated using the root sum of squares (RSS) approach, which accounts for the compounding effects of independent errors, as shown in **Equation 6**:

$$E_{Total} = \sqrt{(E_{CFU})^2 + (E_D)^2 + (E_V)^2} \quad [\text{Eq. 6}]$$

This equation provides a comprehensive assessment of variability in CFU per plate enumeration, integrating errors from microbial recovery fluctuations, dilution-related inaccuracies, and volume-dependent microbial distribution effects. By optimizing experimental parameters—such as en-

sure adequate CFU counts per plate, minimizing dilution steps, and using appropriate test volumes—overall error can be minimized, improving the reliability and reproducibility of microbial enumeration in quality control applications.

Conclusion

In biopharmaceutical manufacturing, ensuring the accuracy and reproducibility of bioburden testing is essential for maintaining product quality and regulatory compliance. Variability in microbial enumeration arises from multiple factors, including microbial distribution, test volume selection, and dilution techniques. The Poisson distribution governing microbial counts underscores the inherent variability in CFU enumeration, particularly at low CFU levels. Consequently, optimizing test volume and minimizing dilution errors are critical steps in improving test reliability. The application of statistical models, such as RE estimation and RSS calculations, provides a systematic approach to quantify and mitigate sources of error in microbial enumeration. By adhering to regulatory guidelines and implementing rigorous quality control measures, biopharmaceutical manufacturers can enhance the precision of bioburden testing, ensuring consistent microbial monitoring across manufacturing processes.

Ultimately, optimizing bioburden testing methodologies through validated enumeration techniques and robust statistical modeling contributes to improved microbial control strategies. This, in turn, supports the production of high-quality, safe biopharmaceutical products while ensuring regulatory compliance and patient safety.

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