

Accuracy of Plate Counts

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"Microbiology Topics" discusses various topics in microbiology of practical use in validation and compliance. We intend this column to be a useful resource for daily work applications.

Reader comments, questions, and suggestions are needed to help us fulfill our objective for this column. Please send your comments and suggestions to column coordinator Scott Sutton at scott.sutton@microbiol.org or journal managing editor Susan Haigney at shaigney@advanstar.com.

IMPORTANT POINTS

The following key points are discussed:

- Microbiological data are inherently variable. The "plate count" is at best an interpretation of an approximation of the number of cells present.
- The linear range for common bacterial counts on standard sized plates is established.
- Unusual plate count situations occur and should be covered by a standard operating procedure (SOP).
- Rounding and averaging rules can play a role in the deduced bacterial count. These rules should be in agreement with the United States Pharmacopeia and described in the SOP.
- Plate counting can have a significant impact on compliance with product specifications and process controls.

WHAT ARE MICROBIOLOGICAL DATA?

Most microbiologists would claim that the recorded number of colony forming units (CFU) were data. However, that number is someone's (skilled technician) interpretation of the number of colonies on the plate. Experience has shown that different technicians (each skilled) can and frequently do observe different

counts on the same sample. The data recorded in the lab notebook are an interpretation of the number of colonies on the plate. However, these are the best data available to us. It must also be remembered that the CFU is only an estimate of the number of cells present. It is a skewed estimate at best as the only cells able to form colonies are those that can grow under the conditions of the test (e.g., incubation media, temperature, time, oxygen conditions). Even those do not represent a single cell, but rather those that happened to be well separated on the plate and so can be distinguished after growth. A colony could arise from one cell or several thousand.

Microbiology has a well-deserved reputation for being highly variable. Our lax attention to precision and accuracy in our measurements helps further this perception. We have allowed specifications for environmental monitoring, raw material bioburden, in-process bioburden, and finished product bioburden to be imposed by regulation without regard for the ability of the methods to support those specifications.

A second reason for concern about plate count accuracy is the introduction of microbiological methods into the lab. Being obsessive by training, we are trying to establish arbitrary measures of accuracy and precision in this exercise that the traditional methods cannot come close to matching. A good example of this is the *European Pharmacopoeia* "Precision" requirement for an alternate method (quantification) to have a relative standard deviation (RSD) in the range of 10-15% (1). While this requirement might be fortunately achieved with dilutions that have plate counts in the 150-250 CFU/plate range, at lower plate counts the target value imposed by regulation will virtually guarantee a long, difficult, and quite possibly unsuccessful validation exercise.

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COUNTABLE RANGE ON A PLATE

The general ranges in common acceptance for countable numbers of colonies on a plate are 30-300 and 25-250. The origin of those ranges is worth examination.

Literature

Breed and Dotterrer published a seminal paper on this topic in 1916. They set out to determine the "limit in the number of colonies that may be allowed to grow on a plate without introducing serious errors in connection with the proposed revisions of standard methods of milk analysis" (2). They note that "the kind of bacteria in the material under examination will have an influence on the size of the colonies, and consequently, on the number that can develop on a plate." Breed and Dotterrer also note that food supply can be an issue, colonies close to each other on the plate may merge, and neighbor colonies may inhibit growth or conversely stimulate growth. "Because of these and other difficulties, certain plates in any series made for a given sample are more satisfactory for use in computing a total than are others. The matter of selecting plates to be used in computing a count becomes, therefore, a matter requiring considerable judgment" (2). Breed and Dotterrer chose their countable plates from triplicate platings of each dilution, requiring acceptable plates to be within 20% of the average. On this analysis, plates with more than 400 CFU were unsatisfactory, as were those of less than 30 CFU, with best results in the range of 50-200 CFU/plate.

Tomasiewicz et al. provides an excellent review of the continued evolution of the appropriate number of CFU per plate from milk (3). The authors took data from colony counts of raw milk from three different experiments (each dilution plated in triplicate) and used the data to determine a mean-squared-error of the estimate for all plates. Their recommendation at the end of the study was for a countable range of 25-250 CFU/plate in triplicate. It is interesting to note that although the authors note that CFU follow a Poisson distribution, no mention is made of any data transformation used to approximate a normal distribution prior to the use of normal statistical analytical tools. Tomasiewicz et al. provide the following excellent cautionary advice:

"The data presented herein are not necessarily applicable to other systems. For automated equipment, the optimum range may well vary with the instrument. Furthermore, even if automation is not used, appropriate numbers of colonies that should be on a countable plate can vary widely, depending on many other variables. With soil fungi for example..." (3).

The compendia have recently harmonized a microbial enumeration test (4). The compendia recommend to "Select the plates corresponding to a given dilution and showing

the highest number of colonies less than 250 for TAMC and 50 for TYMC. In determination of the resistance of biological indicators, the *United States Pharmacopeia (USP)* recommends a range of 20 to 300 colonies, but not less than 6" (5). However, the most complete description of the countable range is found in USP informational chapter <1227> :

"The accepted range for countable colonies on a standard agar plate is between 25 and 250 for most bacteria and Candida albicans. This range was established in the food industry for counting coliform bacteria in milk. The range is acceptable for compendial organisms, except for fungi. It is not optimal for counting all environmental monitoring isolates. The recommended range for Aspergillus niger is between 8 to 80 cfu per plate. The use of membrane filtration to recover challenge organisms, or the use of environmental isolates as challenge organisms in the antimicrobial effectiveness testing, requires validation of the countable range." (6).

ASTM provides countable ranges of 20-80 CFU/membrane, 20-200 for spread plates, and 30-300 for pour plates (7). The US Food and Drug Administration *Bacterial Analytical Manual (BAM)* recommends 25-250 CFU/plate as a countable range (8).

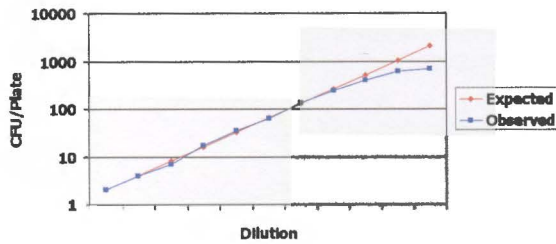
Upper Limit

The upper limit of plate counts is dependent on a number of factors, as described previously. The major issues include the colony size and behavior (e.g., swarming), and the surface area of the plate. The size particularly comes into play with plating a membrane for determination of CFU, as the surface area of that membrane is so much smaller than that of a standard plate.

TNTC (too numerous to count) can be reported out several ways. ASTM (7) recommends reporting this as ">upper limit". For example, a 1:10 dilution with more than 200 CFU on a spread plate would be reported as >2,000 CFU/mL (or gram). FDA's BAM recommends counting the colonies from the dilution with plates giving counts closest to 250, counting a portion of the plate, estimating the total number and then using that number as the estimated aerobic count. The United States Department of Agriculture (USDA) recommends using a grid to segment the counting area, then determining the average CFU/grid and multiplying this average CFU/grid by the number of grids on the plate (9). It is not clear to the author how either of these methods is greatly superior to guessing.

The reason there is an upper limit to CFU/plate is that the colonies begin to compete for space and nutrients, skewing the count. In my opinion, this is an invalid plating and needs to be done correctly at a later date. Note that I am strenuously avoiding the use of the word retest. This

Figure 1: Estimated vs. observed plate counts.



result invalidates the plating, and therefore, the test was not performed correctly. I know this is a hardship to the lab personnel, who were trying to reduce the plating load initially by not plating out sufficient dilutions. However, making a mistake initially is not a reasonable excuse to avoid doing it correctly after the mistake is recognized. If the lab wishes to use this “estimated count,” it should, at a minimum, have it clearly described in the “counting CFU” standard operating procedure (SOP) with a rationale as to when the plate counts are not critical and can be estimated in this fashion.

There are methods available if you should want to accurately determine the upper limit for a unique plating surface or a unique colony type. One is presented in the USP informational chapter <1227> (5) that is based on a pair-wise comparison of counts from a dilution series. This is based on the assumption that at the upper limit the observed numbers of CFU will fall off the expected numbers at some point (see Figure 1). This divergence will become significant at some point—that defines the upper limit of CFU/plate.

Lower Limit

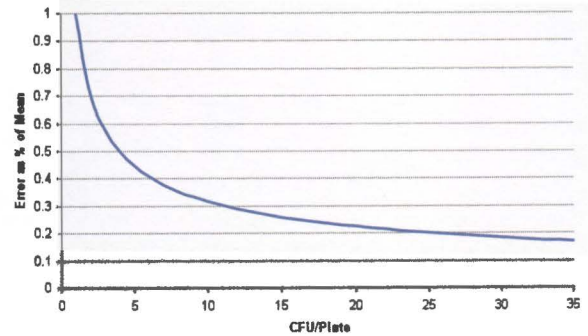
A central concern in this determination is the reporting of the limit of quantification (LOQ) (which is what we are really interested in reporting) against the limit of detection (LOD) (1 CFU). This is an important distinction if we are being held to specifications in the lower range.

ASTM recommendations focus on the LOD and urges the user to report that answer if no colonies are recovered (i.e., <10 CFU/mL for a 1:10 dilution) (7). If countable colonies are present, but below the countable range, count them anyway and report an estimated count.

USP does not have a specific recommendation on how to report these low numbers, but does note “lower counting thresholds for the greatest dilution plating in series must be justified” (6).

The FDA BAM recommends a different reporting format (8). In the FDA BAM method, all counts are recorded in the raw data, but the information is reported as <LOQ. For example, a 1:100 dilution that yields counts of 18 and 12

Figure 2: Error estimates of low plate counts.



would be reported as <2,500. This is, in my opinion, the prudent course. The crux of the argument is that experimental studies have shown very poor accuracy in plate counts below 25 (see above). Theoretically we can argue that because the CFU follow the Poisson distribution, the error of the estimate is the square root of the average (USP <1227>). This leads to graphs such as in Figure 2, which shows us that as the CFU/plate drops below the countable range, the error as a percent of the mean increases rapidly. This confusion between the LOD and the LOQ for plate counts has led to some very difficult situations.

UNUSUAL SITUATIONS

The following situations and questions should be taken into consideration.

Two Dilutions with Countable Colonies

Ideally you would never see two separate dilutions with counts in the countable range, as the countable ranges cover a 10-fold range of CFU. However, this is microbiology. ASTM recommendations urge both dilutions be taken into account, determining the CFU/mL (or gram) separately for each and averaging the results for the final result (7). Breed and Dotterrer (2) also used several dilutions if the numbers fit the quality control (QC) requirements. FDA BAM has no recommendations in this situation. While argument can be made to use all counts, this is a stronger argument if triplicate plates are used and QC limits in place to discard erroneous plates.

A strong argument can also be made to take the dilution providing the larger number of CFU in the countable range. This approach minimizes two concerns: errors in the estimates increase with increasing serial dilutions, and errors in the estimates increase with decreasing plate counts. Use of the smaller dilution (e.g., 1:10 vs. 1:100) could be justified from this perspective. Alternately, the USDA procedure recommends determining the deduced CFU (taking into account the dilution factor) for each dilution separately

and averaging the two estimates (9). Whichever method used should be documented and justified in the “Counting CFU” SOP.

What If All Plates Have Fewer Colonies Than the Minimum of the Countable Range?

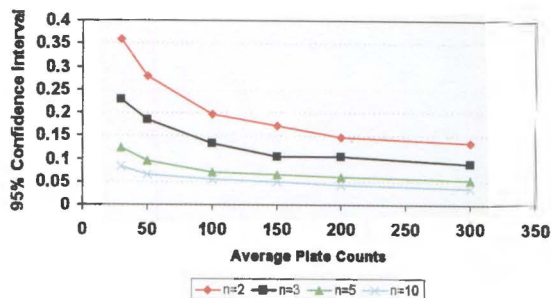
If the average of the plates is below the LOD, then report the result as less than the LOD. For example, plating out duplicates of 1 mL of a 1:10 dilution the LOD is 10 CFU/mL. If no colonies grew on the 1:10 dilution plates, or if one colony grew on one plate (average of 0.5 CFU/plate), this should be reported as <10 CFU/mL. The second situation is where the plates have colonies but less than the countable range. The FDA BAM recommends reporting this as <25 x Dilution Factor. In other words, if the best we had was a 1:1000 dilution with an average of 16 CFU/plate, this would be reported as <25,000. USDA recommends calculating the CFU as normal, but noting this as an estimate. While this particular situation may seem academic, it is one that has significance for determination of adherence to quality specifications.

QC Limits on Replicate Plate Counts

Periodically there are recommendations to establish quality control limits on replicate plate counts. Breed and Dotterer required valid plate counts from triplicate plates to provide estimates of CFU/mL within 20% of the mean (2). In other words, all plates were counted, and each plate’s CFU count was used to estimate the original CFU/mL. Each estimate was evaluated, and if the estimate for each plate was within 30% of the mean, it was deemed acceptable.

Establishment of QC limits for plate counts works best if you have at least three replicate plates for each dilution and you understand the relationship between accuracy and the number of CFU/plate. Weenk developed these considerations in a understandable discussion in relation to media growth promotion studies (10). His analysis assumed a 5.5% dilution error from pipetting and estimated the ability of parallel plating experiments (new media vs. standard) to distinguish between two populations. The results are shown in Figure 3 (recalculated from Weenk’s equations). This treatment clearly shows the effects of increasing the number of plates in replicate—increasing our ability to distinguish between similar populations. In a similar manner, we are able to distinguish smaller differences (the counting becomes more accurate) as the number of CFU/plate increases. Therefore, trying to establish a QC guideline for CFU/replicate (e.g., each replicate must be within 30% of the mean) may be problematic at lower CFU/plate counts. The method used to QC individual plate counts, if used, should be documented and justified in the SOP, along with the response to finding variant counts.

Figure 3: Accuracy as a function of CFU/Plate and number of replicate plates.



Can I Plate 10 x 1 mL Samples to Plate a Total of One 10 mL Sample?

There have been suggestions that a larger volume of material may be plated across several plates, and the results reported out for the larger volume (e.g., plating 10 x 1 mL samples on 10 different plates and then reporting it as if a 10 mL sample was plated). This approach is flawed in that it ignores several sources of variability in plating including sampling, growth, and counting errors (10, 11). The correct interpretation for this situation is that you have just plated 1 mL 10 times, not 10 mL once. The numbers might be averaged, but they cannot be added.

ROUNDING AND AVERAGING

The number of significant figures must be considered in a discussion of rounding and averaging. For raw colony counts, common practice determines that the CFU observed determine the significant figure, and that the average is one decimal to the right of that number. Sticklers for accuracy will report the geometric mean rather than the arithmetic mean given the Poisson distribution followed by CFU. In reporting, it is common practice to report as scientific notation using two significant figures. This requires rounding.

USP (12) and ASTM (7) both round up at five if five is the number to the right of the last significant figure. FDA BAM has a more elaborate scheme, rounding up if the number is six or higher and down if four or lower. If the number is five, BAM looks to the next number to the right and rounds up if it is odd and down if it is even.

This is one of those issues where you do want everyone to do these calculations the same way. Be sure to include direction and its justification in the “Counting CFU” SOP if it does not already exist in a separate SOP.

SPECIFICATIONS AND ENVIRONMENTAL MONITORING CONTROL LEVELS

If you are faced with a finished product bioburden of NMT (Not More Than) 100 CFU/gram, and your method suit-

ability study requires a 1:100 dilution of the product to overcome any antimicrobial effects, then how are you to test it? Common practice is to perform the 1:100 dilution and a pour plate of 1 mL in duplicate. If two colonies grow on each plate, the product fails specification. This common practice is scientifically unsupportable—it confused the LOD with the LOQ for the plate count method.

Let's take a look at environmental monitoring alert and action levels for aseptically produced products. Hussong and Madsen (13) published a thoughtful review of this topic where they argue that the levels of acceptable CFU for many room classifications are below the noise level plate count technology (e.g., in the range of 1-2 CFU/m³). In addition, environmental data are extremely variable, much more than controlled lab studies as the numbers of microorganisms, the physiological state of the isolates, even the species are completely out of the control of the investigator. In addition, the numbers do not conform to a normal distribution, as there are sporadic counts with a count of "zero" CFU predominating. They conclude that because the numbers are unreliable, the trend in the data is the only important consideration, and that EM counts cannot be used for product release criteria. A separate treatment of this subject was presented by Farrington (14), who argues that the relationship between environmental monitoring data and finished product quality is a widely held but unproven belief, compounded by the problems in accuracy with the low counts generated by plate count methodology.

CONCLUSIONS

In conclusion, all methods have limitations. One of the major limitations to the plate count method is the relatively narrow countable range (generally considered to be 25-250 CFU bacteria on a standard petri dish). The current prevailing confusion between the LOD (1 CFU) and LOQ (25 CFU) for the plate count method creates a larger degree of variability in microbiology data than is necessary. An unfortunate regulatory trend in recent years is to establish expectations (e.g., specifications, limits, levels) for data generated by the plate count method that the accuracy of the method cannot support. This is a real opportunity for modification of current practice to approach the goal of "science-based regulations."

REFERENCES

1. EP, "5.1.6 Alternative Methods for Control of Microbiological Quality," *European Pharmacopoeia*, Vol. 5.5 pp 4131-4142, 2006.
2. Breed, R and WD Dotterer, "The Number of Colonies Allowable On Satisfactory Agar Plates," *J Bacteriol.* 1:321-331, 1916.

3. Tomaszewicz, D.M. et al., "The Most Suitable Number of Colonies On Plates for Counting," *J Food Prot.* 43(4):282-286, 1980.
4. USP, "<61> Microbial Examination of Nonsterile Products: Microbial Enumeration Tests," *USP 34*, United States Pharmacopeia, pp. 52-56, 2011.
5. USP, "<55> Biological Indicators-Resistance Performance Tests," *USP 34*, United States Pharmacopeia, pp. 50-52, 2011.
6. USP, "<1227> Validation of Microbial Recovery from Pharmacopoeial Articles," *USP 34*, United States Pharmacopeia, pp. 783-786, 2011.
7. ASTM, D5465-93 (1998) *Standard Practice for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods*, 1998.
8. FDA, Maturin, LJ and JT Peeler, "Chapter 3: Aerobic Plate Count," *Bacteriological Analysis Manual*, <http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/Bacteriological-AnalyticalManualBAM/ucm063346.htm>, (accessed June 28, 2011)
9. USDA, Laboratory Guidebook MLG 3.01 Quantitative Analysis of Bacteria in Foods as Sanitary Indicators, 2011. http://www.fsis.usda.gov/PDF/MLG_3_01.pdf (accessed June 28, 2011).
10. Weenk, G.H., "Microbiological Assessment of Culture Media: Comparison and Statistical Evaluation of Methods," *Int J Food Microbiol.*, 17:159-181, 1992.
11. Jennison, MW and GP Wadsworth, "Evaluation of the Errors Involved In Estimating Bacterial Numbers by the Plating Method," *J Bacteriol.* 39:389-397, 1940.
12. USP, "General Notices: 7.20 Rounding Rules," *USP 34*, United States Pharmacopeia, p. 8, 2011.
13. Hussong, D and RE Madsen, Analysis of Environmental Microbiology Data From Cleanroom Samples, *Pharm Technol. Aseptic Proc Issue*: 10-15, 2004.
14. Farrington, JK., "Environmental Monitoring in Pharmaceutical Manufacturing—A Product Risk Issue," *Amer Pharm Rev.* 8(4):26-30, 2005. **JVT**

ARTICLE ACRONYM LISTING

BAM	Bacterial Analytical Manual
CFU	Colony Forming Units
FDA	US Food and Drug Administration
LOD	Limit of Detection
LOQ	Limit of Quantification
RSD	Relative Standard Deviation
SOP	Standard Operating Procedures
TAMC	Total Aerobic Microbial Count
TNTC	Too Numerous to Count
TYMC	Total Yeast and Mold Count
USDA	United States Department of Agriculture
USP	United States Pharmacopeia