

Protocol: Estimating bacterial loads from plating samples at different dilutions

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Overview

This protocol provides details on how you can estimate the bacterial load in samples based on data from plating those samples at different dilutions. The question being answered through this protocol of data analysis is: How much viable (i.e., replicating) bacteria are in each of your samples?

You can find out by **plating** the sample at different **dilutions** and counting the **colony-forming units (CFUs)** that are cultured on each plate. You put a sample on a plate with a medium they can grow on and then give them time to grow. The idea is that individual bacteria from the original sample end up randomly around the surface of the plate, and any that are viable (able to reproduce) will form a new colony that, after a while, you'll be able to see.

To count the number of colonies, you need a **“just right” dilution** (likely won't know what this is until after plating) to have a **countable plate**. If you have **too high** of a dilution (i.e., one with very few viable bacteria), randomness will play a big role in the CFU count, and you'll estimate the original with more variability. If you have **too low** of a dilution (i.e., one with lots of viable bacteria), it will be difficult to identify separate colonies, and they may compete for resources. (The pattern you see when the dilution is too low (i.e., too concentrated with bacteria) is called a *lawn*—colonies merge together). To translate from diluted concentration to original concentration, you can then do a back-calculation, incorporating both the number of colonies counted at that dilution and how dilute the sample was.

“Quantitative estimation of the number of viable microorganisms in bacteriological samples has been a mainstay of the microbiological laboratory for more than one-hundred years, since Koch first described the technique (Koch, 1883). Serial dilution techniques are routinely used in hospitals, public health, virology, immunology, microbiology, pharmaceutical industry, and food protection (American Public Health Association, 2005, Hollinger, 1993, Taswell, 1984, Lin and Stephenson, 1998) for microorganisms that can grow on bacteriological media and develop into colonies.” (Ben-David and Davidson 2014)

“The objective of the serial dilution method is to estimate the concentration (number of colonies, organisms, bacteria, or viruses) of an unknown sample by counting the number of colonies cultured from serial dilutions of the sample, and then back track the measured counts to the unknown concentration.” (Ben-David and Davidson 2014)

“Generally speaking, the methods which have been employed may be classified into (1) the direct and (2) the indirect. In the former the organisms are counted directly under the microscope, in the latter the number of bacteria present is calculated from an enumeration of the colonies which develop when an aliquot part of the emulsion in question is mixed with a nutrient medium in a Petri dish, and incubated for a variable period of time. The former is designed to record the total number of organisms present, the latter only the number which happens to be viable at the moment of sampling.” (Wilson 1922)

“The indirect or viable count has, as a rule, been performed by a modification of Koch's original plating method. . . . The modifications of Koch's method have been concerned with the medium used, the question of preliminary dilution, the methods of dilution and the exact technique of counting the plates. The majority of observers appear to have used agar, but [a few] seem to have

preferred gelatin, though in some cases both media were employed. With regard to preliminary dilution, the earlier workers generally preferred to plate out the original emulsion, while of late the tendency has been in the opposite direction The method of dilution has been subject to considerable variation; on the whole volumetric pipettes have been the most popular, but [some] used dropping pipettes, while [others] resorted to the use of a standard platinum loop. . . . The important question of the counting of the plates has naturally depended largely on whether or no a preliminary dilution of the emulsion was made. Where the number of colonies was very great, microscopic counting was adopted Where on the contrary, dilution was employed, the use of the microscope was no longer necessary, and counting was performed with the naked eye or with a magnifying glass . . . ” (Wilson 1922)

“In perusing the results of previous workers, it was striking to observe the peculiar lack of attention which was paid to the estimation of the experimental error involved in the methods employed. Probably this is to be attributed to the fact that in many cases in which the enumeration of bacteria was undertaken, a relative, rather than an absolute accuracy was essential. It was felt that the successful accomplishment of this object could only be obtained by working out a technique in which the errors inherent in every step should be known with certainty.” (Wilson 1922)

“The plate count method for estimating bacterial populations is satisfactory for many comparative purposes if *relative* rather than *absolute* numbers of cells are wanted, although in some cases, because of clumping, plate counts may not bear a constant relation to total counts even during the logarithmic growth phase (Jennison, 1937). This lack of agreement may be overcome, at least with some organisms, by proper shaking to break up clumps of cells (Ziegler and Halvorson, 1935).” (Jennison and Wadsworth 1940)

“A dilution assay is an experiment for estimating the concentration or frequency of target entities in a sample, in situations in which accurate counts of the organism are too difficult or costly to obtain. The original sample is divided into subsamples at lower concentrations by dilution. These subsamples may be further sampled to obtain replicate plates, tubes, or wells at each concentration level. Each replicate is then scored for the presence or absence of the target entity. This determination of positivity or negativity of each replicate may require an auxiliary test or procedure. For instance, the presence of bacteria may be deduced from the appearance of colonies after plates have been incubated for a time. . . . The idea of a dilution assay is to choose a sufficiently broad range of dilutions that a transition from positive to negative results is virtually ensured as one proceeds through the dilution sequence. The dilutions at which the transition occurs contain information on the concentrations of target entities in the original sample. We take estimation of this concentration as the primary purpose of the assay.” (Myers, McQuay, and Hollinger 1994)

Laboratory description

These data result from plating each sample at several different dilutions and counting the colony-forming units (CFUs) that are cultured on each plate. Each sample was on a plate with a medium they can grow on and then given [time] to grow. On each plate, the individual bacteria from the original sample should have ended up spread randomly around the surface of the plate, and any that are viable (able to reproduce) formed a colony that, by the end of the procedure, can be counted.

[Image of plating]

Each sample was taken from a single mouse from the experiment. These mice came from different experimental groups, with several replicates (mice) per experimental group. The experimental groups in this case were:

[table of experimental groups]

[Different type of mycobacteria] “The number of viable mycobacterial cells in each single-cell suspension was determined by standard plate counting as a reference method. Briefly, the undiluted stock cell suspension (1.0 ml) was added to 9.0 ml of 10 mM PBS (pH 7.2). Tenfold serial dilutions were made in 10 mM PBS (pH 7.2), with vortexing between each dilution step.

One hundred microliters from each dilution was plated onto each of three 7H10 agar plates supplemented with 10% oleic acid-albumin-dextrose-catalase and 2 microgram/ml of mycobactin J. Colony counts (CFU) were determined after the incubation of plates at 37 degrees C for 10 weeks. MGIT ParaTB tubes were inoculated in triplicate with 100 microliter of the same serial dilutions. To evaluate the effect of the MGIT tube inoculum volume, one set of tubes was inoculated with 100 microliter and another was inoculated with 1,000 microliters from each of the serial dilutions of multiple *M. paratuberculosis* strains.” (Shin et al. 2007)

Data description

Data are collected for this process into a spreadsheet that is set up in a “tidy” format used by our laboratory to collect this type of data. Here are the first few rows of the data:

```
## # A tibble: 6 x 4
##   group replicate dilution  CFUs
##   <dbl> <chr>      <dbl> <dbl>
## 1     2 2-A          0     26
## 2     2 2-C          0      0
## 3     3 3-A          0      0
## 4     3 3-C          0      0
## 5     4 4-A          0      0
## 6     4 4-B          0      0
```

Each row represents the number of bacterial colonies counted after plating a certain sample at a certain dilution. Columns are included with values for the experimental group of the sample (**group**), the specific ID of the sample within that experimental group (**replicate**, e.g., 2-A is mouse A in experimental group 2), the dilution level for that plating (**dilution**), and the number of bacterial colonies counted in that sample (CFUs).

Reading data into R

The data are stored in a comma-separated plain text file called “cfu_data.csv.” They can be read into R using the following code:

```
library(tidyverse)
cfu_data <- read_csv("cfu_data.csv")
head(cfu_data)
```

```
## # A tibble: 6 x 4
##   group replicate dilution  CFUs
##   <dbl> <chr>      <dbl> <dbl>
## 1     2 2-A          0     26
## 2     2 2-C          0      0
## 3     3 3-A          0      0
## 4     3 3-C          0      0
## 5     4 4-A          0      0
## 6     4 4-B          0      0
```

You will need to be sure that the data file (“cfu_data.csv” in this case) is in the working directory of your R session. You can check the current working directory in R with the call `getwd()`, and you can list all files in the current working directory with the call `list.files()`. These function calls can be useful to check to make sure that this file is in your working directory and, if not, to identify which directory on your computer file system you should move it to.

Once you run this command, the data will be available in your R session in the object `cfu_data`. You can see the first few rows by running:

```
head(cfu_data)
```

```
## # A tibble: 6 x 4
##   group replicate dilution  CFUs
##   <dbl> <chr>      <dbl> <dbl>
## 1     2 2-A         0     26
## 2     2 2-C         0      0
## 3     3 3-A         0      0
## 4     3 3-C         0      0
## 5     4 4-A         0      0
## 6     4 4-B         0      0
```

You can get a summary of the data by running:

```
summary(cfu_data)
```

```
##      group      replicate      dilution      CFUs
##  Min.   : 2.000   Length:89      Min.   :0.000   Min.   : 0.000
## 1st Qu.: 4.000   Class :character 1st Qu.:1.000   1st Qu.: 0.000
## Median : 5.000   Mode  :character Median :2.000   Median : 0.000
## Mean   : 5.933                      Mean   :1.539   Mean   : 1.944
## 3rd Qu.: 8.000                      3rd Qu.:3.000   3rd Qu.: 0.000
## Max.   :10.000                     Max.   :3.000   Max.   :52.000
```

Exploring the data and quality checks

It is helpful to explore the data once you read it in, to check for quality control issues and other characteristics of the data from a particular experiment. This section provides code examples for this quality control.

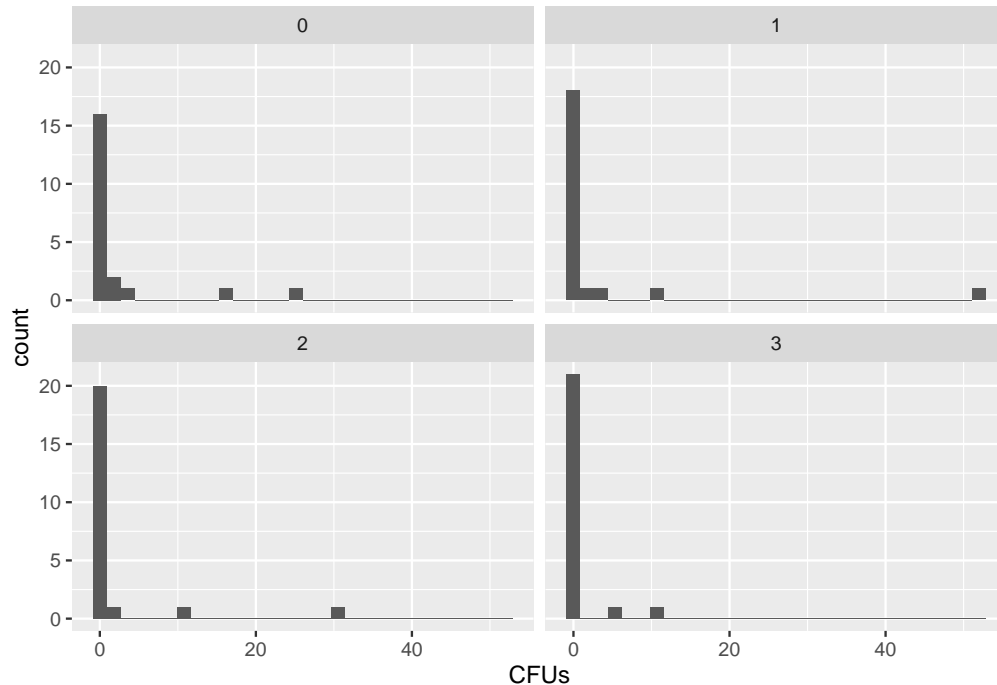
First, it can be helpful to ensure that the data includes the number of experimental groups, and the number of replicates within each group, that you are expecting. First, we can determine that there are eight experimental groups, with between eight and twelve samples (replicates) in each group:

```
cfu_data %>%
  group_by(group) %>%
  count()
```

```
## # A tibble: 8 x 2
## # Groups:   group [8]
##   group     n
##   <dbl> <int>
## 1     2    11
## 2     3    10
## 3     4    12
## 4     5    12
## 5     7    12
## 6     8    12
## 7     9    12
## 8    10     8
```

It is also helpful to see the distribution of CFUs at each dilution level, which can be plotted with the following code:

```
cfu_data %>%
  ggplot(aes(x = CFUs)) +
  geom_histogram() +
  facet_wrap(~ dilution)
```



In this case, most bacterial counts are zero at all dilution levels. However, there are more non-zero CFUs at dilution level 0 and more as you move to higher dilution levels. The highest CFU count at any dilution level is a little over 50, for dilution level 1. Most non-zero CFU counts are at or below 30, regardless of dilution level.

Identifying a good dilution for each sample

To count the number of colonies, you need a “good” dilution to have a **countable plate**. You won’t be able to pick a “good” dilution for each sample until you have plated several dilutions and see approximately how many colonies form on each. This is why a plating experiment will include plates for each sample at several dilution levels of the sample.

It is important to use a “good” dilution to estimate the bacterial load in the original sample, because there are challenges and pitfalls to trying to count CFUs if the dilution is too high or too low.

If you try to count for a plate that has too high of a dilution (i.e., the sample has been diluted a lot, and so has very few viable bacteria), randomness will play a big role in the CFU count, and you’ll estimate bacterial load in the original sample with more variability than desired. If you have too low of a dilution (i.e., one that has not been diluted much and has lots of viable bacteria), it will be difficult to identify separate colonies, and they may compete for resources. (The pattern you see when the dilution is too low (i.e., too concentrated with bacteria) is called a *lawn*—colonies merge together).

Typically, there will be some standard range of CFUs that are indicative of a plate having a “good” dilution for counting. For example, a laboratory group may specify that plates with between [x] and [x] CFUs are ideal for counting CFUs, and so use the dilution for each sample that provides a count in this range when estimating bacterial load in the original sample. The exact range used to identify plates with “good” dilutions may range from laboratory group to laboratory group. It may also depend on the type of bacteria being plated.

In this protocol, we are describing how to analyze data from experiments with *Mycobacterium tuberculosis*. This is a very slow-growing bacteria (Pathak et al. 2012), and so we typically identify “good” dilutions using a lower range of CFUs than other laboratories might when working with faster-growing bacteria. The trade-off, of course, is that there will likely be more variability in our bacterial load estimates, since they tend to be based on plates with lower average counts. However, we balance this limitation with the practical

limitation of how long it takes for this type of bacteria to grow, and so need to settle for using lower CFUs in some cases than might be ideal if working with faster-growing bacteria.

“Due to the slow growth rate and pathogenicity of mycobacteria, enumeration by traditional reference methods like colony counting is notoriously time-consuming, inconvenient and biohazardous.” (Pathak et al. 2012)

“Traditionally, quantification of mycobacteria is done by seeding serial dilutions of bacterial suspensions on suitable media such as Middlebrook 7H10 agar or Lowenstein Jensen followed by counting colony-forming units (CFU). However, this method is hampered by the long generation time and the tendency of mycobacteria to aggregate, resulting in multiple founders of a single colony and an underestimation of the correct number of bacteria. Typically, the time required for visible colonies to appear on 7H10 agar is 2–3 weeks for *M. tuberculosis* and *M. a. avium*, while it takes about 4–8 weeks for *M. a. paratuberculosis*. In addition, plating enough dilutions to make sure the results can be reliably counted is a tedious task that gives piles of plates with biohazardous bacteria. A further disadvantage of the colony counting method is that it cannot be reliably conducted on frozen samples, which may be both more practical and desirable in several research settings.” (Pathak et al. 2012)

“*Mycobacterium tuberculosis* culture, a critical technique for routine diagnosis of tuberculosis, takes more than two weeks.” (Ghodbane, Raoult, and Drancourt 2014)

“A major problem when dealing with tuberculosis has been a difficulty in diagnosis due to slow growth of mycobacterial cultures, which subsequently explains the slow process of evaluating the susceptibility of this microorganism to antibiotics. Using current tools, a primary culture is obtained in two to four weeks on average and antibiotic susceptibility is determined after an additional two to four weeks. Therefore, four to eight weeks are needed to obtain an isolate and determine its susceptibility to antibiotics.” (Ghodbane, Raoult, and Drancourt 2014)

“Quantification of viable bacteria is a crucial foundation for many types of research. This seemingly simple task can be challenging, expensive, and imprecise for *Mycobacterium paratuberculosis*, a slowly growing organism (>24-h generation time) with a strong tendency to form large clumps. Studies of environmental survival, resistance to pasteurization or disinfectants, and quantification of the pathogen in milk and feces from infected animals are just a few examples that require precise and sensitive quantification of viable *M. paratuberculosis* cells.” (Shin et al. 2007)

“The importance of serial dilution and colony counting is reflected by the number of standard operating procedures and regulatory guidelines describing this methodology. In all of these guidelines the optimal number (\hat{n}_j) of colonies to be counted has been reported (Park and Williams, 1905, Wilson, 1922, Jennison and Wadsworth, 1940, Tomasiewicz et al., 1980, FDA, 2001, Goldman and Green, 2008) as 40–400, 200–400, 100–400, 25–250, 30–300. It is interesting to note that these references do not specify the area in which the colonies grow, nor the diameter of the particular organism assayed. The result is that titration and counting colonies is done within a range that may be inadequate, and may introduce considerable error.” (Ben-David and Davidson 2014)

“The main challenge in serial dilution experiments is the estimation of the undiluted microorganisms counts n_0 from the measured \hat{n}_j . There are two competing processes (Tomasiewicz et al., 1980) that affect the accuracy of the estimation: sampling errors and counting errors. Sampling errors are caused by the statistical fluctuations of the population. For example, when sampling an average of 100 colonies, the fluctuations in the number of the population are expected to be $\pm\sqrt{100}$ when the sampling process is governed by a Poisson probability (Poisson and Binomial distributions are often used in statistical analysis to describe the dilution process (Hedges, 2002, Myers et al., 1994)) where the standard deviation equals square-root of the mean; the relative error (ratio of the standard deviation to the mean) is $\sqrt{100}/100 = 0.1$. Thus, the larger the sample size is, the smaller the relative sampling error; hence, one would like to use a dilution plate with the largest number (i.e., the least diluted sample, $j \rightarrow 1$). However, as the number of colonies

increases, counting error is introduced due to the high probability of two (or more) colonies to merge (due to overcrowding) and become indistinguishable, and be erroneously counted as one colony. An optimum (a ‘sweet spot’) between these two processes (sampling and counting error) needs to be found for using the optimal dilution (i.e., the optimal j th plate) with which to estimate n_0 . Cells can grow into colonies in various ways. Wilson (1922) states that when two cells are placed very close together only one cell will develop, and when two cells are situated at a distance from each other both cells may grow and then fuse into one colony. Either way, the end result is the appearance of one colony which causes counting error.” (Ben-David and Davidson 2014)

“It is clear that if three tubes are put up from an emulsion containing a comparatively small number of bacilli, the chances of obtaining a representative sample must be smaller than if an emulsion be employed which contains a much larger number of bacilli. Similarly with the tubes themselves. If only a few bacilli are introduced, the chances of obtaining a correct idea of the exact number are smaller than if a large number of bacilli are introduced. Thus the greater the number of colonies per tube, the less is the error of sampling. That this is not a mere theoretical consideration is shown from an examination of the data accumulated during the progress of this work. . . the percentage deviation of each individual tube from the arithmetic mean was considerably greater in the case when a small number of colonies developed than in the case when a large number of bacilli were inoculated. In other words the sampling error in the former instance was large, in the latter comparatively small.” (Ben-David and Davidson 2014)

“We now come to consider the second factor determining the optimum number of bacilli to be inoculated in putting up viable counts by the tube method, namely the error of overcrowding. More or less in proportion as the error of sampling decreases as the number of developing colonies increases, so the error of overcrowding increases as the number of developing colonies increases. The two vary in opposite directions; the greater the number of colonies the less the sampling error; the fewer the number of colonies, the less is the overcrowding error. A point must be chosen between the two which will permit of the minimum combined error experienced. Before this could be done, however, it was necessary to ascertain the actual effect of overcrowding on the development of colonies in tube preparations. As mentioned above, this overcrowding error is one which seems to have been neglected by the majority of observers, or at any rate, not clearly recognized. It is obvious that the greater the number of bacilli distributed in a given space, the less is the interval between each of them, and the greater the chance of two being coincident. In every case in which two bacilli are coincident or are placed very close together only one colony will develop. Further, when two bacilli are situated at such a distance from each other that each is able to develop, yet at such a distance that continued development of both will result in fusion, it is clear that a simple colony must arise. Whether one continues to grow and the other desists or whether both develop, the result is the same—namely, the appearance of one colony in place of two bacilli. On pure a priori grounds one would expect this overcrowding factor to be of considerable importance in determining the number of colonies which will develop in a given space. One would expect it to play but a small part so long as comparatively few bacilli were inoculated, but as the number of the latter increased so should the percentage which fails to develop into colonies become greater.” (Ben-David and Davidson 2014)

“Considering the plating method per se, the total error of the mean plate count of a given dilution of cells is chiefly made up of two rather distinct sources of deviations: (a) the distribution or sampling error, sometimes inaccurately called the counting error, (i.e., variation in number of colonies, due to sampling, between replicate plates of the given dilution), and (b), the dilution error, (i.e., the errors of pipetting involved in reaching the given dilution). . . . It is customary to measure the reliability of the plate count by calculating only the distribution error, and assuming that the dilution error is small, constant, and unimportant. We shall show, however, that at best this dilution error is of about the same order of magnitude as the distribution error, and is, therefore, equally deserving of consideration in arriving at the total error of plate counts. Furthermore, the dilution error increases with higher dilutions, whereas the distribution error does not. Obviously, one must take into account both sources of variation in evaluating the total

error, as, for example, in a problem involving significance of differences, in which the same dilution might be employed.” (Jennison and Wadsworth 1940)

“The size of the distribution error depends upon the number of replicate plates counted, within limits, other conditions being the same. To obtain a small distribution error, a dilution giving the proper number of colonies per plate (100–400) for enumeration must be available, in order to minimise errors of overcrowding and sampling (Wilson, 1922), and a sufficient number of replicate plates (3–5) to give a precise mean must be used (Wilson and Kullman, 1931).” (Jennison and Wadsworth 1940)

“So far as evaluating the distribution error is concerned, this is usually done by calculating the standard deviation of the mean (standard error) of the replicate plates, assuming that the variation between such plates is that of random samples. Under good experimental conditions, this coefficient of variation will average ± 4 –5 per cent (Jennison, 1937). In order to test whether observed variations between replicate plates are due to chance or to technique, the χ^2 (‘chi square’) test may be used (Wilson and Kullmann, 1931). The calculated value of χ^2 will be distributed in a known manner if the replicate samples are from a Poisson series, that is, if their variation is that of random samples from the same population. Fisher, Thornton, and MacKenize (1922), and Fisher (1938), have shown that a Poisson distribution is obtained in parallel plate counts made under standardized experimental conditions. Both the χ^2 test and calculation of the standard error of replicate plates apply only to a given dilution; they do not account for errors involved in arriving at that dilution.” (Jennison and Wadsworth 1940)

“The size of the dilution error will depend upon errors in volume of dilution blanks, the variation in delivery of pipettes, and upon the number of dilutions made. We shall assume that dilutions are made in powers of 10, using 9 mL and 99 mL dilution blanks and 1 mL pipettes. With the same percentage error in 99 mL and 9 mL blanks it is, of course, better to use the former in preference to the latter, since fewer are required to reach a given dilution. The average error in volume of blanks, and in delivery of pipettes under experimental conditions must be known.” (Jennison and Wadsworth 1940)

“Almost every textbook, laboratory manual and methods volume in microbiology contains the statement that plates for counting bacteria should contain, when possible 30–300 colonies. The ‘30–300’ concept has been so ingrained in our thinking that the limits are rarely questioned. . . . The ‘30–300’ concept originated with two publications by Breed and Dotterer (7,8); the text and tables presented in both publications are identical. The authors summarized results of a few early studies and then proceeded to more clearly define the problem and provide a solution.” (Tomasiewicz et al. 1980)

“Plates with over 500 colonies under-estimate the true count owing to the overcrowding error. With careful workers the actual error of counting probably does not become appreciable till there are about 300 colonies per plate, and for some distance above this limit it will probably be counterbalanced by the diminished sampling error. If many places, however, have to be counted, the fatigue error, which seems to be mainly responsible for the failure of the sampling error to decrease with increasing numbers of colonies in accordance with theoretical expectations, becomes appreciable.” (Wilson and others 1935)

“For automated equipment (10), the optimum counting range may well vary with the instrument, particle (colony) size limits, range of colony sizes, etc. 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, maxima of from 25–100 colonies per plate have been suggested (17). Coliform analyses demand another range (24).” (Tomasiewicz et al. 1980)

Estimating bacterial load for each sample

“Given an unknown sample which contains n_0 colony forming units (CFUs), a series of J dilutions are made sequentially each with a dilution factor α . From each of the J dilutions a fraction α_p^{-1} is taken and spread (plated) on an agar plate (assay) where colonies are counted. Thus, in general there are two dilution factors: α and α_p . For example, $\alpha = 10$ indicates a 10-fold dilution, e.g., by diluting successively 0.1 ml of sample into 0.9 ml of media; and $\alpha_p = 1$ means that the entire volume is spread (plated) on the agar plate. For an experiment with a larger dilution factor α_p , multiple plates may be spread at the same dilution stage. For example, $\alpha_p = 20$ represent a 5% plating of the dilution, and thus up to 20 replicates could be created. At each dilution the true number of colonies is $n_j = n_0 \alpha^{-j} \alpha_p^{-1}$ and the estimated number is \hat{n}_j . The estimated quantities are denoted with a ‘hat’ (estimated quantities can be measured quantities, or quantities that are derived from measured or sampled quantities); symbols without a ‘hat’ denote true quantities (also known as population values in statistics) that do not contain any sampling or measurement error. In this work both n_j and n_0 are ‘counts,’ i.e., number of colonies. Knowing the aliquot volume, one can easily convert counts to concentration (for example CFU/ml).” (Ben-David and Davidson 2014)

“A simple method to estimate the number of colonies n_0 in the unknown sample from the counted number of colonies at the j th assay is presented. Our method is easy to implement. The method selects the optimal count (i.e., a best single plate) with which n_0 is estimated. There are only a few inputs needed: the incubation plate size, the microbial colony size, and the dilution factors (α and α_p).” (Ben-David and Davidson 2014)

Outputting final estimates

References

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