Protocol: Estimating bacterial loads from plating samples at different dilutions

3/1/2021

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 complete 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.

> "The most widely used method for determining drug efficacy [for TB] in mice remains enumeration of the bacterial load in lungs and spleens, by counting the colony forming units (CFU) of the organ homogenates on agar plates." (Franzblau et al. 2012)

> "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 ot 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 transistion from positive to negative results is virtually ensured as one proceeds through the dilution sequence. The dilutions at which the transition occurse 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)

"The plate count method is based on viable cell counts. The plate count method is performed by diluting the original sample in serial dilution tubes, followed by the plating of aliquots of the prepared serial dilutions into appropriate plate count agar plates by the pour plate or spread plate technique. The pour plate technique utilizes tempered molton plate count agar poured into the respective plate and mixed with the diluted aliquot sample in the plate, whereas the spread plate technique utilizes the addition and spreading of the diluted aliquot sample on the surface of the preformed solid plate count agar in the respective plant. ... These prepared plate count agar plates are then optimally incubated, and the colonies observed on these plate count agar plates are then counted as the number of CFUs. The counting of CFUs assumes that every colony is separate and founded by a single viable microbial cell. The total colony counts obtained in CFUs from the incubated agar plates and the respective dilution factor used can then be combined to calculate the original number of microorganisms in the sample in CFUs per mL. The typical counting ranges are 20–250 CFUs or 30–300 CFUs per standard plate count agar plate. Additional

considerations for counting CFUs are counting of plate spreaders, too numerous to count (TNTC) reporting and statistics, rounding and averaging of observed plate counts, limit of detection, and limit of quantification of plate countes (77–79). There are also optimal condition assumptions for the plate count method as changes to the plate count agar nutrient level or temperature can affect the surface growth of bacteria (80, 81). Primary equipment and materials used for this method are serial dilution tubes (bottles); Petri plates or dishes; pipettes; specific growth medium, diluents, and reagents; incubator and water bath with appropriate optimal temperature setting; commercial colony counter (manual or automate); and plate spreader or rod. Total bacteria and fungi can be enumerated separately using the plate count method based on the type of culture medium utilized (82–86). Specific or selective culture medium can also be used in place of the standard plate count agar media for more specific microbial enumeration (87). Sources of error using this method are improper or inadequate preparation of the test samples, serial dilution error, suboptimal incubation conditions, undercounting due to cell aggregation or clumping, and analyst error in the colony counting or calculation of observed results." (Goldman and Green 2015)

"The plate count method is primarily used in the enumeration of samples with high microorganism numbers of microorganisms that do not grow well in liquid media. Plate count methods are used in the areas of food, pharmaceutical, environmental including drinking water applications, and biofilm testing. Modified or alternative versions of the plate count methods have also been developed to further enhance the use of the standard plate count method approach to estimate bacteria or fungi by utilizing the roll tube method, drop plate method, spiral plate count method, Petrifilm, SimPlate, replicate organism detection and counting (RODAC) plate for environmental surface sampling, dip slide or dipstick paddle method, and adhesive sheet method." (Goldman and Green 2015)

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. For each animal, the spleen was homogenized in phosphate buffer saline (PBS). This homogenate was plated on 7H11 agar plates at four serial dilutions, from undiluted to a dilution of 5^{-3}. The plates were incubated for 3–5 weeks at 37^oC, and then colony-forming units (CFUs) were counted 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]

The CFU solution, in each case, was resuspended in 0.5 milliliters [of . . .]. For each plate, 100 microliters were plated. Half of the spleen was used for each sample.

The dilution factor in this experiment is 5 (i.e., a five-fold dilution from each dilution level to the next).

[Plating in quarters?]

[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)

"Mtb is a member of the slow-growing pathogenic mycobacterial species, characterized by a 12- to 24-hour division rate and prolonged culture period on agar of up to 21 days. Why Mtb grows so slowly is not well understood. Proposed mechanisms include limitation of nutrient uptake through the highly impermeable cell wall and slow rates of RNA synthesis (96). During experimental infections, its metabolism can shift from an aerobic, carbohydrate-metabolizing mode to one that is microaerophilic and lipid metabolizing (25). Mycobacteria are facultative intracellular bacteria that multiply within phagocytic cells, particularly macrophages and monocytes. Although many mycobacterial species are environmental, Mtb is strictly parasitic." (Sakamoto 2012)

"For the testing of single compounds as well as short term mouse experiments, some investigators mentioned plating whole lungs while others plate the homogenate of a single lung lobe from each animal. After long treatment regimens when low bacterial numbers are expected, generally the whole lung is homogenized and a sizeable fraction of up to one-half of the homogenate is plated. One group homogenizes whole lungs in a total of 2.5 mL PBS and then plates the entire homogenate on five 7H11 plates (0.5 mL per plate). Another group uses whole lungs and total spleen homogenized in 4 mL PBS supplemented with 0.05% Triton X-100." (Franzblau et al. 2012)

"Several issues regarding the endpoints and the mouse models themselves were discussed. Regarding accurate endpoints in treatment trials, the method used for enumerating bacteria from organ homogenates should be carefully (re)considered given the possibility that some non-culturable bacteria in samples of animal tissues may not form colonies on solid agar. Liquid media, such as BACTEC or MGIT, was suggested as a method to enhance sensitivity for finding low numbers of CFU and to gain some insight into the state of the bacillus at the time point examined. Liquid testing using the MGIT system is also now the method of choice for sputum evaluation to assess culture conversion for clinical trials. Several automated liquid culture systems have shown greater sensitivity than the traditional solid-media cultures the acknowledged increased in the mycobacterial recovery rate of liquid media, which is likely due to a more mycobacterial populations being able to recover in liquid culture than on solid media. This is supported by in vivo and in vitro observations that subpopulations of M. tuberculosis with different states of metabolic activity co-exist in old liquid cultures, as well as in liquid cultures with bacterial growth from chronic infected mice that do not grow on solid media." (Franzblau et al. 2012)

"Another topic of discussion in at least two laboratories was the issue of drug carry-over at the time of enumeration of bacterial colonies in the organs. CLF and TMC-207 both have long half-lives, high tissue distribution and tissue binding, and therefore drug might still be present at the time of sacrificing the animals. The first indication that drug carry-over is an issue is observed when dilutions of organ homogenates do not have the expected reduction in bacterial number. Another indication might also be the lack of correlation between the bacterial number and the gross pathology observation. Several methods have been described to reduce the carry-over of drug in agar plates, including using LJ medium or 7H11 with 5% bovine serum albumin (BSA) for TMC-207, and using 0.4% activated charcoal for CLF. Relapse studies will then show the true sterilizing potential of these drugs and drug combinations." (Franzblau et al. 2012)

"It was pointed out that colony forming units (CFU) measures by plating organ homogenates on solid agar are often inaccurate since they do not necessarity include just single bacilli but rather small clumps or conglomerations. There was discussion around the hypothesis that these clusters represent a type of biofilm. Hatfull et al. found these M. tuberculosis biofilms in vitro to consist of bacteria surrounded by a layer of free mycolic acids. Although the biofilm hypothesis was not accepted by everyone, it was agreed that the extracellular bacteria in these micro-environments

are unique and should be treated by drug treatment. Another topic of discussion was about the drug refractory nature of these persisting bacilli being a drug penetration issue, by drugs not getting through this fibrous rim and extracellular lipid matrix. The ability to determine the penetration of TB drugs into a granuloma was seen as an important gap in the current knowledge about TB drugs." (Franzblau et al. 2012)

"The activity of an investigational drug or regimen has been in recent years mostly determined by the reduction of colony forming units (CFU) of M. tuberculosis by dilution of organ homogenates on solid agar 7H11 plates. Plating of the organ homogenates has always been the gold standard for quantifying drug efficacy in vivo. However, drug discovery efforts are often times held up by this time consuming step requiring an incubation period of the bacterial plates of 3–4 weeks. For early drug discovery efforts, more efforts are being investigated lately for indirect, but more rapid, methods to 'measure' the bacterial load: such as the luciferase readout or fluorescence. These novel detection methods will undoubtedly accelerate the TB drug discovery process by delivering an immediate readout on the efficacy of an experimental compound at either time of sacrifice (for luciferase readout) and even in liveanimals in real time (fluorescence). Thorough validation, however, is not available as of yet and would be required before these indirect methods can replace the enumeration by CFU for all drug classes." (Franzblau et al. 2012)

"Another discussion took place at several occasions during this project, pointing out that perhaps not all bacteria can be identified, cultured or visualized by current methods. The discussion started with the recent introduction of liquid culture media for the diagnostic evaluation of clinical specimens for suspected tuberculosis. The question then became whether drugs or regimens which preferentially kill certain bacillary populations would give rise to a 'flawed' readout by only culturing certain subsets of bacteria on solid agar. If liquid cultures of organ homogenates allowthe growth of TB subpopulations that will not grow on solid media, the parallel evaluation in liquid and in solid cultures might offer an opportunity to study drug effects on subpopulations in different metabolic states. ... This question might even be more relevant in animal models with a greater variety in granulomatous lesion types where bacilli are located in different environments (such as in necrotic, closed or cavitary lesions) and hence might have different metabolic stages." (Franzblau et al. 2012)

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`).

In cases where the bacteria were too numerous to count, that dilution level is excluded from the data.

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

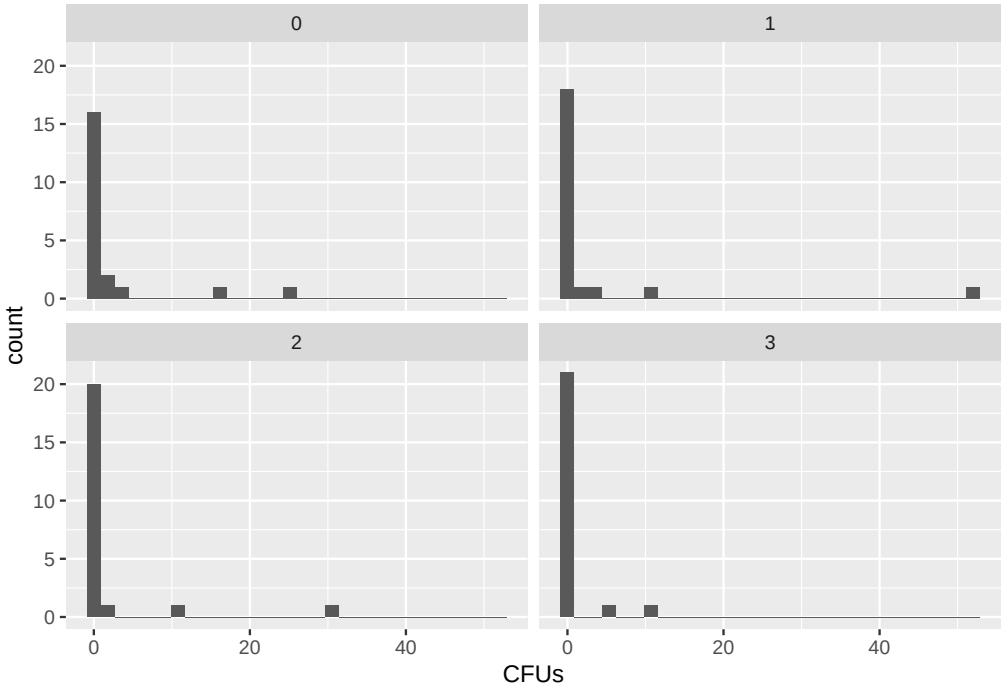
groups, with between two and three samples (replicates) in each group:

```
cfu_data %>%
  group_by(group) %>%
  summarize(n_replicates = length(unique(replicate)))
```

```
## # A tibble: 8 x 2
##   group n_replicates
## * <dbl>        <int>
## 1     2            3
## 2     3            3
## 3     4            3
## 4     5            3
## 5     7            3
## 6     8            3
## 7     9            3
## 8    10            2
```

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

In this experiment, we plated at dilution levels of 0, 1, 3, and 3, with a dilution factor of 5. In other words, we have the following dilutions for each dilution level:

Dilution level	Dilution at that level
0	10^{0}
1	$10^{-0.5}$
2	10^{-1}
3	$10^{-1.5}$

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 complete 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.

[Quadrant plating also resulting in a lower ideal count?]

We used the following criteria to pick the best dilution in each sample to use to back-calculate the bacterial load in the original sample:

1. If CFU counts at all dilutions were 0, use the lowest dilution (level 0 dilution); and
2. If some of the dilutions gave non-zero CFU counts, use the dilution with a CFU count closest to 25; and
3. If there are ties in terms of which dilution gave a CFU closest to 25, take the plate with the lowest dilution level of those tied.

Here is the code we used to identify the best dilution level for each sample, based on these criteria:

```
cfu_data <- cfu_data %>%
  # For each dilution, calculate how far the CFUs counted on the plate are
  # from 25.
  mutate(diff_from_25 = abs(CFUs - 25)) %>%
```

```
  # For each original sample (ID-ed by the 'replicate' column), determine
  # first if CFUs are 0 at all dilutions and second which dilution
  # had a CFU count closes to 25. Finally, include a check to see if there
  # are non-zero ties for the sample, in terms of plates with non-zero CFU counts
  # equally close to 25.
  group_by(replicate) %>%
  mutate(all_zeros = all(CFUs == 0),
         closest_to_25 = dilution[which.min(diff_from_25)],
         ties_closest_to_25 = sum(diff_from_25 == min(diff_from_25)) > 1)
```

You can check some samples in these data to make sure it's identifying the right dilution level. First, here is a sample where all CFUs are zero (replicate 3-A). The code has correctly identified that the CFUs for these samples are all zeros, marking `TRUE` in the `all_zeros` column.

```
cfu_data %>%
  filter(replicate == "3-A")
```

```
## # A tibble: 4 x 8
## # Groups:   replicate [1]
##   group replicate dilution  CFUs diff_from_25 all_zeros closest_to_25
##   <dbl> <chr>        <dbl> <dbl>        <dbl> <lgl>             <dbl>
## 1     3 3-A              0     0           25 TRUE                  0
## 2     3 3-A              1     0           25 TRUE                  0
## 3     3 3-A              2     0           25 TRUE                  0
## 4     3 3-A              3     0           25 TRUE                  0
## # ... with 1 more variable: ties_closest_to_25 <lgl>
```

Here is an example where some dilutions resulted in non-zero CFU counts, from replicate 2-B. In this case, there were non-zero CFU counts at dilution levels 1, 2, and 3. The closest to 25 was for dilution level 2, since $|25 - 10| = 15$, which is lower than $|25 - 52| = 27$ or $|25 - 5| = 20$.

```
cfu_data %>%
  filter(replicate == "2-B")
```

```
## # A tibble: 3 x 8
## # Groups:   replicate [1]
##   group replicate dilution  CFUs diff_from_25 all_zeros closest_to_25
##   <dbl> <chr>        <dbl> <dbl>        <dbl> <lgl>             <dbl>
## 1     2 2-B              1    52           27 FALSE                 2
## 2     2 2-B              2    10           15 FALSE                 2
## 3     2 2-B              3     5           20 FALSE                 2
## # ... with 1 more variable: ties_closest_to_25 <lgl>
```

As a quality check, we can see if there are any samples where there were non-zero ties in terms of which dilution level gave a CFU count closest to 25:

```
cfu_data %>%
  filter(ties_closest_to_25 & !all_zeros)
```

```
## # A tibble: 0 x 8
## # Groups:   replicate [0]
## # ... with 8 variables: group <dbl>, replicate <chr>, dilution <dbl>,
## #   CFUs <dbl>, diff_from_25 <dbl>, all_zeros <lgl>, closest_to_25 <dbl>,
## #   ties_closest_to_25 <lgl>
```

These results show that there are no samples where there are non-zero ties in terms of which dilutions gave CFU counts closest to 25, so in this case, that is not a criteria we need to consider further in identifying the

best dilution level for each sample.

Now that we've identified the best dilution level for each replicate, we can reduce the data, creating a new dataset with only a single row for each sample. Each sample will have a row that provides the best dilution level for that sample and the CFU count at that dilution level:

```
cfu_data_reduced <- cfu_data %>%
  # Limit to the row with the best dilution for each sample
  filter(dilution == closest_to_25) %>%
  # Remove the grouping tag applied in the code earlier
  ungroup() %>%
  # Limit to just the columns of data we need for further steps
  select(group:CFUs)

# Print this reduced data out as a table
cfu_data_reduced %>%
  knitr::kable()
```

group	replicate	dilution	CFUs
2	2-A	0	26
2	2-C	0	0
3	3-A	0	0
3	3-C	0	0
4	4-A	0	0
4	4-B	0	0
4	4-C	0	0
5	5-A	0	0
5	5-B	0	0
5	5-C	0	0
7	7-B	0	0
7	7-C	0	0
8	8-A	0	17
8	8-B	0	0
8	8-C	0	4
9	9-A	0	0
9	9-B	0	0
9	9-C	0	1
10	10-A	0	0
10	10-B	0	0
7	7-A	1	2
2	2-B	2	10
3	3-B	2	30

For this experiment, many of the samples had 0 CFUs at all dilution levels. For the samples with non-zero CFU counts, the best dilutions were often at dilution level 0 (i.e., undiluted sample), although in a few cases the best dilutions for samples were at dilution levels of 1 ($10-0.5$ dilution) or 2 (10^{-1} dilution). The CFU counts at the best dilution levels were, for non-zero CFU samples, between 1 and 30. We may want to keep in mind that the sampling error is likely pretty high for the CFU counts that are lower than about 10 in these samples.

> "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, $j \to 1$). 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 jth 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 proporation 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 colculation 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 Dotterrer (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 1935)

"For automated equipment (10), the optiumum 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

As a reminder, here are some of the experimental parameters used in this experimental that will be needed to back-calculate estimates of bacterial load in the original samples:

Experimental parameter	Value in this experiment
Dilution factor	0.5
Volume used to resuspend CFU solution	0.5 mL
Volume plated	100 μL
Proportion of organ used in sample	0.5

We can load these parameter values into R so they're available in the following calculations:

```
dilution_factor <- 0.5      # So a dilution level of 2 corresponds to a 10^-1 dilution
volume_resuspend <- 0.5     # in milliliters
volume_plated <- 100        # in microliters
prop_organ_in_sample <- 0.5 # Half the spleen was included in the sample
```

First, we can calculate the actual dilution for each sample by taking the dilution factor and raising it to the power of the dilution level. For example, the dilution at level 2 should be $0.5^2 = 0.25$, while the dilution at level 0 (undiluted) should be $0.5^0 = 1$).

```
cfu_data_reduced <- cfu_data_reduced %>%
  mutate(dilution_value = dilution_factor^dilution)

cfu_data_reduced
```

```
## # A tibble: 23 x 5
##    group replicate dilution  CFUs dilution_value
##    <dbl> <chr>        <dbl> <dbl>          <dbl>
##  1     2 2-A              0    26              1
##  2     2 2-C              0     0              1
##  3     3 3-A              0     0              1
##  4     3 3-C              0     0              1
##  5     4 4-A              0     0              1
##  6     4 4-B              0     0              1
##  7     4 4-C              0     0              1
##  8     5 5-A              0     0              1
##  9     5 5-B              0     0              1
## 10     5 5-C              0     0              1
## # ... with 13 more rows
```

[bacterial load per mL]

Next, we calculated the bacterial load in the original sample. To do this, we multiplied the dilution value, calculated in the previous step, by the CFU count at that dilution, as well as by the resuspension volume and divided by the volume plated, adding a multiplicative factor of 1000 to account for the difference in units between those experimental parameters (milliliters and microliters, respectively):

```
cfu_data_reduced <- cfu_data_reduced %>%
  mutate(bact_load_in_sample = CFUs *
           dilution_value * volume_resuspend * 1000 /
           volume_plated)
cfu_data_reduced
```

```
## # A tibble: 23 x 6
##    group replicate dilution  CFUs dilution_value bact_load_in_sample
##    <dbl> <chr>        <dbl> <dbl>          <dbl>                <dbl>
##  1     2 2-A              0    26              1                  130
##  2     2 2-C              0     0              1                    0
##  3     3 3-A              0     0              1                    0
##  4     3 3-C              0     0              1                    0
##  5     4 4-A              0     0              1                    0
##  6     4 4-B              0     0              1                    0
##  7     4 4-C              0     0              1                    0
##  8     5 5-A              0     0              1                    0
##  9     5 5-B              0     0              1                    0
## 10     5 5-C              0     0              1                    0
```

```
## # ... with 13 more rows
```

Finally, to get an estimate of the bacterial load in the full organ, we can back-calculate based on the proportion of the organ that was included in the original sample. In this experiment, we used half of the spleen, so the estimated bacterial load in the full organ should be approximately twice that estimated in the sample (since $1/0.5 = 2$). We calculated this in R:

```
cfu_data_reduced <- cfu_data_reduced %>%
  mutate(bact_load_in_organ = bact_load_in_sample *
           (1 / prop_organ_in_sample))
cfu_data_reduced
```

```
## # A tibble: 23 x 7
##    group replicate dilution  CFUs dilution_value bact_load_in_sample
##    <dbl> <chr>        <dbl> <dbl>          <dbl>                <dbl>
##  1     2 2-A              0    26              1                  130
##  2     2 2-C              0     0              1                    0
##  3     3 3-A              0     0              1                    0
##  4     3 3-C              0     0              1                    0
##  5     4 4-A              0     0              1                    0
##  6     4 4-B              0     0              1                    0
##  7     4 4-C              0     0              1                    0
##  8     5 5-A              0     0              1                    0
##  9     5 5-B              0     0              1                    0
## 10     5 5-C              0     0              1                    0
## # ... with 13 more rows, and 1 more variable: bact_load_in_organ <dbl>
```

We have also included a column with log(CFUs), since these are often used in further data analysis for this type of experiment. This is because ... . We used log_{10}, to be consistent with conventions in the field (the default `log` function in R takes the natural log, so this required using the `log10` function).

```
cfu_data_reduced <- cfu_data_reduced %>%
  mutate(log_cfus_in_organ = log10(bact_load_in_organ))

cfu_data_reduced
```

```
## # A tibble: 23 x 8
##    group replicate dilution  CFUs dilution_value bact_load_in_sample
##    <dbl> <chr>        <dbl> <dbl>          <dbl>                <dbl>
##  1     2 2-A              0    26              1                  130
##  2     2 2-C              0     0              1                    0
##  3     3 3-A              0     0              1                    0
##  4     3 3-C              0     0              1                    0
##  5     4 4-A              0     0              1                    0
##  6     4 4-B              0     0              1                    0
##  7     4 4-C              0     0              1                    0
##  8     5 5-A              0     0              1                    0
##  9     5 5-B              0     0              1                    0
## 10     5 5-C              0     0              1                    0
## # ... with 13 more rows, and 2 more variables: bact_load_in_organ <dbl>,
## #   log_cfus_in_organ <dbl>
```

> "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 jth 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)

Some of the colony-forming units may have resulted from more than one bacterial cell (e.g., a clump of bacteria) being plated at that spot on the plate.

This technique tends to be more suitable for estimating the relative size of bacterial loads, for example, to compare across several samples, rather than the absolute bacterial load in a sample.

Final output

This protocol describes the pre-processing of data collected through a serial dilution viable plate count for each sample from our experiment. Our input was the CFUs counted for each plate at each of four dilution levels. Our output is an estimate of the bacterial load in the spleen from each sample in the experiment.

Our final data includes a row for each of the samples in the experiment—in this case, each sample represents one animal, and there were several [two or more?] animals (replicates) for each of [x] experimental groups. For each sample, our final data provides a single CFU estimate, representing our best estimate of the bacterial load in the whole spleen of that animal.

"All respondents [from labs working on tuberculosis] use statistical methods in conjunction with their animal trials and most do not consult with a statistician. Treatment effects are mostly analyzed by a one-way ANOVA, followed by a T-test, Tukey or Dunnett's test. For relapse studies a Fisher Exact or Chi-square test is applied to compare relapse proportions between groups." (Franzblau et al. 2012)

"Statistical methods are of key importance and different methods are used depending on the question asked. Methods used were found to be very similar across laboratories, and often assistance of a statistician was provided to answer new questions. Power analysis prior to the experiments is required to determine the number of animals used in the experiment. The primary data analysis for mouse models is usually a one-way analysis of variance (ANOVA) of the log-10 CF bacterial loads, with t-distribution based contrasts comparing individual treatments. A Dunnett's test is an appropriate method for comparing all new compounds to untreated controls, while controlling the overall error rate in that set of ccomparisons is set at the usual 0.05 level. Also Bonferroni and Tukey statistical tests are appropriate tests for a pairwise comparison between treatment groups. Evidence of differential relapse based on detection (yes/no) of TB bacteria would be established using a Fisher's exact test comparing rates of relapse between two experimental groups. In experiments with n = 5–10 per group, power to identify significantly different relapse rates is very low. Therefore in order to increase the statistical power, more treatment groups are implemented with various lengths of treatment, or a higher relapse rate is aimed for in order to see significant differences between different treatment regimens. Relapse information obtained from mouse models should always be interpreted with great care and seen as trends in relapse differences between treatments." (Franzblau et al. 2012)

References

Ben-David, Avishai, and Charles E Davidson. 2014. "Estimation Method for Serial Dilution Experiments." *Journal of Microbiological Methods* 107: 214–21.

Franzblau, Scott G, Mary Ann DeGroote, Sang Hyun Cho, Koen Andries, Eric Nuermberger, Ian M Orme, Khisimuzi Mdluli, et al. 2012. "Comprehensive Analysis of Methods Used for the Evaluation of Compounds Against Mycobacterium Tuberculosis." *Tuberculosis* 92 (6): 453–88.

Ghodbane, Ramzi, Didier Raoult, and Michel Drancourt. 2014. "Dramatic Reduction of Culture Time of Mycobacterium tuberculosis." *Scientific Reports* 4 (1): 1–4.

Goldman, Emanuel, and Lorrence H Green. 2015. *Practical Handbook of Microbiology*. CRC press.

Jennison, Marshall W, and George P Wadsworth. 1940. "Evaluation of the Errors Involved in Estimating Bacterial Numbers by the Plating Method." *Journal of Bacteriology* 39 (4): 389.

Myers, Lawrence E, Lisa J McQuay, and F Blaine Hollinger. 1994. "Dilution Assay Statistics." *Journal of Clinical Microbiology* 32 (3): 732–39.

Pathak, Sharad, Jane A Awuh, Nils Anders Leversen, Trude H Flo, and Birgitta Åsjø. 2012. "Counting Mycobacteria in Infected Human Cells and Mouse Tissue: A Comparison Between qPCR and CFU." *PLoS One* 7 (4): e34931.

Sakamoto, K. 2012. "The Pathology of Mycobacterium Tuberculosis Infection." *Veterinary Pathology* 49 (3): 423–39.

Shin, Sung Jae, Jun Hee Han, Elizabeth JB Manning, and Michael T Collins. 2007. "Rapid and Reliable Method for Quantification of Mycobacterium Paratuberculosis by Use of the BACTEC MGIT 960 System." *Journal of Clinical Microbiology* 45 (6): 1941–48.

Tomasiewicz, Diane M, Donald K Hotchkiss, George W Reinbold, Ralston B Read, and Paul A Hartman. 1980. "The Most Suitable Number of Colonies on Plates for Counting." *Journal of Food Protection* 43 (4): 282–86.

Wilson, GS. 1922. "The Proportion of Viable Bacteria in Young Cultures with Especial Reference to the Technique Employed in Counting." *Journal of Bacteriology* 7 (4): 405.

———. 1935. "The Bacteriological Grading of Milk." *The Bacteriological Grading of Milk.*, no. 206.