

# Protocol: Estimating bacterial loads from plating samples at different dilutions

Henao-Tomayo Research Laboratory

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## Overview

In our laboratory, we often need to estimate the **bacterial load** of *Mycobacterium tuberculosis* in organs—including lungs and spleens—of animals from experiments. These measurements help us assess how well a vaccine or drug has worked in comparison to controls.

We typically estimate bacterial load in an animal organ using the **plate count method** with **serial dilutions**. Serial dilutions allow you to create a highly diluted sample without needing a massive amount of diluent: as you increase the dilution one step at a time, you can steadily bring the samples down to lower bacterial loads per volume. This method is common across laboratories that study tuberculosis drug efficacy as a method for estimating bacterial load in animal organs (Franzblau et al. 2012) and is a well-established method across microbiology in general, dating back to Koch in the late 1800s (Wilson 1922; Ben-David and Davidson 2014).

With this method, we homogenize part of the organ, and then create several increasingly dilute samples. Each dilution is then spread on a plate with a medium in which *Mycobacterium tuberculosis* can grow and left to grow for several weeks at a temperature conducive to *Mycobacterium tuberculosis* growth. The idea is that individual bacteria from the original sample end up randomly spread across the surface of the plate, and any bacteria that are **viable** (able to reproduce) will form a new colony that, after a while, you'll be able to see (Wilson 1922; Goldman and Green 2015). At the end of this incubation period, you can count the number of these **colony-forming units (CFUs)** on each plate.

To count the number of CFUs, you need a “**just right**” **dilution** (and you 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, which isn’t ideal. 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).

Once you identify a good dilution for each sample, the CFU count from this dilution can be used to estimate the bacterial load in the animal’s organ. To translate from diluted concentration to original concentration, you do a back-calculation, incorporating both the number of colonies counted at that dilution and how dilute the sample was (Ben-David and Davidson 2014; Goldman and Green 2015).

More details on each step of this process are described later in this document. Broadly, this document provides an overview of our experimental process—for background in understanding the pre-processing of these data—and then focuses on how we pre-process the CFU counts from plating to generate estimates of the bacterial load in the animal’s organ.

## Laboratory description

In this section, we describe more of the details of the laboratory procedure we use for creating serial dilutions, plating them, and counting CFUs on them after a suitable incubation period. This section is not meant to

Table 1: Experimental groups in the example data for this pre-processing protocol.

group	route	drug	vaccination
1	I.P.	control	PBS
2	I.P.	control	BCG
3	I.P.	Drug 1	BCG
4	I.P.	Drug 2	BCG
5	I.P.	Drug 1 + Drug 2	BCG
6	Oral	control	PBS
7	Oral	control	BCG
8	Oral	Drug 1	BCG
9	Oral	Drug 2	BCG
10	Oral	Drug 1 + Drug 2	BCG

serve as a full laboratory protocol, but rather to provide adequate details that are helpful to understand when pre-processing the data that result from this process.

In a typical experiment in this laboratory, we will measure bacterial load under several experimental conditions (e.g., no vaccine, BCG vaccine, novel vaccine) at several time points. For each set of experimental conditions, we will have several **replicates**—several animals exposed to that set of conditions. These animals are sacrificed at the appropriate time point for the experiment, and the organs are used for several experimental measurements. Typically, some part of the animal’s lungs and spleen will be used to estimate bacterial load in that organ by homogenizing part of the organ and then plating the homogenate and several serial dilutions and counting the CFUs on each plate following an incubation period.

In this data pre-processing protocol, we are using example data from a research project that our laboratory is currently conducting. This project tests novel vaccines to protect against *Mycobacterium tuberculosis*. The experimental groups in this case are given in Table 1.

Figure 1 provides an overview of the experimental process. For each animal, half of the spleen was homogenized in 500 microliters phosphate buffer saline (PBS) to create a homogenate. While only part of the organ was used in this experiment, in experiments where we expect bacterial counts to be very low (e.g., long treatment regimens), it may make more sense to use a larger portion of—up to the whole—organ and to plate a very large proportion of the homogenate (Franzblau et al. 2012), to be able to get higher CFU counts and reduce sampling error in these counts. The homogenate was diluted several times, and both the homogenate and each dilution were plated on a quarter of a 7H11 agar plate. Each dilution resulted from taking a 100  $\mu$ L aliquot from the previous test tube (either homogenate or the previous dilution) and resuspending it in 400  $\mu$ L PBS, for a total liquid volume of 500  $\mu$ L. From each test tube, 100  $\mu$ L were plated. Figure 2 shows a photograph of the resulting plating, and you can see that each sample was plated on a quarter of the plate. The plates were incubated for 3–5 weeks at 37°C, and then colony-forming units (CFUs) were counted on each plate and recorded in the data used as input to this protocol. *Mycobacterium tuberculosis* is very slow-growing, with a rate of division that is between 12 and 24 hours (Sakamoto 2012), so this incubation period is much longer than would be needed for plate counts for most other types of bacteria.

### Method limitations

As a conclusion for the experimental description, it is useful to keep in mind some limitations of this method. There are a number of advantages to this experimental procedure that result in our laboratory using it. However, there are some constraints and limitations that should be kept in mind, as well.

First, the bacterial load estimates that are generated from counting CFUs through plating are likely better suited to estimating *relative* loads across different samples, rather than as a measure of *absolute* bacterial cell counts (Wilson 1922; Jennison and Wadsworth 1940).

Next, because *Mycobacterium tuberculosis* grows so slowly, it takes an inconveniently long time to determine

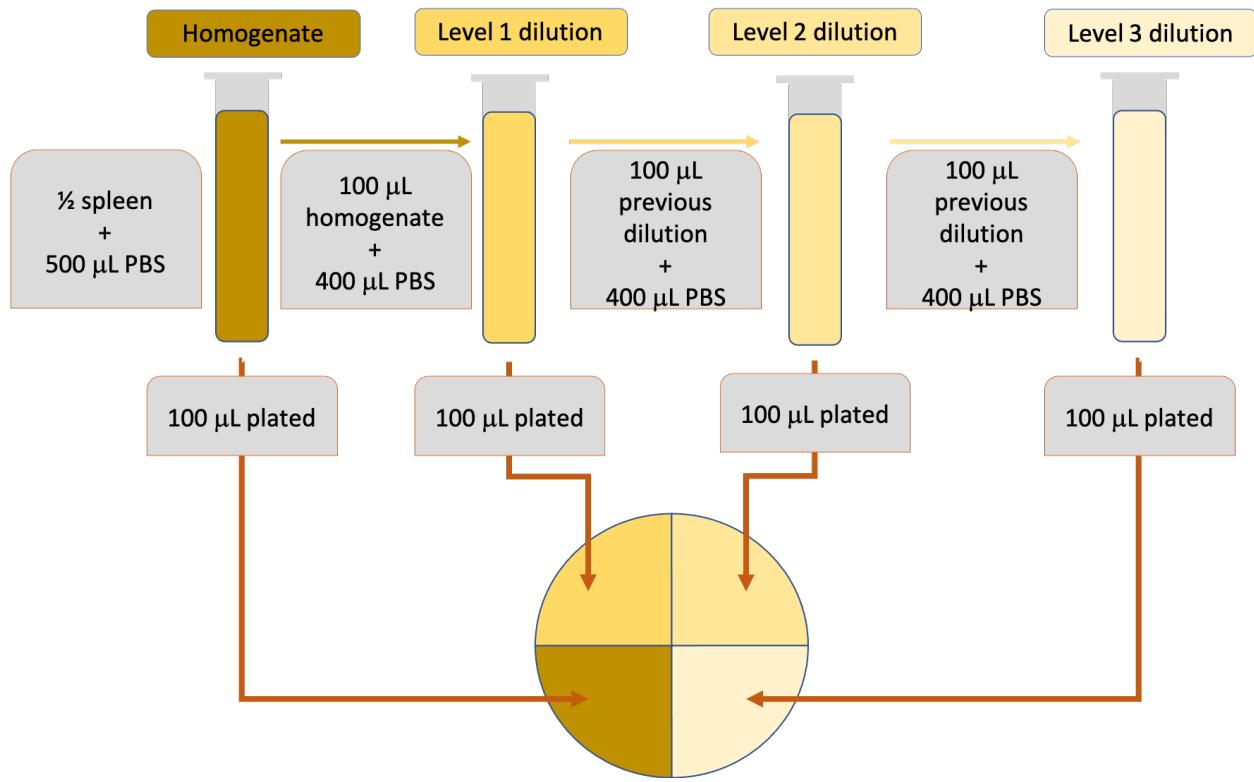


Figure 1: Visual overview of the dilution and plating process for this experiment. For each animal, half the spleen was homogenized in 500 microliters phosphate buffer saline (PBS) for plating ('Homogenate' tube in graphic). Three serial dilutions were created by resuspending 100 microliters from the homogenate or previous dilution in 400 microliters PBS ('Level 1 dilution', 'Level 2 dilution' and 'Level 3 dilution' tubes in graphic). From each tube, 100 microliters were plated in one quarter of a 7H11 agar plate (circle at bottom of graphic). After 3–5 weeks of incubation at 37°C, colony-forming units were counted from each quarter of the plate and recorded. These are the input data for this protocol.

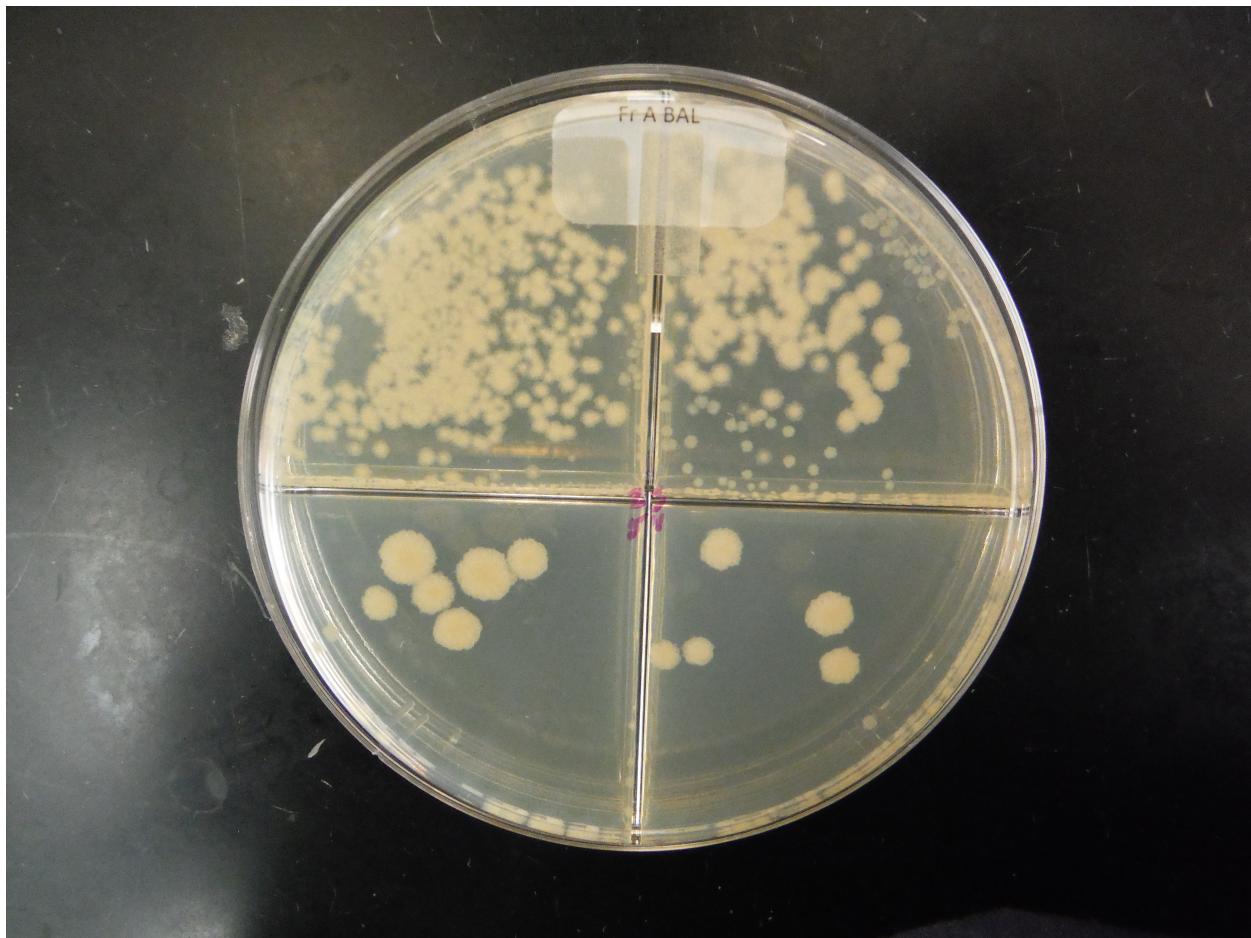


Figure 2: Example of a plate from this process. Each plate is divided into quarters, with a single sample (i.e., from a specific tube shown in Figure 1) spread in each quarter of the plate. This shows the plate after enough time has passed following plating for colony forming units (CFUs) to grow. In this example, CFUs can easily be counted in the bottom two quadrants of the plate, but may be too numerous to count in the top two quadrants.

bacterial loads by plating (Franzblau et al. 2012; Pathak et al. 2012). Since these bacteria are dangerous, the incubation of the plates must be done in a secure laboratory, and so a key limitation is that the process takes up valuable laboratory space over several weeks (Pathak et al. 2012). Further, the incubation process adds several weeks to the time needed to generate results from an experiment (Franzblau et al. 2012). Faster methods are being explored to cut out the 3–4 weeks of waiting required for plating; some new methods that are faster include luciferase readout and fluorescence (Franzblau et al. 2012).

Next, cells in the original sample can be prone to clumping (Jennison and Wadsworth 1940; Franzblau et al. 2012), mycobacteria in particular (Pathak et al. 2012). This can make it more likely that bacterial cells land at the same spot or nearby on a plate. When bacterial cells land at the same spot or very close together on a plate, they will likely result in a single colony forming, rather than one per viable bacterial cell. This can happen both when two colonies form too close together to distinguish them separately, and so are counted as a single colony, and also when one of two nearby colonies is stunted in its growth because the other colony takes needed growth resources at that spot on the plate. Both situations result in the CFU count underestimating the load of bacterial cells in the original sample (Goldman and Green 2015). There are methods, like shaking the sample, that can be used in preparing the dilutions and plates that can help prevent clumping (Jennison and Wadsworth 1940).

Next, the CFUs may provide a count only of viable bacteria, not of all bacteria cells in the original sample (Wilson 1922; Goldman and Green 2015), and this count will often focus on faster-growing bacteria, and may not capture the load of subpopulations of *Mycobacterium tuberculosis* in the sample that are at lower metabolic activity states. Some laboratories use liquid culture as an alternative to solid agar to encourage better growth of subpopulations of *Mycobacterium tuberculosis* that are in different metabolic activity states (Franzblau et al. 2012).

Further, there is some chance, when studying drug efficacy, that some drug activity persists during the incubation period, after plating (Franzblau et al. 2012). In this case, the drug activity may slow or hinder incubation of the colonies during plating, and so result in an underestimate of the bacterial load at the time the animal was sacrificed. One survey of multiple TB research laboratories suggested some potential approaches to limit this problem, including “using LJ medium or 7H11 with 5% bovine serum albumin (BSA) for [the drug] TMC-207, and using 0.4% activated charcoal for [the drug] CLF” (Franzblau et al. 2012).

Finally, there are several sources of error that can affect bacterial load estimates during this process. **Sampling error** is introduced by the role of randomness in CFU counts from sample to sample, and plays a particularly important role in cases where CFUs are low (Ben-David and Davidson 2014). **Counting error**, conversely, is linked with difficulties in correctly counting large numbers of CFUs, as well as counting difficulties introduced with overcrowding of colonies on a plate, and so plays a large role when CFUs are high (Ben-David and Davidson 2014; Goldman and Green 2015). There are opportunities for error, as well, in preparing the dilutions, plating them, and incubating them (Goldman and Green 2015). **Dilution error**, for example, is introduced by the process of pipetting from one dilution level to another, since small errors in volume can occur each time a sample is pipetted (Jennison and Wadsworth 1940).

## Data description

In our laboratory, we collect data from plating serial dilutions in a plain text file, recording the data using a spreadsheet program that is set up so it is easy to convert it into a “tidy” data format once it is read into R. Here are the first few rows of the data:

```
## # A tibble: 6 x 6
##   group replicate dilution_0 dilution_1 dilution_2 dilution_3
##   <dbl> <chr>      <chr>      <chr>      <dbl>      <dbl>
## 1     2 2-A       26         10          0          0
## 2     2 2-B       TNTC        52         10          5
## 3     2 2-C       0          0          0          0
## 4     3 3-A       0          0          0          0
## 5     3 3-B       TNTC        TNTC       30         10
## 6     3 3-C       0          0          0          0
```

Each row represents a replicate from the experiment, providing the number of bacterial colonies counted after plating a samples from that replicate at each dilution. Columns are included with values for the experimental group of the replicate (`group`), the specific ID of the replicate within that experimental group (`replicate`, e.g., 2-A is mouse A in experimental group 2), and the CFUs counted at each dilution level (`dilution_0`, `dilution_1`, `dilution_2`, and `dilution_3`). In cases where the bacteria were too numerous to count, this is marked in the cell with “TNTC.”

## 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 6
##   group replicate dilution_0 dilution_1 dilution_2 dilution_3
##   <dbl> <chr>      <chr>      <chr>      <dbl>      <dbl>
## 1     2 2-A       26         10          0          0
## 2     2 2-B       TNTC        52         10          5
## 3     2 2-C       0          0          0          0
## 4     3 3-A       0          0          0          0
## 5     3 3-B       TNTC        TNTC        30         10
## 6     3 3-C       0          0          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 6
##   group replicate dilution_0 dilution_1 dilution_2 dilution_3
##   <dbl> <chr>      <chr>      <chr>      <dbl>      <dbl>
## 1     2 2-A       26         10          0          0
## 2     2 2-B       TNTC        52         10          5
## 3     2 2-C       0          0          0          0
## 4     3 3-A       0          0          0          0
## 5     3 3-B       TNTC        TNTC        30         10
## 6     3 3-C       0          0          0          0
```

As soon as you read these data into R, you will want to convert them to a “tidy” data format, as this will make it easier to do other work with the data. In this case, that will require transforming the data so there’s a single row for the CFUs recorded for each dilution level for each replicate. As we “tidy” the data, we’ll also get rid of cases where the CFUs were too numerous to count, as well as ensure that the column that gives CFU counts is in a numeric data type, so that we can do numerical calculations on values in that column.

You can use the following code to make this transformation.

```
cfu_data <- cfu_data %>%
  # Convert all dilution columns to be numeric (this will change the "TNTC"
```

```

# markers to missing---NA)
mutate_at(c("dilution_0", "dilution_1", "dilution_2", "dilution_3"),
          .funs = as.numeric) %>%
# Pivot the dilution columns so that there's one column giving the
# dilution level (previous the column name) and one giving the CFUs at that
# dilution (previously the cell values for the columns)
pivot_longer(cols = starts_with("dilution"),
             names_to = "dilution",
             values_to = "CFUs") %>%
# Clean up the `dilution` column so it gives only the numeric level
# of the dilution, and make sure that the column is in a numeric data type
mutate(dilution = str_extract(dilution, "[0-9]+"),
       dilution = as.numeric(dilution))

```

The data are now in a tidy format:

```
cfu_data
```

```

## # A tibble: 92 x 4
##   group replicate dilution  CFUs
##   <dbl>    <chr>     <dbl>   <dbl>
## 1 1        2 2-A      0     26
## 2 2        2 2-A      1     10
## 3 3        2 2-A      2      0
## 4 4        2 2-A      3      0
## 5 5        2 2-B      0     NA
## 6 6        2 2-B      1     52
## 7 7        2 2-B      2     10
## 8 8        2 2-B      3      5
## 9 9        2 2-C      0      0
## 10 10      2 2-C      1      0
## # ... with 82 more rows

```

You can now get a summary of the data by running:

```
summary(cfu_data)
```

```

##      group      replicate      dilution      CFUs
##  Min.   : 2.000  Length:92   Min.   :0.00  Min.   : 0.000
##  1st Qu.: 3.000  Class :character  1st Qu.:0.75  1st Qu.: 0.000
##  Median : 5.000  Mode  :character  Median :1.50  Median : 0.000
##  Mean   : 5.826                    Mean   :1.50  Mean   : 1.944
##  3rd Qu.: 8.000                    3rd Qu.:2.25  3rd Qu.: 0.000
##  Max.   :10.000                   Max.   :3.00  Max.   :52.000
##                               NA's   :3

```

## 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

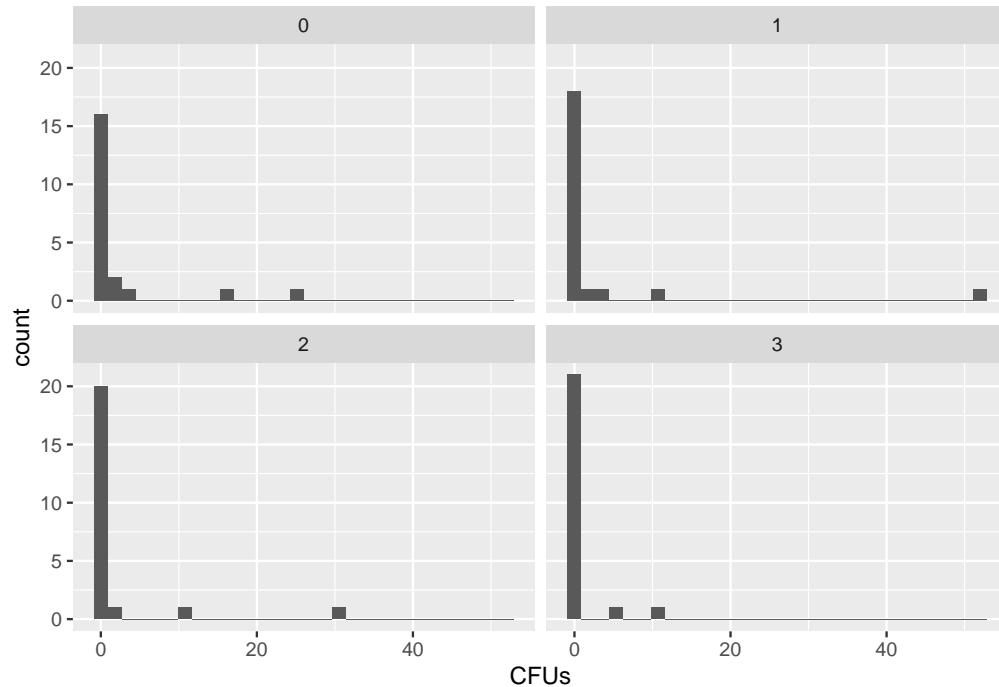
```

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 fewer 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 CFUs for a sample, you need a “good” dilution for that sample, one at which you have a chance 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. As mentioned earlier, there are several potential sources of error when estimating bacterial load from CFUs from plating, and this search for an optimal dilution tries to minimize those errors. The potential for sampling error is higher at lower CFU counts, where randomness of the underlying Poisson process governing the bacterial cells in a sample can introduce substantial variation across estimates from the same sample (Ben-David and Davidson 2014). Conversely, the potential for counting errors and errors from overcrowding are higher at higher CFU counts (Ben-David and Davidson 2014; Wilson 1935)—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. Further, when there are a lot of CFUs, there can be counting errors, in which the person counting the colonies miscounts or loses track when counting.

Typically, there will be some standard range of CFUs that are indicative of a plate having a “good” dilution for counting. Several sources recommend ranges of CFUs for a countable plate; depending on the source, these suggested ranges are often 30–300 CFUs (Goldman and Green 2015; Ben-David and Davidson 2014; Tomasiewicz et al. 1980), although some sources have also recommended ranges of 25–200, 40–400, 200–400, and 100–400 CFUs (Ben-David and Davidson 2014).

Since we plate each sample in a quarter of a plate, rather than across the whole plate, we use a range appropriate for this smaller surface area. We identify optimal dilutions for each sample as that in which the CFU is as close as possible to 25. This is similar to a full-plate count of 100 ( $25 * 4 = 100$ ), in terms of the amount of resources and chance of overcrowding on the plate at that dilution. The lower CFU number also helps reduce counting error, although it does present a higher chance for sampling error in the estimates, which we consider a reasonable trade-off as there are space constraints for growing plated bacteria within our BSL-3 facility.

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

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 closest 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: 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     2 2-B          0     NA        NA FALSE           2
## 2     2 2-B          1     52        27 FALSE           2
## 3     2 2-B          2     10        15 FALSE           2
## 4     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 CFU count at the best dilution level for each sample in these example data.

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:

```
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-B	2	10
2	2-C	0	0
3	3-A	0	0
3	3-B	2	30
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-A	1	2
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

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 homogenate; see Figure 1), although in a few cases the best dilutions for samples were at dilution levels of 1 ( $\frac{1}{5}$  dilution) or 2 ( $\frac{1}{25}$  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.

## Estimating bacterial load for each sample

Now that we have identified a good dilution level for each sample, we will use this to back-calculate to estimate the bacterial load in each of the experimental animals' spleens.

We first need to determine the dilution and dilution factor for each sample. Figure 1 shows the process of diluting and plating the samples. Based on this information, we can determine the dilution and dilution factor for each of the tubes. We can also determine the percent of liquid in each tube that was plated.

First, we can determine the **dilution** in each tube. The **dilution** for any particular diluted sample is:

$$\text{Dilution} = \frac{\text{Volume of aliquot}}{\text{Total volume}}$$

The total sample volume includes the volume of the aliquot (100  $\mu\text{L}$ ) and the volume of PBS in which this aliquot was diluted (400  $\mu\text{L}$ ). We can therefore calculate the dilution for each level of dilution (as compared

to the undiluted homogenate) in this experiment as:

```
volume_aliquot <- 100      # in microliters
volume_pbs <- 400          # in microliters

dilution <- volume_aliquot / (volume_aliquot + volume_pbs)
dilution

## [1] 0.2
```

The **dilution factor** for any particular sample is:

$$\text{Dilution factor} = \frac{\text{Concentration in undiluted sample}}{\text{Concentration in diluted sample}} = \frac{1}{\text{Dilution}}$$

Therefore, the dilution factor for this experiment is:

```
dilution_factor <- 1 / dilution
dilution_factor
```

```
## [1] 5
```

In other words, for each level of dilution, there is a five-fold dilution of the sample.

These following general equations apply for determining the total dilution in any of the tubes:

$$\text{Dilution factor in tube} = 5^x$$

$$\text{Dilution in tube} = \frac{1}{5^x}$$

where  $x$  is the dilution level in the tube.

As you move through the levels of dilution, each level will become diluted by an additional factor of 5 compared to the homogenate in the first tube (Figure 1):

Dilution level	Dilution factor in tube	Dilution in tube
0	$5^0 = 1$	1
1	$5^1 = 5$	$\frac{1}{5}$
2	$5^2 = 25$	$\frac{1}{25}$
3	$5^3 = 125$	$\frac{1}{125}$

We can apply these principles to get the dilution factor for the optimal dilution we identified for each sample in the previous section:

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

cfu_data_reduced %>%
  knitr::kable()
```

group	replicate	dilution	CFUs	dilution_factor_in_tube
2	2-A	0	26	1
2	2-B	2	10	25
2	2-C	0	0	1
3	3-A	0	0	1
3	3-B	2	30	25
3	3-C	0	0	1
4	4-A	0	0	1
4	4-B	0	0	1
4	4-C	0	0	1
5	5-A	0	0	1
5	5-B	0	0	1
5	5-C	0	0	1
7	7-A	1	2	5
7	7-B	0	0	1
7	7-C	0	0	1
8	8-A	0	17	1
8	8-B	0	0	1
8	8-C	0	4	1
9	9-A	0	0	1
9	9-B	0	0	1
9	9-C	0	1	1
10	10-A	0	0	1
10	10-B	0	0	1

Next, we can measure the proportion of each tube that was used for plating. With plating, there is a dilution factor both for diluting to each tube, then a further dilution factor to account for how much of each tube is spread on a plate to incubate (Ben-David and Davidson 2014)—we have already calculated the dilution factor for dilution to each tube, and so we now need to calculate the dilution factor for plating a sample from the tube. The second dilution factor is the inverse of the proportion of each tube that is plated (Ben-David and Davidson 2014). From each tube, 100  $\mu\text{L}$  is taken to plate, out of 500  $\mu\text{L}$  of liquid in the tube (100  $\mu\text{L}$  of aliquot from the previous dilution or homogenate and 400  $\mu\text{L}$  PBS), so you are plating 20% of the liquid in each tube:

$$100\mu\text{L} * \frac{1}{500\mu\text{L}} = 0.2$$

Following this logic, we can calculate the dilution factor for the plate at the selected dilution, as the total dilution on that plate will be the product of the dilution factor in that tube and the dilution factor of plating:

```
# Calculate dilution factor for plating process
volume_plated <- 100      # in microliters
frac_plated <- volume_plated / (volume_aliquot + volume_pbs)
plate_dilution_factor <- 1 / frac_plated
plate_dilution_factor

## [1] 5

# Calculate dilution factor on plate for each sample compared to
# homogenate in first tube
cfu_data_reduced <- cfu_data_reduced %>%
  mutate(dilution_factor_on_plate = dilution_factor_in_tube *
    plate_dilution_factor)

# Print out the results
```

```
cfu_data_reduced %>%
  knitr::kable()
```

group	replicate	dilution	CFUs	dilution_factor_in_tube	dilution_factor_on_plate
2	2-A	0	26	1	5
2	2-B	2	10	25	125
2	2-C	0	0	1	5
3	3-A	0	0	1	5
3	3-B	2	30	25	125
3	3-C	0	0	1	5
4	4-A	0	0	1	5
4	4-B	0	0	1	5
4	4-C	0	0	1	5
5	5-A	0	0	1	5
5	5-B	0	0	1	5
5	5-C	0	0	1	5
7	7-A	1	2	5	25
7	7-B	0	0	1	5
7	7-C	0	0	1	5
8	8-A	0	17	1	5
8	8-B	0	0	1	5
8	8-C	0	4	1	5
9	9-A	0	0	1	5
9	9-B	0	0	1	5
9	9-C	0	1	1	5
10	10-A	0	0	1	5
10	10-B	0	0	1	5

Now, we can use the CFUs counted on the plate to estimate the concentration of viable bacteria in the homogenate by multiplying the counted CFUs by the total dilution factor on that plate:

```
# Estimate CFUs in homogenate
cfu_data_reduced <- cfu_data_reduced %>%
  mutate(cfu_in_homog = CFUs * dilution_factor_on_plate)

# Print results out as a table
cfu_data_reduced %>%
  knitr::kable()
```

group	replicate	dilution	CFUs	dilution_factor_in_tube	dilution_factor_on_plate	cfu_in_homog
2	2-A	0	26	1	5	130
2	2-B	2	10	25	125	1250
2	2-C	0	0	1	5	0
3	3-A	0	0	1	5	0
3	3-B	2	30	25	125	3750
3	3-C	0	0	1	5	0
4	4-A	0	0	1	5	0
4	4-B	0	0	1	5	0
4	4-C	0	0	1	5	0
5	5-A	0	0	1	5	0
5	5-B	0	0	1	5	0
5	5-C	0	0	1	5	0
7	7-A	1	2	5	25	50
7	7-B	0	0	1	5	0
7	7-C	0	0	1	5	0
8	8-A	0	17	1	5	85
8	8-B	0	0	1	5	0
8	8-C	0	4	1	5	20
9	9-A	0	0	1	5	0
9	9-B	0	0	1	5	0
9	9-C	0	1	1	5	5
10	10-A	0	0	1	5	0
10	10-B	0	0	1	5	0

Finally, to get an estimate of the bacterial load in the full organ, we need to back-calculate from the concentration of bacteria we estimated in the homogenate. To do this, we need to account for two factors: the volume of PBS that was used to create the homogenate and the proportion of the organ that was included in the homogenate.

The homogenate was created using 500 µL of PBS to resuspend the portion of the spleen, so the bacterial concentration that we estimated in the homogenate provides an estimate of CFUs per 500 µL. To estimate the total number of viable bacteria in the original homogenate, we'll need to multiply the concentration of bacteria in the homogenate, which we calculated previously, by this volume.

Next, to get an estimate of bacteria load in the full organ, we need to backcalculate 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 can calculate to account for these two factors, allowing us to estimate the bacterial load in the entire spleen of the animal:

```
prop_organ_in_homog <- 0.5 # Half the spleen was included in the homogenate
volume_resuspend <- 500    # The homogenate was created by resuspending the
                           # organ part in this many microliters of PBS

# Estimate the bacterial load in the original organ based on the estimated
# bacterial concentration in the homogenate
cfu_data_reduced <- cfu_data_reduced %>%
  mutate(cfu_in_organ = cfu_in_homog * volume_resuspend *
        (1 / prop_organ_in_homog))

# Print the results as a table
cfu_data_reduced %>%
  select(-group) %>%
```

```
knitr::kable()
```

replicate	dilution	CFUs	dilution_factor_in_tube	dilution_factor_on_plate	cfu_in_homog	cfu_in_organ
2-A	0	26	1	5	130	130000
2-B	2	10	25	125	1250	1250000
2-C	0	0	1	5	0	0
3-A	0	0	1	5	0	0
3-B	2	30	25	125	3750	3750000
3-C	0	0	1	5	0	0
4-A	0	0	1	5	0	0
4-B	0	0	1	5	0	0
4-C	0	0	1	5	0	0
5-A	0	0	1	5	0	0
5-B	0	0	1	5	0	0
5-C	0	0	1	5	0	0
7-A	1	2	5	25	50	50000
7-B	0	0	1	5	0	0
7-C	0	0	1	5	0	0
8-A	0	17	1	5	85	85000
8-B	0	0	1	5	0	0
8-C	0	4	1	5	20	20000
9-A	0	0	1	5	0	0
9-B	0	0	1	5	0	0
9-C	0	1	1	5	5	5000
10-A	0	0	1	5	0	0
10-B	0	0	1	5	0	0

## 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 replicate (animal) in the experiment.

From the previous steps, we can remove the columns that we no longer need, to create a simpler format for the output data:

```
cfu_final <- cfu_data_reduced %>%
  select(group, replicate, cfu_in_organ)
cfu_final

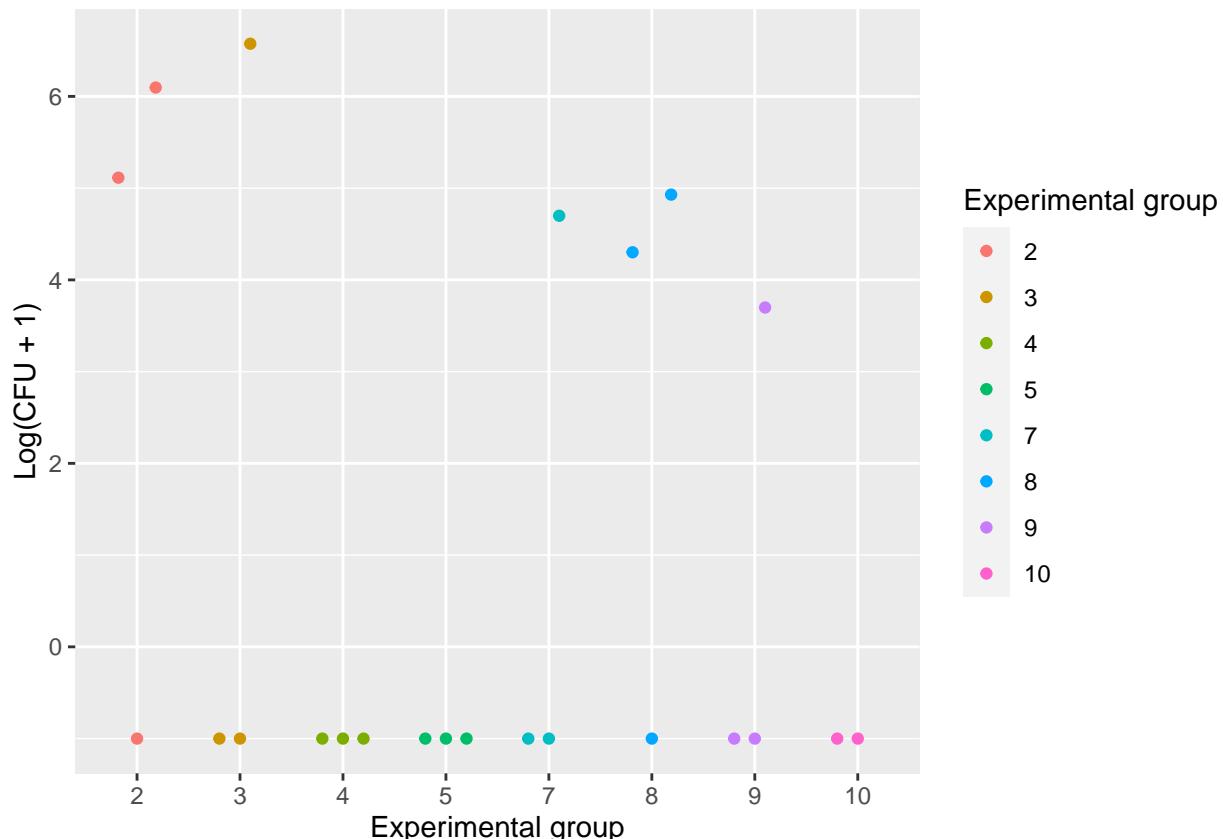
## # A tibble: 23 x 3
##   group replicate cfu_in_organ
##   <dbl> <chr>          <dbl>
## 1 1     2 2-A        130000
## 2 2     2 2-B        1250000
## 3 2     2 2-C          0
## 4 3     3 3-A          0
## 5 3     3 3-B        3750000
## 6 3     3 3-C          0
## 7 4     4 4-A          0
## 8 4     4 4-B          0
## 9 4     4 4-C          0
## 10 5    5 5-A          0
```

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

Our final data includes a row for each of the samples in the experiment—in this case, each sample represents one animal (replicate), with a single CFU estimate representing our best estimate of the bacterial load in the whole spleen of that animal.

You can visualize this data to explore patterns in it. For example, you can compare the CFU estimates across each experimental animal, grouping animals that are replicates for the same experimental group together:

```
library(ggbeeswarm)
cfu_final %>%
  ggplot(aes(x = as.factor(group),
             y = log10(cfu_in_organ + 0.1),
             color = as.factor(group))) +
  geom_quasirandom() +
  labs(x = "Experimental group",
       y = "Log(CFU + 1)",
       color = "Experimental group")
```



In this example plot, the log-10 values of the CFUs are shown, since the counts can otherwise be hard to compare. Since some of the CFU counts in the samples are 0, a small constant (0.1) is added to each value before taking the log, since the log of 0 is undefined.

We output this final data to a plain text file (a csv) using the following code:

```
write_csv(cfu_final, file = "processed_cfu_estimates.csv")
```

These data can now be used for further analysis and combined with other pre-processed experimental data. Through a survey of tuberculosis research laboratories, for example, the following was found regarding standard ways to analyze resulting data, and could be considered in next steps with these output:

"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 comparisons is set at the usual 0.05 level. Also Bonferroni and Tukey statistical tests are appropriate tests for a pairwise comparison between treatment groups." (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.
- 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.
- 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.
- 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.