



Research Article

**An Excel-based Calculator for Measuring Bacterial Concentration**

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**Abstract:** It is essential to know the initial bacterial count in microbiological studies. The classical plate count method is one of the most reliable methods. However, it is time-consuming. This study offers a fast method based on turbidity. This study describes how to create a calculator to be used for calculation of the bacteria concentration with spectrophotometric data in Excel 2010. In the study, McFarland standard and spread plate methods were used as standards for the control of spectrophotometric measurements. Bacteria used in the experiment were grown in suitable media and spectrophotometric measurements were performed. Then, using the Excel application, a calculator was created with a bio analytical approach to determine the linear relationship between turbidity and bacterial count. Bacteria counts were calculated by entering the absorbance data into the calculator, and the results were verified using cultural methods. As a result, an Excel-based calculator was created that can be used to calculate the number of bacteria from broth media.

**Bakteriyel Konsantrasyonu Ölçmek için Excel Tabanlı Bir Hesap Makinesi**

**Makale Bilgileri**

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**Anahtar Kelimeler**

Bakteriyel hesaplama,  
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McFarland hesap makinası,  
McFarland standartları

**Öz:** Mikrobiyolojik çalışmalarda başlangıç bakteri sayısının bilinmesi esastır. Klasik plaka sayım yöntemi en güvenilir yöntemlerden biridir ancak zaman alıcıdır. Bu çalışma, bulanıklığa dayalı hızlı bir yöntem sunmaktadır. Bu yöntemle, sıvı kültürler için büyüme ortamında kör olarak bakteri içermeyen büyüme ortamı kullanılarak doğrudan bir ölçüm yapılabilir. Bu çalışma Excel 2010'da spektrofotometrik verilerle bakteri sayısının hesaplanmasında kullanılacak hesap makinasının nasıl oluşturulacağını anlatmaktadır. Çalışmada spektrofotometrik ölçümlerin kontrolü için standart olarak McFarland standardı ve yayma plaka yöntemi kullanılmıştır. Denemede kullanılan bakteriler uygun besi ortamında büyütülmüş, spektrofotometrik ölçümleri gerçekleştirilmiştir. Daha sonra Excel uygulaması kullanılarak bulanıklık ve bakteri sayısı arasındaki linear ilişkiyi belirlemek için biyoanalitik yaklaşımla hesap makinası oluşturulmuştur. Yapılan ölçümlerdeki absorbans verileri hesap makinasına girilerek bakteri sayıları hesaplanmış, bulunan bakteri sayıları kültürel yöntemler kullanılarak doğrulanmıştır. Sonuç olarak, sıvı besi ortamlarından bakteri sayısının hesaplanmasında kullanılacak Excel tabanlı bir hesap makinası oluşturulmuştur.

## 1. Introduction

Bacterial concentration is an important step in microbiological research. McFarland Turbidity standards are a widely used method proposed by McFarland (McFarland, 1907). Bacterial measurement with McFarland standard is done with comparing prepared Standard with bacterial culture in a spectrophotometer. McFarland standard is prepared by mixing different volumes of 1% BaCl<sub>2</sub> and 1% H<sub>2</sub>SO<sub>4</sub> (Table 1) to give turbidity values at 625 nm (Isenberg, 2004). 0.5 McFarland standard should give approximately 0.1 Abs at 625 nm.

Table 1. McFarland preparation table and bacterial concentrations (Isenberg, 2004)

McFarland Standart	1% BaCl <sub>2</sub> (ml)	1% H <sub>2</sub> SO <sub>4</sub> (ml)	Bacterial concentration (cfu ml <sup>-1</sup> )
0.5	0.05	9.95	1.5×10 <sup>8</sup>
1	0.1	9.9	3.0×10 <sup>8</sup>
2	0.2	9.8	6.0×10 <sup>8</sup>
3	0.3	9.7	9.0×10 <sup>8</sup>
4	0.4	9.6	1.2×10 <sup>9</sup>
5	0.5	9.5	1.5×10 <sup>9</sup>
6	0.6	9.4	1.8×10 <sup>9</sup>
7	0.7	9.3	2.1×10 <sup>9</sup>
8	0.8	9.2	2.4×10 <sup>9</sup>
9	0.9	9.1	2.7×10 <sup>9</sup>
10	1.0	9.0	3.0×10 <sup>9</sup>

The calibration curve in the bio analytical approach is a linear relationship between concentration (independent variable) and response (dependent variable) using a least-squares approach. This relationship is made to anticipate the unknown concentrations of the content in a sophisticated matrix. (Prichard & Barwick, 2003; Moosavi & Ghassabian, 2018). The quality of a bio analytical approach is extremely reliant on the linearity of the calibration curve (Almeida et al., 2002). A linear calibration curve is a clear implication of assay performance in a validated analytical range. Other aspects of the calibration curve, including the regression model, the slope of the line, weighting, and correlation coefficient, need to be fully evaluated (Moosavi & Ghassabian, 2018).

This Excel-based calculator was prepared by creating a linear calibration curve with absorbance values and bacterial concentrations corresponding to each McFarland standard. To do a calibration curve we need to compare the readings of a spectrophotometer to known values called standards (McFarland standard). With this we achieve a series of data to develop a calibration curve. Using two data pairs as reference points and draw a line between them we could select any point on the line that correlate to the value spectrophotometer shows and we could find the corresponding bacterial concentration. The line is filling the data between known points so we can be logically sure with our estimated bacterial concentration without measuring a standard for that reading.

Hayashi et al. (2005) studied detection limit of the calibration curve for competitive ELISA. They deduced slope of log-dose B/B<sub>0</sub> at L<sub>D</sub>=[relative standard deviation of blank responses]/0.13 corresponds to the dose at which relative standard deviation of dose estimates is % 30. Sofalvi and Schueler (2021) assessed the bioanalytical method validation data using heteroscedastic seven-point linear calibration curves by customized Excel template. They developed an excel template that can calculate all needed numerical and statistical validation results with minimal data. Pesti et al. (2022) used statistical methods to improve the results of calibration curve in bioassays by determining the zinc bioavailability in broiler chickens. They found the abductive method has advantage on other statistical methods: estimation is consistent with the theory. When error is small (R<sup>2</sup>>0.95) the advantage is minimum but when the error is large, like in many growing organisms, method increases the accuracy of calculations.

Excel allows us to devise the data pairs in a chart, add a trend line, and present equation on the chart. It is also possible to measure the line's formula using SLOPE and INTERCEPT functions. When we use SLOPE and INTERCEPT in the formula, the value we will be calculated based on measurement.

## 2. Material and Methods

*Photobacterium damselea* subsp *piscicida*, and *Vibrio anguillarum* were used as the test bacteria. Bacteria were isolated from sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) samples. After fenotypic identification molecular identification were performed. Bacterial DNA was isolated with Qiagen DNeasy Blood and Tissue kit according to manufacturer's instructions. Real Time PCR reactions were performed using Rotor-Gene Q thermal cycler with Microbial DNA qPCR assay kit designed for *Photobacterium damselea* subsp *piscicida* and *Vibrio anguillarum*. Samples with strong positive ( $Ct \leq 25$ ) results were chosen for further experimentation.

After identification, bacteria were inoculated in Tryptic Soy Broth (Merck) at 25 °C for 24 hours. Serial dilutions were made from  $10^0$  to  $10^{-9}$ . Each dilution was measured with spectrophotometer at 625 nm. We made inoculations from each dilution on Tryptic Soy Agar (Merck). Agar plates were inoculated at 25 °C for 48 hours. After inoculation colonies were counted to calculate viable bacterial concentration. Same procedure was used for both bacteria.

McFarland standards were prepared with  $BaCl_2$  and  $H_2SO_4$  as given in Table 1. McFarland standards were used as control for both calibration curve and bacterial concentration. Each standard's absorbance was measured and a calibration curve was formed. Measurements were performed with the Hach Lange DR5000 spectrophotometer. Each experiment was done in triplicate.

### 2.1. Drawing calibration curve

The calibration curve was prepared according to Prichard & Barwick (2003). To draw a calibration curve, at least three replicates of each standard are recommended. This model's expectation is that the measurement error is the same and normally distributed for each sample. If application of this assumption is not possible, an extended or weighted least-squares analysis will be recommended. The distribution properties of the residuals (the difference between predicted and measured value) are predicted to be normal and centered on zero (Kolmogorov–Smirnov test; Moosavi & Ghassabian, 2018).

First, we will draw a calibration curve using eleven data pairs of McFarland standard. First, we prepare our standards according to Table 1. Then we measured 0.5 McFarland standard at 625 nm. The absorbance value should be  $0.1 \pm 0.05$ . After successfully measuring first standard we measure the remaining ten and made a chart on Excel (Table 2) with the corresponding colony forming units (cfu).

Table 2. McFarland chart of cfu-absorbance values. X-values are cfu and Y-values are absorbance readings. MF: McFarland standards. Absorbance values are mean values of three independent measurements

MF	X-Value	Y-Value
0.5	150000000	0.125
1	300000000	0.257
2	600000000	0.441
3	900000000	0.507
4	1200000000	0.651
5	1500000000	0.779
6	1800000000	0.934
7	2100000000	1.123
8	2400000000	1.39
9	2700000000	1.515
10	3000000000	1.589

Then we draw an XY Scatter chart with X and Y values. After this, we add a linear trend line. Closer the R-Squared value to 1 means the more accurate results. You can customize the chart's title and add axis titles as appropriate for your study (Figure 1).

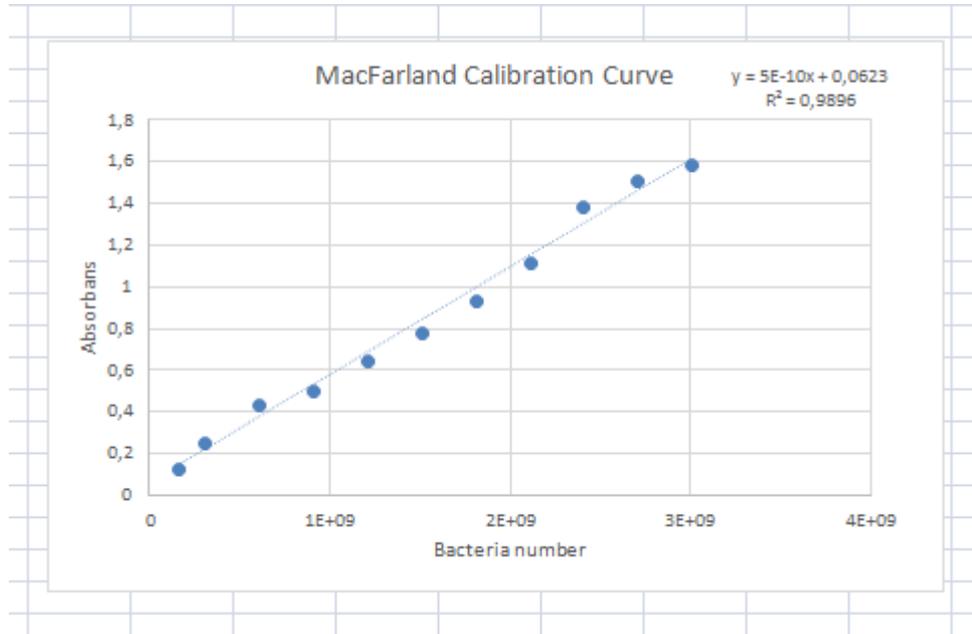


Figure 1. McFarland calibration curve after customizing.

The third step is to compute the R-squared statistics and equation with SLOPE, INTERCEPT, and CORREL functions (Bresolle et al., 1996; Cuadros-Rodríguez et al., 1996; Edwards, 2019). Add ‘Slope’, ‘Intercept’ and ‘R-squared’ titles to three different cells.

To calculate the slope, select the cell below the slope title. Chose Formulas>More Functions>Statistical>SLOPE.

In the function choose Y-values cells. Do the same for X-values. The order of Y and X values are important in this function. The formula will look like the equation below. Cell names can be different in your Excel file depending on your choice of the cells.

$$= SLOPE(D3:D13, C3:C13) \tag{1}$$

The SLOPE value should match the value of the equation on the chart. Next to calculate INTERCEPT (Gu et al., 2014).

In the function choose Y-values cells. Do the same for X-values. The order of Y and X values are also important in this function. The final formula will look like the equation below. Cell names can be different in your Excel file depending on your choice of the cells.

$$= INTERCEPT(D3:D13, C3:C13) \tag{2}$$

The value of the INTERCEPT should match the y-intercept shown in the equation on the chart. Next to calculate CORREL.

In the function arguments choose Y-values cells or select ‘Array 1’ field. Do the same for X – values for ‘Array 2’. The order of Y and X values are not important in this function. Do not forget to square your R value. The equation will look like the equation below. Cell names can be different in your Excel file depending on your choice of the cells.

$$= CORREL(D3:D13, C3:C13)^2 \tag{3}$$

After performing this CORREL value should match the R<sup>2</sup> value on the chart.

The fourth step is to organize formulas. We will use the slope, intercept and R-squared to predict the concentration of our bacteria in the solution. In next step, we will organize the formulas necessary to enter an absorbance to get the corresponding bacterial concentration or vice versa.

## 2.2. Checking the calculator

The formula to calculate the absorbance is “Absorbance=(SLOPE\*CFU)+INTERCEPT,” so to find the “Absorbance”, we multiply the “CFU” with SLOPE and then add the INTERCEPT value. If you put 0 in the CFU you should get the intercept value so we knew the formula works correctly. The formula will look like this:

$$= B20 * I25 + C20 \tag{5}$$

B20 is the cell of the slope, I25 is the cell of the CFU value we entered and C20 is the cell of the intercept.

To find the “CFU” we need to subtract the INTERCEPT from “Absorbance” and divide the result by the SLOPE. So, we have to formulate "CFU=(Absorbance-INTERCEPT)/SLOPE". The formula will look like this:

$$= (I30 - C20)/B20 \tag{6}$$

I30 is the cell of the absorbance we entered, C20 is the cell of the intercept and B20 is the cell of the slope.

The viable plate count method was used to control the accuracy of the calculator. Ten-fold dilutions of the bacterial broth were prepared and measured with a spectrophotometer and corresponding CFU values were calculated with our calculator. Then inoculations were made from the same dilutions on plate count agar. Calculation results and colonies counted on the plate were compared.

## 3. Results

After finishing all procedures in Excel, the final look of the calculator should be like Figure 2.

Slope	Intercept	r-squared
5,1803E-10	0,06234514	0,989568

Enter X to find Y	
CFU	0
Absorbance	0,06234514

Enter Y to find X	
Absorbance	0,699
CFU	1228991695

Figure 2. The final look of the calculator.

After preparing the charts and necessary formulae if you enter CFU Excel will calculate the corresponding absorbance or if you enter absorbance Excel will calculate corresponding CFU. After preparing this calculator viable plate count method was used to control the accuracy of the calculator (Table 3).

Table 3. Results of the accuracy test of the calculator compared to the viable plate count method

Absorbance	Calculated CFU	Counted CFU
0.644	$1.1 \times 10^9$	$1.2 \times 10^9$
0.512	$8.6 \times 10^8$	$9.0 \times 10^8$
0.436	$7.2 \times 10^8$	$7.4 \times 10^8$
0.322	$5.0 \times 10^8$	$4.9 \times 10^8$
0.230	$3.2 \times 10^8$	$3.0 \times 10^8$

#### 4. Discussion and Conclusion

The experimental results seldom fit the mathematical results so there will be differences between the experimental and the calculated values which are called residuals. The sum of squared residuals demands to be reduced to minimal to have the best estimation of the model parameters, and "method of least squares" can be used (Moosavi & Ghassabian, 2018).

Escher et al. (2018) studied the advantages of linear concentration-response curves for in-vitro bioassays with environmental samples. They recommend using all of the concentrations for cell viability. On lower doses the advantage of the system is greater. The main theoretical warning is a normal distribution with standard deviation of the effect data or a slope is a prerequisite for concentration response curves to be linear up to 30 %. Farhat et al. (2018) used flow cytometry and ATP to measure bacterial growth. Their results showed consistent bacterial growth in all tested samples. They suggested a fast and accurate approach to measure bacterial growth in samples. But they didn't control their results with cultural methods. In my opinion checking with cultural methods will produce more solid results since they are being used as standard bacterial counting methods according to ISO regulations.

One of the most common methods to measure bacterial concentration is the viable plate count. One of the disadvantages of the viable plate count method is the assumption that each colony forms from one cell. Some species grow in clusters. This results in the underestimation of the actual concentration of the bacteria. McFarland method measures turbidity instead of counting colonies (Zapata & Ramirez-Arcos, 2015). The viable plate count method is a time-consuming method. Depending on the bacteria it might take several days or even weeks to get results. McFarland method doesn't need extra inoculation steps, you can get results after the initial growth time and with a calculator, you can measure the values between standards. When you are performing a measurement in spectrophotometer use your solvent (growth medium, physiologic saline water etc.) as blank.

The major limitation of this method is the serial dilutions because manually prepared dilutions aren't completely fit for linear calibration curves. However, automated dispensers can be used to solve the problem. Also an expert can manually prepare dilutions suitable for linear calibration curves. Other limitation of this method is; this method is suitable for the bacteria which can be homogenized during the measurement. Because some bacteria can form clusters and do not show homogeneous distribution in the growth media, measuring a heterogeneous solution will give false results. If you are using a fresh culture this method will give accurate numbers but in older cultures I don't suggest using this method since it doesn't discriminate between live and dead cells. If you want to determine the number of live cells one of the plate count methods will be more suitable.

Calculating the bacterial concentration is essential in most of the microbiologic studies. This calculator provides a quick measurement of the bacterial concentration. It must be noted that this calculator gives a close estimation of the actual bacterial concentration depending on the standards prepared. For best results standard preparation and measurement steps must be carefully carried out.

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