

Using a Disk Diffusion Assay to Introduce Statistical Methods

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Abstract

Students perform a disk diffusion assay according to the protocol recommended by the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards). They learn about the stringent guidelines, including media preparation, culture preparation, inoculation with a swab, proper placement of antibiotic disks, and incubation conditions, that are necessary for accurate interpretation of results. The protocol is modified to include a second, more concentrated, inoculum. Differences in the diameters of the zones of inhibition produced by the two inocula are compared with the *t* test. Each student in a group of four makes independent measurements of the zones of inhibition, and the compiled data for each group are examined for differences using the analysis of variance test (ANOVA). Enumeration data is used to check the accuracy of dilutions.

Activity

Invitation for User Feedback. If you have used the activity and would like to provide feedback, please send an e-mail to MicrobeLibrary@asmusa.org. Feedback can include ideas which complement the activity and new approaches for implementing the activity. Your comments will be added to the activity under a separate section labeled "Feedback." Comments may be edited.

INTRODUCTION

Learning Objectives.

At the completion of this activity, students will

- understand the importance of using standardized methods (i.e., antibiotic resistance analysis using the disk diffusion method) and recognize important variables involved in antibiotic resistance analysis.
- better understand the scientific method and its application to experimental investigations.
- be more familiar with hypothesis testing and the use of inferential statistics.
- be able to use spreadsheets for simple statistical functions.
- have improved various lab skills, such as aseptic technique, preparing dilutions, making viable counts, inoculating spread plates, and using pipettes.

This exercise requires the use of mathematics at both an elementary and more advanced level. Thus, the students' math skills and the application of common statistical tests will be enhanced by this exercise.

Basic microbiology laboratory skills include:

- Properly use aseptic techniques for the transfer and handling of microorganisms and instruments, including
 - Sterilizing and maintaining sterility of transfer instruments
 - Performing aseptic transfer
- Use appropriate microbiological media and test systems, including
 - Isolating colonies
 - Maintaining pure cultures
 - Accurately recording macroscopic observations

- Estimate the number of microbes in a sample using serial dilution techniques, including
 - Correctly choosing and using pipettes and pipetting devices
 - Correctly spreading diluted samples for counting
 - Estimating appropriate dilutions
 - Extrapolating plate counts to obtain the correct CFU or PFU in the starting sample
- Use standard microbiology laboratory equipment correctly, including
 - Using the standard metric system for weights, lengths, diameters, and volumes
 - Lighting and adjusting a laboratory burner
 - Using an incubator

Background.

Students should have basic knowledge of and skills with aseptic techniques and dilutions. Students should have a working familiarity with algebra. Experience with spreadsheets would be beneficial but is not essential.

PROCEDURE

Materials.

(per group of four students)

- 32 Mueller-Hinton agar plates
- Sterile cotton swabs
- 1-ml sterile pipettes
- Sterile tubes
- Test tube racks
- Overnight culture of *Escherichia coli* on tryptic soy agar
- *E. coli* culture adjusted to 0.5 McFarland standard
- Tubes of sterile saline for dilutions (4 x 9.9 ml, 6 x 9.0 ml, plus some for adjusting culture to McFarland standards)
- McFarland standards (0.5, 1, 2, 4)
- Gentamicin antibiotic disks (GM10)
- Sulfamethoxazole-trimethoprim antibiotic disks (SXT)
- Forceps
- 95% ethanol in a beaker
- Bunsen burner
- Vortex
- Ruler or caliper
- Magnifier
- Sterile bent (L-shaped) plastic rods (i.e., hockey sticks)
- Ice in small buckets or beakers

Instructor and Lab Preparation.

Although the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards) documents can be used to obtain this protocol (5), most manufacturers, such as Becton Dickinson BBL whose products were used in this exercise, enclose the standardized protocol as a package insert along with their antibiotic disks (2).

1. Sterilize and prepare all media and materials at least one day before the exercise.
2. On the day before the exercise, inoculate a streak plate of tryptic soy agar (or any other nonselective medium) with *E. coli*, one per group plus one for you. Incubate overnight at 37°C.
3. Prepare at least one set of McFarland standards (0.5, 1, 2, 4) per lab (6). On the day of the lab or prior to that, let each group know the experimental cell density that they will make (1, 2, or 4 McFarland standard). Let them know that they will be using the experimental culture they make for comparison with a control culture, adjusted to the 0.5 McFarland standard, that you will provide.
4. Just prior to the lab, prepare tubes of *E. coli* with turbidity equal to the 0.5 McFarland standard by suspending colonies in sterile saline. Make enough of the cell suspension so that each group will have 5 ml of the inoculum. Each group of students will prepare the other inoculum to the McFarland standard assigned to them.

Student Version.

Day 1 (Inoculations)

1. Divide into groups of four students each.
2. Each group will be given a culture diluted to 0.5 McFarland standard. This is the cell concentration recommended in the Clinical and Laboratory Standards Institute (CLSI) protocol and will be considered the control culture in this experiment. You will also be assigned another cell concentration based on the McFarland standard (1, 2, or 4) and will need to make the appropriate suspension from an *E. coli* plate culture using sterile saline. Aseptically transfer colonies from the plate into a tube of sterile saline, vortexing after each addition until the colony is completely dispersed. Continue adding cells until the turbidity of your suspension matches the appropriate McFarland standard. (Hint: looking at a line of print through the standard and the cell suspension is a good way to compare the density.) The cell suspension you make will be considered the experimental culture. Keep both cultures on ice to inhibit growth and keep cell concentrations from changing during the course of the experiment.
3. Each group will need two tubes of 9.9-ml sterile saline and three tubes of 9-ml sterile saline to enumerate each of the two cell cultures. (Four tubes of 9.9-ml and six tubes of 9-ml saline, total.) Label each series of tubes with the culture density (0.5 McFarland and your other density). Label the first 9.9-ml tube 10^{-2} and the second 9.9-ml tube

- 10⁻⁴. Label the three 9-ml tubes 10⁻⁵, 10⁻⁶, and 10⁻⁷. These tubes will be used to dilute the control and experimental culture to make inocula for spread plates.
4. Plate counts should be performed by diluting the two cultures with the saline dilution blanks (step 3, above). Start by transferring 0.1 ml from the control culture to the tube marked "10⁻²" using good aseptic technique, then vortex that dilution. Using a new, sterile pipette, transfer 0.1 ml from the 10⁻² dilution to the tube marked "10⁻⁴" and vortex that tube. Transfer 1 ml from the "10⁻⁴" tube to the tube labeled "10⁻⁵." Continue in this manner, inoculating the last two dilutions. Repeat this entire process with the experimental culture.
 5. Use your dilutions to inoculate spread plates in order to determine the viable cell count in your cultures. For the control culture and experimental cultures adjusted to the 1 McFarland standard, inoculate duplicate plates each with 0.1 ml from the 10⁻⁴, 10⁻⁵, and 10⁻⁶ dilutions. With the experimental cultures adjusted to the 2 or 4 McFarland standard, inoculate duplicate plates each with 0.1 ml from the 10⁻⁵, 10⁻⁶, and 10⁻⁷ dilutions. Use a sterile hockey stick to spread the inoculum over the entire surface of the plate, using a fresh hockey stick for each dilution with each culture.
 6. For the disk diffusion assay, each group should swab 10 Mueller-Hinton agar plates from the control culture. Repeat this, inoculating 10 more Mueller-Hinton agar plates from the experimental culture. Swabbing should be done by following the CLSI protocol (2, 5):
 - A. Dip a sterile cotton swab into the appropriate liquid culture.
 - B. Press the swab against the inside of the tube to squeeze out excess fluid.
 - C. Lightly rub the swab over the entire surface of the Mueller-Hinton plate.
 - D. Rotate the plate 60° and swab the surface a second time.
 - E. Rotate the plate 60° and swab the surface a third and final time.
 - F. Roll the swab on the agar around the inside edge of the plate.

You can use the same swab to inoculate every plate in the series but you must dip it in the liquid culture before swabbing each plate. Be sure to properly dispose of the cotton swab following inoculation.

7. After 5 minutes, but no more than 15 minutes, the two different antibiotic disks should be placed on the agar surface of each of the 10 plates from each culture, at least 24 mm from each other and 10 mm from the edge of the plates, and tapped down with sterile forceps. (Placement may be done using an automatic dispenser or by using loose disks in a sterile petri plate, placing the disks with sterile forceps.) Sterilize forceps by dipping the tips in 95% ethanol and igniting the ethanol by rapid passage through a flame. (Keep the tip pointed down, away from the ethanol reservoir, and do not hold the forceps in the flame!) Invert the plates and incubate at 37°C for 18 hours.

Day 2 (Record data)

1. After 18 hours, the plates with the antibiotic disks should be removed from the incubator, and the diameters of the zones of inhibition should be measured in mm by each student in the group (i.e., four sets of measurements for the control culture and four sets of measurements for the experimental culture). The use of a magnifier, such as those on colony counters, greatly improves accuracy. Record the results in your lab notebook and share your data with the other members of your group.
2. Count the colonies on the enumeration plates, recording your counts. Calculate the viable cell concentration in the two McFarland cultures and determine if their ratio matches the ratio between the McFarland values for the cultures.

Day 3 (Analyze data)

1. Determine if the sizes of the zones of inhibition with the two McFarland cultures are the same or different for each antibiotic using the *t* test. Typical student data is shown in [Table 1](#). This can be done manually using a calculator or with a spreadsheet (recommended, see Appendix). The null hypothesis (no difference between the diameters of the zones of inhibition for different concentrations of *E. coli* in the inocula) is accepted if the calculated *t* value is less than the critical *t* value with an alpha of 0.5 and 18 degrees of freedom. A one-tailed test is appropriate since a higher concentration of *E. coli* may produce a zone of inhibition that is equal to or smaller than a zone resulting with a lower concentration of cells. Thus the critical value of *t* is 1.734.
2. Determine if the individual measurements of the zones of inhibition made by each group member are the same or different for each antibiotic using ANOVA. Student data for a control culture with trimethoprim-sulfamethoxazole is shown in [Table 2](#). The null hypothesis that there is no significant difference between measurements of the diameter of the zones of inhibition made by different students is accepted if the calculated *F* value is less than the critical *F* value with an alpha value of 0.5 and 3 degrees of freedom, that is 2.866.

Instructor Version.

1. *Lecture background.* Antibiotic resistance is becoming an ever-increasing concern. Thus, the ability to accurately assess the level of resistance of clinical isolates is of great importance, ultimately affecting patient outcome. Antibiotic resistance testing using the disk diffusion technique was developed in the 1940s soon after the discovery of the first antibiotics. In 1966, Bauer et al. (1) published their paper which helped standardize this protocol. Today, CLSI provides periodic updates of this protocol (5) and tables (4) so when performing this assay results are reproducible from day to day and from lab to lab if using the same isolate and the same antibiotics. That is, if the directions are followed, the same results will always be obtained with the same isolate, regardless of where or when the analysis is performed.

In this exercise we have modified the CLSI disk diffusion assay so that various concentrations of the inoculum are used in addition to the standard inoculum concentration. After incubation for each inoculum, the diameter of the zone of inhibition of each antibiotic disk is measured. Comparisons are made of the effect of inocula concentration on the diameter of the zone of inhibition. By testing this variable (i.e., different concentrations of inocula) we hope to show the importance of following a standardized protocol and to illustrate the use and the value of statistical analysis in microbiology. The null hypothesis, that is the hypothesis of no difference, is that the diameters of the zones of inhibition will be the same regardless of *E. coli* concentration in the inocula. The hypothesis is tested using the *t* test. In addition, each student in the group measures the

zones of inhibition and their results are compared. In this instance, the null hypothesis, that there is no difference between their measurements, is tested with ANOVA.

Students should read the protocol and organize their groups before the start of the experiment. In preparation for this exercise, we recommend that the instructor have them address the following points:

- Draw a flow diagram of the experiment noting how you would make the dilutions and then perform the plate counts.
- This experiment investigates the effect of bacterial concentration in the disk diffusion assay. What other variables could be investigated and which statistical tools would be appropriate for analysis?
- What are the modes of action of the antibiotics used? Would they be expected to act against gram-positive or gram-negative organisms or both?
- Do you think that it would make a difference if more than one person inoculated the plates for antibiotic testing? What statistical test would you use to test your hypothesis?
- How is resistance or sensitivity to an antibiotic determined using the disk diffusion assay? Does this have clinical relevance? What is the measurement that is used to determine this? Would a measurement of the same magnitude imply resistance (or sensitivity) if the organism tested was a different species?
- Would you expect bacteria in the stationary phase of growth to be more sensitive or resistant to an antibiotic as compared to the same species in the exponential phase? (Hint: would preincubation of plates with the antibiotic disks in place affect the size of the zone around the disk?)
- List some ways in which antibiotic resistance can be transferred from one bacterium to another. Briefly describe each mechanism.

2. Before beginning the lab work, a preliminary lecture outlining the objectives, describing the laboratory techniques, and discussing how the data will be analyzed would be helpful. The emphasis should be on what are likely to be new techniques, for example inoculating spread plates with a swab, as described above, and diluting cultures to a McFarland standard. It is important that swabbing be done with a light touch or small channels will occur, making it difficult to measure the zone of inhibition. Diluting to the McFarland standard seemed to be particularly stressful. The hint given above, to read a line of print through the standard and the culture, is very helpful. Although students may want to use a spectrophotometer, it should be emphasized that the method was developed using the 0.5 McFarland standard and that this is another skill to learn. (For your benefit, the absorbance of a 0.5 McFarland standard at 625 nm in a cuvette with a 1-cm light path should be 0.08 to 0.10.)

3. Plates should be incubated at 37°C as soon as the antibiotic disks are in place. Preincubation at room temperature will skew results.

4. A general lecture over the importance of statistics to data analysis may be used to introduce discussion of analyzing class data. Several statistics books may be used as a reference but *Introductory Biological Statistics* (3) is recommended. Key points to consider:

- Select an alpha level of 0.05. This is the level usually selected as it offers a good compromise to avoid Type I (rejecting a null hypothesis that is true) or Type II (not rejecting a null hypothesis that is false) errors.
- The *t* test is a powerful and robust test for determining if two populations have different means when samples are independent and random and the measured variable is continuous and normally distributed.
- ANOVA (analysis of variance) is more appropriate (and less error prone) than running multiple *t* tests when there are more than two samples to compare. In ANOVA there are two types of variance to consider: error variance (within-groups variance) and treatment variance (between-groups variance). Since the zones of inhibition being measured by each group member are the same, the within-groups variance should be the same. Therefore, any difference between the variances of the measurements would be due to the treatment (individual making the measurement). ANOVA can indicate a difference between the treatments but additional tests are necessary to find which treatments are significantly different.

5. It is recommended to perform calculations with a spreadsheet rather than a calculator because

- spreadsheets record the data entered, decreasing input mistakes and allowing the data to be distributed in a clear format among coworkers and to instructors.
- spreadsheets allow several analyses to be performed from one set of entered data.
- spreadsheets simplify analyses, reducing mistakes.
- spreadsheets are a powerful analytical instrument that have become a common scientific tool and, as such, should be part of any education in science.

6. A tutorial on using Microsoft Excel to analyze the data in this exercise is available in the Appendix.

7. It may be possible to gather the entire class' data and plot cell concentration versus zone of inhibition. It provides an opportunity to discuss graphing and may demonstrate if the relationship between zone size and concentration is linear or logarithmic.

Safety Issues.

E. coli is considered a biosafety level 2 pathogen. Although all students who are taking this class at our institution already have knowledge of safe handling procedures for microorganisms, it is the responsibility of the instructor to review these issues. Information on appropriate handling can be obtained from Centers for Disease Control and Prevention (7).

ASSESSMENT and OUTCOMES

Suggestions for Assessment.

The activity is assessed using written reports prepared by the students. The reports include the raw data, results of statistical analyses, and interpretations of the statistical analyses. Students should also be able to describe, in their own words, the importance of a standardized antibiotic testing protocol.

Field Testing.

This activity is included in Microbiological Procedures, a lab-oriented course at the sophomore-junior level. There are usually 25 to 30 undergraduate microbiology majors enrolled. Based on pre- and posttests, students demonstrated increased understanding of laboratory methods, including bacterial enumeration, the use of standardized protocols, and the appropriate application of the *t* test and ANOVA. Students identified data analysis, especially using spreadsheets, as the best part of this activity.

Student Data.

[Table 1](#) displays typical student data for diameters of zones of inhibition from gentamicin for *E. coli* cultures adjusted to 0.5 and 1 McFarland standards. Results of the *t* test, performed using Microsoft Excel, indicate that the zones with the greater cell density were significantly smaller than the zones with the less dense culture.

[Table 2](#) displays typical student data for measurements of the diameters of zones of inhibition from gentamicin for an *E. coli* culture adjusted to a 0.5 McFarland standard, made separately by four different students. ANOVA (using a spreadsheet) suggests no significant difference between the sets of measurements.

SUPPLEMENTARY MATERIALS**References.**

1. **Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turke.** 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* **45**:493–496.
2. **Becton Dickinson and Company.** 2004. BBL Sensi-Disc antimicrobial susceptibility test discs. 2001/06. BD Diagnostic Systems, Sparks, Md.
3. **Hampton, R. E.** 1994. Introductory biological statistics. WCB/McGraw-Hill, Dubuque, Ia.
4. **National Committee for Clinical Laboratory Standards.** 2004. Performance standards for antimicrobial susceptibility testing: fourteenth informational supplement. M100-S14. National Committee for Clinical Laboratory Standards, Wayne, Pa.
5. **National Committee for Clinical Laboratory Standards.** 2000. Performance standards for antimicrobial susceptibility tests, 7th ed. National Committee for Clinical Laboratory Standards, Wayne, Pa.
6. **Smibert, R. M., and N. R. Krieg.** 1994. Phenotypic characterization, p. 607–654. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), *Methods for general and molecular biology*. American Society for Microbiology, Washington, D.C.
7. **U.S. Department of Health and Human Services Public Health Service, Centers for Disease Control and Prevention, and National Institutes of Health.** 1999. Biosafety in microbiological and biomedical laboratories, 4th ed. U.S. Department of Health and Human Services publication no. (CDC) 99-xxxx.

Appendices.

Appendix 1. [Using Microsoft Excel to Analyze Data from the Disk Diffusion Assay](#)

Table 2. Measurements of the diameter of zones of inhibition made with the same ten samples by each of the four group members. Data were compared using ANOVA.

Disk Diffusion					Anova: Single Factor							
SXT, 0.5												
A	B	C	D	= student	SUMMARY							
29	29	30	27		Groups	Count	Sum	Average	Variance			
32	29	31	31		A	10	298	29.8	0.844444			
29	29	30	27		B	10	302	30.2	1.511111			
30	32	31	31		C	10	304	30.4	0.266667			
30	31	31	30		D	10	298	29.8	2.4			
30	32	30	31									
30	31	31	30									
30	30	30	30									
29	29	30	31		ANOVA							
29	30	30	30		Source of Variation	SS	df	MS	F	P-value	F crit	
					Between Groups	2.7	3	0.9	0.716814	0.548441	2.866265	
29.8	30.2	30.4	29.8	= mean	Within Groups	45.2	36	1.255556				
0.918937	1.229273	0.516398	1.549193	= s.d.	Total	47.9	39					

Using Microsoft® Excel to Analyze Data from the Disk Diffusion Assay

Entering and Formatting Data

Open Excel. Set up the spreadsheet page (Sheet 1) so that anyone who reads it will understand the page (Figure 1).

- Type a **title** in the cell in the upper lefthand corner, cell A1
- **Label column A** as the data from the 0.5 McFarland culture with gentamicin (GM10) in cell A3
- **Label column B** as the data from the other McFarland culture with gentamicin. (In this example, the 1 McFarland culture.)
- Repeat with the trimethoprim/sulfamethoxazole (SXT) data.
- **Enter the appropriate data** (diameter of zones of inhibition in mm) in each column (Fig. 1).

	A	B	C	D
1	Disk Diffusion Assay			
2				
3	GM10, 0.5	GM10, 1	SXT, 0.5	SXT, 1
4	24	24	30	29
5	25	26	31	31
6	24	25	30	32
7	25	24	31	31
8	25	25	31	31
9	24	26	30	34
10	24	25	31	30
11	25	26	30	32
12	24	25	30	32
13	24	25	30	32
14				

Figure 1

Calculating the Mean and Standard Deviation (should this be desired)

You can **calculate the mean and standard deviation** for each column using the functions “=average()” and “=stdev()”, respectively (Fig 2).

3	GM10, 0.5	GM10, 1
4	24	24
5	25	26
6	24	25
7	25	24
8	25	25
9	24	26
10	24	25
11	25	26
12	24	25
13	24	25
14		
15	=average(A4:A13)	
16		

Figure 2

- The **data for each column goes inside the parentheses** and may be entered by left-clicking and dragging the mouse over the values after typing the open parenthesis.
- Finish by typing the **close parenthesis** and hitting the “Enter” button.
- **Copy the functions** (left-click and drag to select, right click and “Copy”) and paste them under the other columns (left-click and drag to select, right click and “Paste”) (Fig 3).

	A	B	C	D
1	Disk Diffusion Assay			
2				
3	GM10, 0.5	GM10, 1	SXT, 0.5	SXT, 1
4	24	24	30	29
5	25	26	31	31
6	24	25	30	32
7	25	24	31	31
8	25	25	31	31
9	24	26	30	34
10	24	25	31	30
11	25	26	30	32
12	24	25	30	32
13	24	25	30	32
14				
15	24.4	25.1	30.4	31.4
16	0.516398	0.737865	0.516398	1.349897

Figure 3

The *t*-test

The easiest way to perform **statistical analyses** with a spreadsheet is to **use the built-in functions**. With Excel, they can be found by **clicking on “Tools”** on the toolbar and **selecting “Data Analysis”** (Fig. 4).

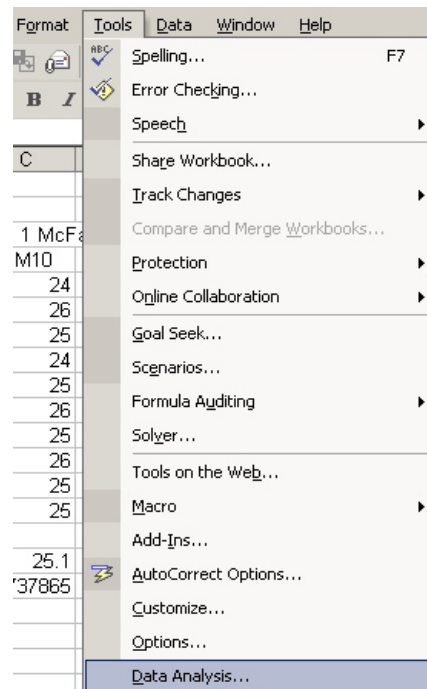


Figure 4

If the “**Data Analysis**” selection is not listed it means that the functions haven’t been installed. To install them, you must have access to the **installation program**, either on CD or through a network.

- Select “**Add-Ins**” under the “**Tools**” menu (Fig. 4).
- When the “Add-Ins” menu comes up, choose “**Analysis ToolPak**” (Fig. 5) and click on “OK.”

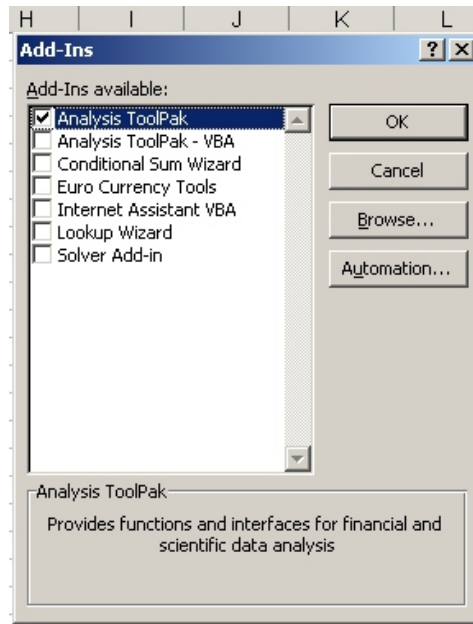


Figure 5

When you select “Data Analysis” on the “Tools” menu (Fig. 5), the “Data Analysis” menu pops up (Fig. 6).

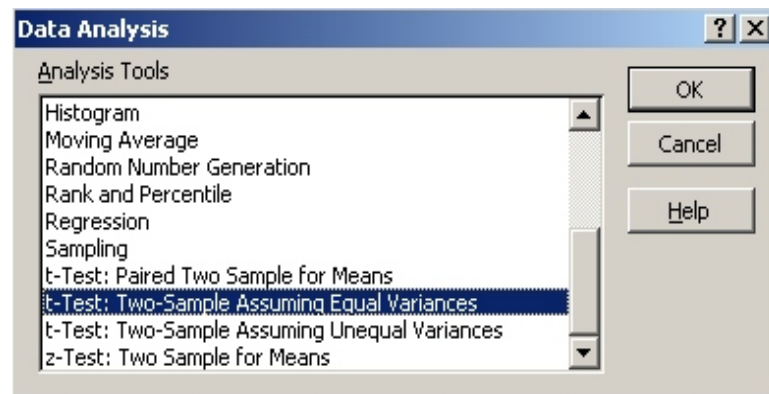


Figure 6

- Scroll down and select “**t-test: Two-Sample Assuming Equal Variances.**”
- After you **click “OK,”** the “t-test: Two-Sample Assuming Equal Variances” menu pops up (Fig. 7) and you need to **select your data.**

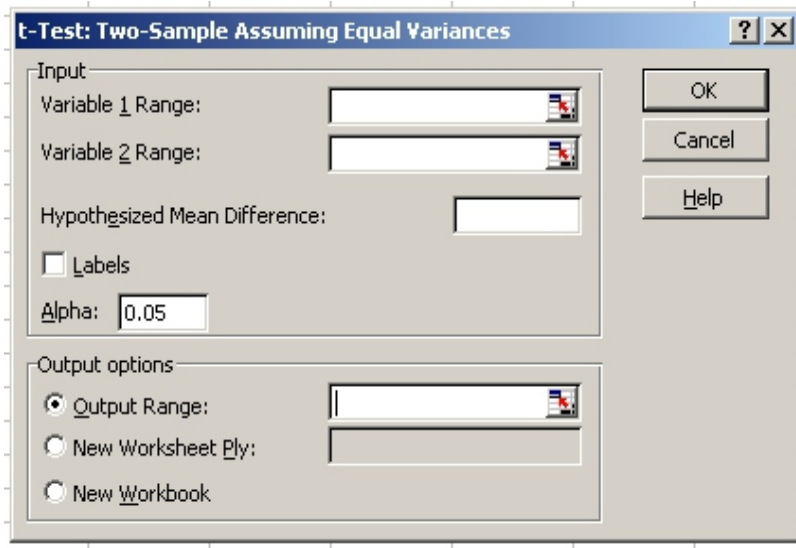


Figure 7

- **Click on the red arrow** on the right side of the box next to the “**Variable 1 Range:**” label.
- A menu pops up for data input (Fig. 8).

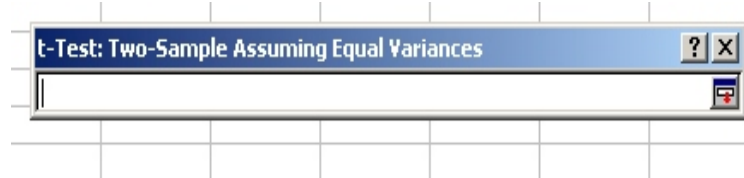


Figure 8

- **Enter data** by dragging the mouse over the values in the appropriate column, for example Column A, cells 3-13. **Hit “Enter” when done.** This will input the values for the zones of inhibition with gentamicin (GM10) on the plates inoculated from the 0.5 McFarland culture, as well as the column label.
- **Repeat this process**, clicking on the arrow for “**Variable 2 Range:**” and entering the other gentamicin data in Column B.
- Leave the “**Hypothesized Mean Difference**” selection blank, check the “**Labels**” box, and leave “**Alpha**” at 0.05.
- **Select a cell** on the spreadsheet, for example F3, where you want the **results of the t-test** to be placed.
- The completed “**t-test: Two-Sample Assuming Equal Variances**” menu should look similar to Figure 9.

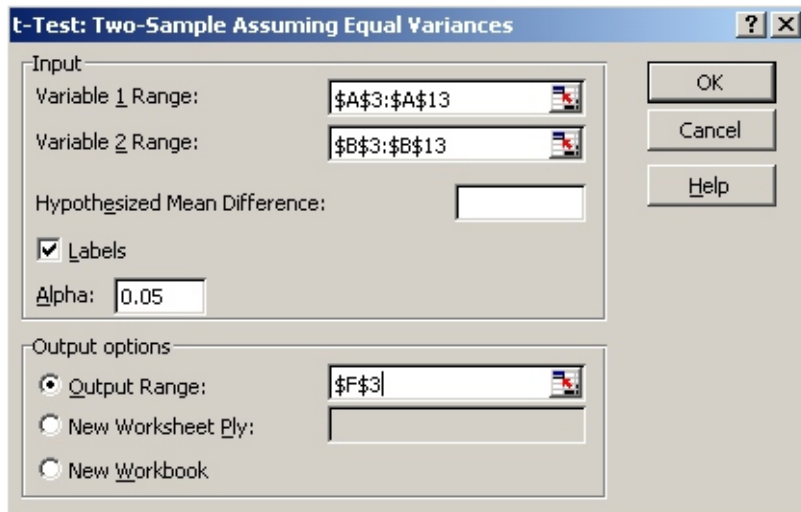


Figure 9

- Click on “OK” and the table of information for the t -test should appear, beginning in cell F3.
- Move the cursor to the right hand border of the Column F label so that the **column resize cursor** appears (Fig. 10).

	F	G	H	I	J
	t-Test: Two-Sample Assuming Equal Variances				
		GM10, 0.5	GM10, 1		
	Mean	24.4	25.1		
	Variance	0.266667	0.544444		
	Observatio	10	10		
	Pooled Va	0.405556			
	Hypothesi:	0			
	df	18			
	t Stat	-2.45786			
	P(T<=t) on	0.012172			
	t Critical on	1.734063			
	P(T<=t) tw	0.024345			
	t Critical tw	2.100924			

Figure 10

- Drag the column to the right until the labels can be read (Fig. 11).

t-Test: Two-Sample Assuming Equal Variances		
	GM10, 0.5	GM10, 1
Mean	24.4	25.1
Variance	0.266667	0.544444
Observations	10	10
Pooled Variance	0.405556	
Hypothesized Mean Difference	0	
df	18	
t Stat	-2.45786	
P(T<=t) one-tail	0.012172	
t Critical one-tail	1.734063	
P(T<=t) two-tail	0.024345	
t Critical two-tail	2.100924	

Figure 11

- **Compare the calculated t value (t Stat) with the critical value for t (t Critical) for $\alpha = 0.05$ and with 18 degrees of freedom (df).** We might expect that with a higher concentration of cells the zone of inhibition would be equal to or smaller than the zone produced with the 0.5 McFarland culture, so a one-tailed t -test would be appropriate. Therefore, if the calculated t value (-2.45786, in this example) is less than the negative critical value for t (-1.734073), we reject the null hypothesis that there is no difference between the zones of inhibition from the 0.5 and 1 McFarland cultures.

ANOVA

To **set up a spreadsheet for ANOVA**, list the measurements of zones of inhibition made by each student according to antibiotic and McFarland culture (Fig. 12). Calculate means and standard deviations, if desired.

	A	B	C	D
1	Disk Diffusion			
2				
3	SXT, 0.5			
4	A	B	C	D
5	29	29	30	27
6	32	29	31	31
7	29	29	30	27
8	30	32	31	31
9	30	31	31	30
10	30	32	30	31
11	30	31	31	30
12	30	30	30	30
13	29	29	30	31
14	29	30	30	30
15				
16	29.8	30.2	30.4	29.8
17	0.918937	1.229273	0.516398	1.549193

Figure 12

For ANOVA, use the “Data Analysis” menu under the “Tools” menu.

- Click on “Tools” on the toolbar
- Select “Data Analysis” (Fig. 4)
- Choose “ANOVA: Single Factor” (Fig. 13).

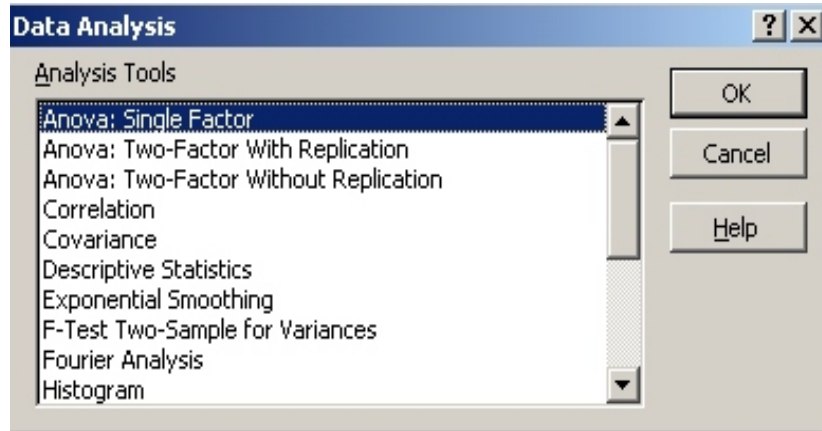


Figure 13

- For “Input Range:” select the **data in all four columns**, including the headings in Row 4.
- Make sure that “Columns” is selected in “Grouped By:.”
- Select “Labels in First Row.”
- “Alpha:” should be 0.05.
- For “Output Range” select a cell near the data. (In this example, F3.)
- When complete, the “ANOVA: Single Factor” menu should look like Figure 14. Click “OK” and a table will be generated, starting with Cell F3 (Fig. 15).

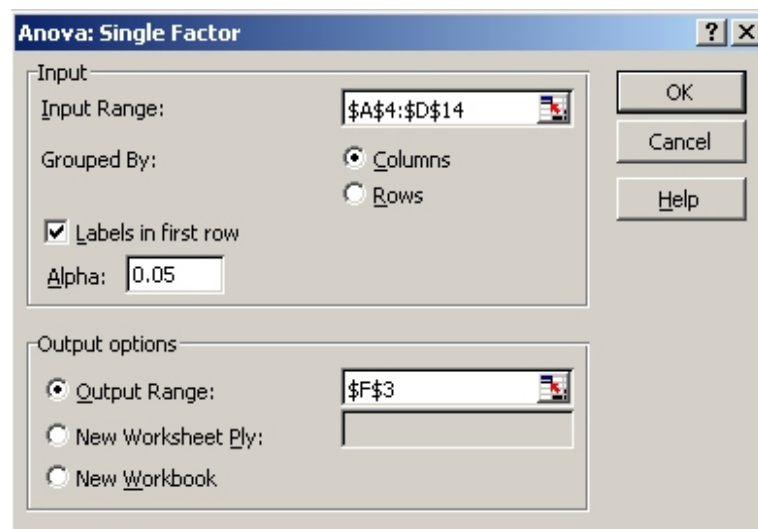


Figure 14

- **Adjust column widths** so that the table looks like Figure 15.

Anova: Single Factor						
SUMMARY						
Groups	Count	Sum	Average	Variance		
A	10	298	29.8	0.844444		
B	10	302	30.2	1.511111		
C	10	304	30.4	0.266667		
D	10	298	29.8	2.4		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2.7	3	0.9	0.716814	0.548441	2.866265
Within Groups	45.2	36	1.255556			
Total	47.9	39				

Figure 15

- **Compare the calculated F value** (0.716814, in this example) with the **critical F value** (F_{crit})(2.866265). Since the F value is less than the critical F value, we cannot reject the null hypothesis that there is no difference between the measurements of zones of inhibition made by the four group members.