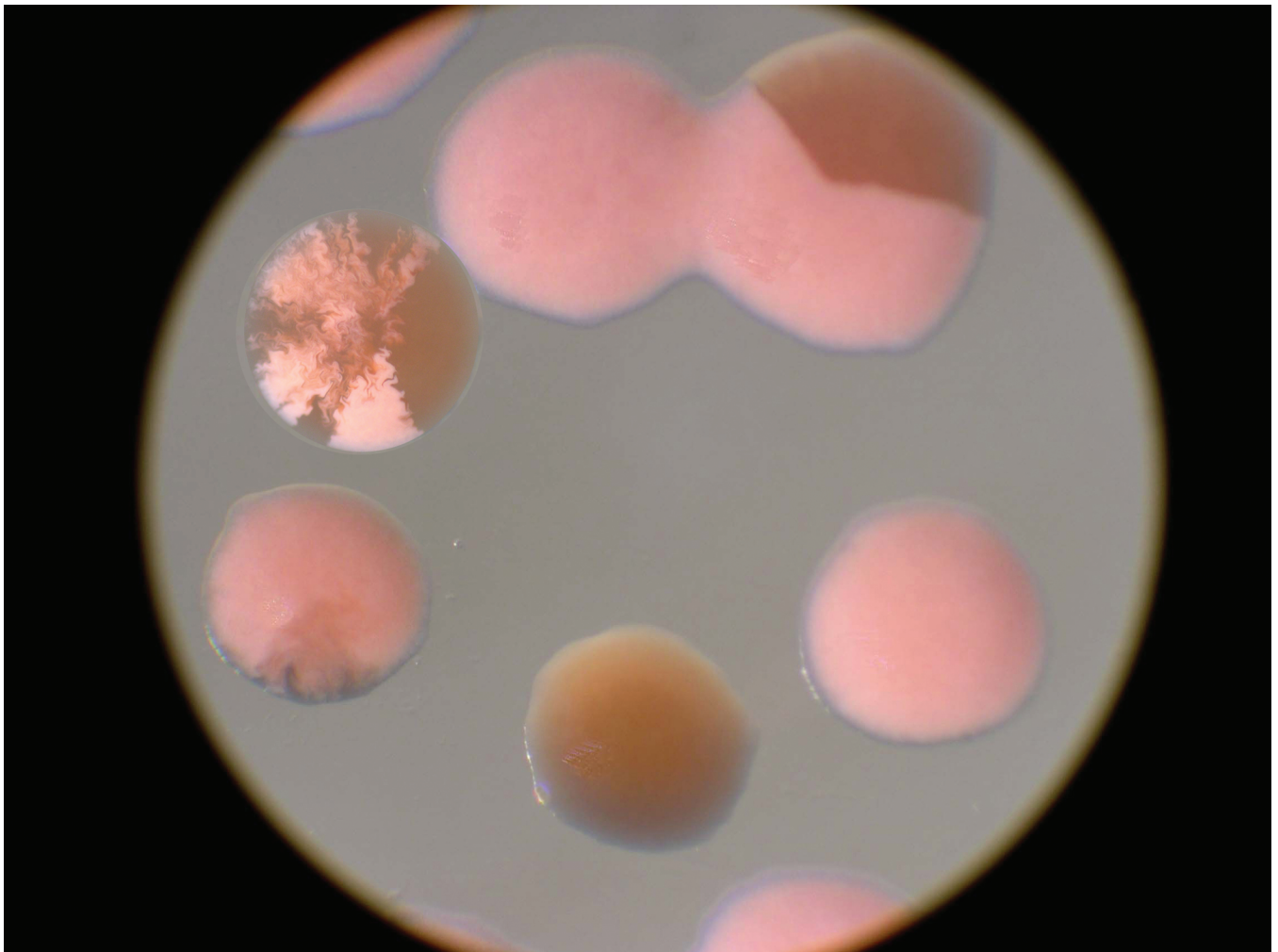


15-4772

Basic Microbiology Skills Part 2

TEACHER'S MANUAL WITH STUDENT INSTRUCTIONS



by Priya and Shiladitya DasSarma

Basic Microbiology Part 2

Teacher's Manual

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About the authors

Priya and Shil DasSarma have studied *Halobacterium* sp. NRC-1 for over 20 years. They are enthusiastic about making these safe, convenient microbes accessible to educators and students at all levels. Visit their website at http://www.carolina.com/life_science/halobacteria for more information, ideas, and contact information.

Basic Microbiology Skills Part 2

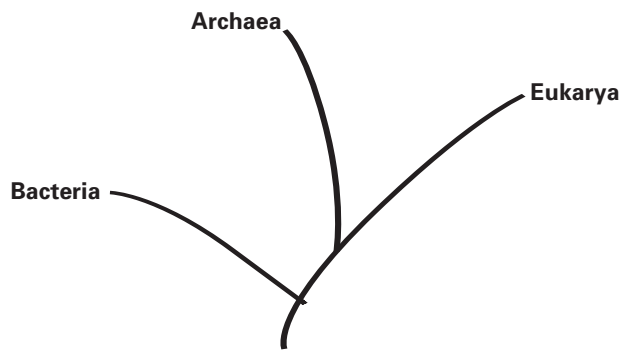
Introduction

Few microbes are as safe and easy to work with in classrooms and school laboratories as *Halobacterium* sp. NRC-1. It is nonpathogenic, and since virtually no other microbes can live in *Halobacterium*'s salty medium, contamination of cultures is not a significant concern. Furthermore, the large body of research about the microbe provides plenty of background information as well as a springboard for further investigations. As a bonus, *Halobacterium* represents an unusual and diverse group of intriguing life forms perhaps previously unknown to students—the archaean extremophiles.

Basic microbiology skills include sterile technique and the methods of maintaining and transferring microbial cultures. Basic Microbiology Skills Part 1 (RN-15-4772) covers the use of sterile technique, the streaking of agar plates, and the incubation and examination of cultures. Basic Microbiology Skills Part 2 covers the preparation of a microbial lawn and the use of serial dilution. A **lawn** is an even covering of microbial cells on a plate. Lawns are often used in testing whether a substance is toxic to particular microbes, or, in the case of motile cells such as *Halobacterium*, whether a substance attracts or repels the cells. **Serial dilution** is a technique frequently used in isolating microbes and purifying cultures. It is particularly useful in isolating pure cultures from samples in which more than one type of microbe was originally present. In this kit, the technique is used to approximate the number of viable cells in a culture.

Generally, microbes divide by **binary fission**, the splitting of one parent cell into two daughter cells, and each doubling represents a new generation. Usually, after approximately 25 generations, a single cell will form a colony visible to the naked eye. In most cases, this single colony is composed of many millions of progeny of the original parental cell. The founding parental cell of a colony is sometimes referred to as a **colony-forming unit** or cfu. The term cfu is often used in quantifying the number of viable cells in a given volume of culture (e.g., 12×10^8 cfu/mL).

More About *Halobacterium*



In recent years, most biologists have come to agree that life forms occur in three main divisions, called domains. The more familiar domains are **Eukarya** (or Eukaryota) and **Bacteria**. Less familiar may be the domain **Archaea**. Like bacteria, archaea lack a nuclear membrane that confines the cell's DNA, but archaea share some important biochemical features with eukaryotes as well. It is thought that the three domains diverged very early in the evolution of life.

Archaea includes a variety of microbes with unusual living requirements or habitat preferences. Many of these microbes are termed extremophiles because they live in environments of extreme temperature, pressure, radiation, or chemical concentrations. One archaean group consists of extreme halophiles (salt lovers). Extreme halophiles are adapted to environments with extremely high salt concentrations, such as salt marshes, natural hypersaline lakes and ponds (e.g., Utah's Great Salt Lake), solar salt production facilities, and brine inclusions in salt crystals in salt mines. *Halobacterium* sp. NRC-1 is one such organism. It requires a salt concentration (3–5 M) that for most microbes would quickly lead to osmotic stress and death. In addition to hypersalinity, *Halobacterium* tolerates high levels of UV and other solar radiation, high temperatures, and low availability of oxygen and nutrients. How does it do it?

Halobacterium thrives in hypersalinity because its cell counterbalances the NaCl content of its environment by taking in KCl. The microbe keeps its internal KCl concentration equivalent to the external concentration of NaCl, thus avoiding osmotic stress.

Halobacterium's tolerance for UV and other solar radiation is due to several factors. Red carotenoid pigments help shield the microbe from solar radiation. In addition, *Halobacterium* has active repair mechanisms that fix damage done to its DNA by UV radiation. UV radiation often causes the formation of thymine-thymine dimers in DNA, resulting in mutations. *Halobacterium* employs a process called photoreactivation, which uses visible light to activate a repair enzyme called photolyase to undo the damage done by UV light.

Halobacterium tolerates low oxygen levels and limited nutrients by using a unique energy-production mechanism that works somewhat like photosynthesis. Instead of chlorophyll, this method of producing energy uses a protein called bacteriorhodopsin, which uses the energy from light to generate ATP. This form of phototrophy, which is found in several halophilic archaea, is thought to be ancient and may predate chlorophyll photosynthesis. In order to remain exposed to the light necessary to drive its energy production, *Halobacterium* produces gas vesicles, internal structures filled with air, which float the cells to the surface of brine.

Many of the extreme conditions either tolerated or required by *Halobacterium* are similar to conditions on Mars, where ancient evaporated seas and salt deposits are likely to exist. Mars is much colder than Earth; however, since salt lowers the freezing temperature of water, it is possible that hypersaline liquid brines exist below the surface of Mars within a temperature range of -20 to -50°C . Halophiles have already been found in Antarctica at similar temperatures. The prospect that other halophiles might be able to survive on Mars or even that they may already live there is, of course, very intriguing to microbiologists and to space scientists.

Materials

Included in the kit

40 petri dishes
 8 bottles *Halobacterium* agar
Halobacterium liquid culture
 60 sterile dropping pipets
 40 sterile spreaders
 NaCl, 10 g
 8 tubes for making serial dilutions
 autoclavable disposal bag

Needed, but not supplied

hand lenses, or dissecting microscope(s)
 wax markers or lab pens
 Bunsen burners
 test tubes
 resealable plastic bags or airtight plastic food storage boxes for incubating plates
 *Incubators, 42° or 37°C

*If incubators are not available, the plates may be incubated on the lab bench. If the plates are incubated at 42°C, results may be obtained in 7 days, and if at 37°C, approximately 1–2 weeks. If the plates are incubated at room temperature on the bench, it may take 2 weeks or more to get results, depending upon the temperature of your classroom.

Note: In some circumstances gloves are worn when practicing sterile technique. In this exercise they are not necessary, but if you want to simulate sterile technique as practiced under more stringent conditions you may wish to have your students wear gloves.

Teacher Preparation

You will need to

- Read through the entire booklet to familiarize yourself with the information and instructions given to the students.
- Photocopy sets of Student Instructions for individuals or teams. You may also photocopy page 4, *More About Halobacterium*, to share with your students.
- Decide how and when to share the Explanation of Results with your students.
- As you consider the two procedures, decide how best to organize students and available supplies. Students learn two main procedures: how to prepare a lawn, and how to use serial dilutions to estimate the number of viable cells in a culture. The instructions assume that each of 32 students will make a lawn and that the serial dilution procedure will be done as a group activity or demonstration. **Note:** The lawns can be used as a source of cells from which to extract DNA (see kit RN-15-4773), or they can be used for further experimentation.

Preparing Media Plates

1. To melt the medium, slightly loosen the cap(s) and set the bottle(s) of medium in a pot of water and bring it to a boil. Make sure the water level is even with the level of the medium in the bottle(s). Leave the bottle(s) in the boiling water until the medium has completely melted. This will take approximately 30 minutes.
2. Allow the medium to cool to 55°C either by allowing the pot of water to cool to that temperature or by letting the bottle(s) sit for several minutes at room temperature. The bottle(s) should feel comfortably hot to the touch when it is around 55°C.
3. Disinfect the work surface by wiping with a disinfectant such as bleach or alcohol. Wash your hands thoroughly. Unpack the petri dishes, being careful not to disturb their sterility. Align the sterile plates along the edge of a clean, level tabletop away from any draft or breeze.
4. Remove the cap and flame the mouth of a bottle of medium. As you pour the plates, lift the lid of each petri dish just enough to pour in the molten medium. Carefully, pour to a depth of about 5 mm per plate. After pouring each plate, replace the lid immediately to prevent contamination.
5. Repeat Step 4 with the other bottle(s) of medium.
6. Let the plates stand undisturbed until they solidify (about 1 hour). Then, let the plates sit out until any condensation on the lid evaporates.
7. Store plates in an airtight container in the refrigerator. (Always store plates in airtight containers—even during incubation. Otherwise, they will dry out and salt crystals will form.)
8. Dispose of the empty bottles in an autoclave disposal bag or soak them overnight in a 10% solution of bleach before discarding.

For the lab procedure and a review of sterile technique, see the Student Instructions.

Answers to Student Questions and Explanation of Results

1. Student's reasoning about and calculations regarding the number of cfu in the original culture should resemble the example below.

Suppose that the plate with the 10^{-5} dilution has 90 colonies on it. This means that the 250 μL (5 drops is about 250 μL) of culture that was plated on that plate contained 90 cfu.

The 250 μL of culture plated onto the plate was a 10^{-5} dilution of the original culture. So, to calculate the number of cfu in 250 μL of the original culture, multiply 90 by 10^5 .

$$90 \times 10^5 = 9.0 \times 10^6$$

There are 9.0×10^6 cfu per 250 μL of the original culture. Now they need to figure out how many cfu there are in a mL. Using proportions is an easy way to figure this out.

$$\frac{9.0 \times 10^6 \text{ cfu}}{250 \mu\text{L}} = \frac{X \text{ cfu}}{1 \text{ mL}}$$

Convert 250 μL to mL and solve for X.

$$X (0.250 \text{ mL}) = (1 \text{ mL})(9.0 \times 10^6 \text{ cfu})$$

$$X = 3.6 \times 10^7 \text{ cfu}$$

2. Answers will vary.
3. This question is worded such that you may provide your students with the following information ahead of time, or you may allow them to discover the phenotypes on their own:

The *Halobacterium* colonies may display the following three phenotypes:

- **Vac⁺, or wild-type, pink colonies.** *Halobacterium* appears pink because of red carotenoid pigments and gas vesicles, internal organelles that refract light and enable the cells to float in a liquid culture. *Halobacterium* converts light energy to chemical energy using the protein bacteriorhodopsin in a process called phototrophy. The buoyancy of the gas vesicles is advantageous because it allows the cells to position themselves near the surface of natural lakes and ponds where the light is most intense. In addition, oxygen for respiration is more plentiful near the surface.
- **Vac⁻, or red colonies.** In these colonies, the genes necessary for creating the gas vesicles are mutated. Without light-refracting gas vesicles, these mutant colonies appear red and translucent. Also due to the absence of gas vesicles, the Vac⁻ mutants sink in liquid culture. *Halobacterium*'s red color is due to its pigments—mainly the red carotenoids, which largely mask the purplish bacteriorhodopsin.
- **Sectored colonies.** These colonies result from mutations that occur during a colony's growth. A sectored colony consists of a mixture of Vac⁺ and Vac⁻ segments and can resemble a pie with different-colored (red or pink) slices.

Why do these colonies look so different from one another? It is because of the presence of insertion sequences (IS elements), or “jumping genes,” which “jump” into other genes at random. The insertion of the IS element often interrupts a gene's coding sequence in a way that causes the gene to become nonfunctional. In a *Halobacterium* cell, when an IS element jumps into one of the genes that normally code for the creation of gas vesicles, the disruption can render the cell and its progeny unable to produce gas vesicles. The absence of gas vesicles results in a phenotypic (observable) change that is due to a genotypic (genetic) change—a change from pink to red cells (from cells producing gas vesicles to ones that do not) that is due to a change in the DNA sequence.

Further Investigation

If you do not use the lawns as a source of DNA for spooling, you may want to have students use them for research projects. For example, students might test the effect of various substances by dropping them directly onto plates with lawns of *Halobacterium* or by placing them on filter paper disks that can be laid on the agar surface. For example, students might place a paper disk soaked in motor oil onto a freshly plated *Halobacterium* lawn. If nearby growth were inhibited or killed (indicated by a zone of clearing around the disk) the oil is toxic to the microbes. Lack of a clear zone would indicate that the microbes were at least tolerant of the oil, if not utilizing it. If the disk became overrun with *Halobacterium*, that would indicate the microbes were attracted to the material and, being motile, moved toward it. Any number of substances could be tested in this way. As a negative control, if your students use paper disks, make sure that they also test how the microbes respond to a disk that has nothing on it.

The DasSarma Team would be happy to discuss projects with students and educators and can be reached by email: dassarma@comcast.net.

Glossary

Archaea a domain of prokaryotic life distinct from domain Bacteria. Archaea includes the extreme halophiles, organisms that grow in high-salt environments; methanogens, anaerobes that produce methane; and thermophiles, which thrive in high-temperature environments.

Bacteria the domain of prokaryotic life distinct from the organisms in the domain Archaea.

Bacteriorhodopsin a purple pigment that enables some microbes to turn solar energy into chemical energy through a form of phototrophy different from photosynthesis.

Binary fission division of a parent cell into two daughter cells.

Clone a genetic duplicate of an organism. A microbial colony consists of clones of the founding cell.

Colony a visible cluster of microbial cells, resulting from division of a founder cell, its daughters, and so on.

Colony-forming unit (cfu) a microbial cell capable of undergoing growth and division that results in a colony.

Domain one of the three broadest categories of life forms in current biological taxonomy: Eukarya, Bacteria, and Archaea.

Eukarya (or Eukaryota) the domain of life that includes the organisms whose cells contain nuclei and membranous organelles.

Lawn an even covering of microbial growth on an agar plate.

Serial dilution a method in which a series of dilutions is made, each successive one beginning with a small portion of the previous dilution; frequently used for quantifying the number of cells in a liquid culture.

**Further
Reading**

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- DasSarma, S. 2004. Genome sequence of an extremely halophilic archaeon, in *Microbial Genomes*, 383–99, C.M. Fraser, T. Read, and K.E. Nelson (eds.), Humana Press, Inc, Totowa, NJ.
- DasSarma, S., and P. Arora. 2002. “Halophiles,” *Encyclopedia of Life Sciences*, Vol. 8, 458–66, London: Nature Publishing Group.
- Ng, W.V., S.P. Kennedy, G.G. Mahairas, B. Berquist, M. Pan, H.D. Shukla, S.R. Lasky, N.S. Baliga, V. Thorsson, J. Sbrogna, S. Swartzell, D. Weir, J. Hall, T.A. Dahl, R. Welti, Y.A. Goo, B. Leithauser, K. Keller, R. Cruz, M.J. Danson, D.W. Hough, D.G. Maddocks, P.E. Jablonski, M.P. Krebs, C.M. Angevine, H. Dale, T.A. Isenbarger, R.F. Peck, M. Pohlschroder, J.L. Spudich, K.H. Jung, M. Alam, T. Freitas, S. Hou, C.J. Daniels, P.P. Dennis, A.D. Omer, H. Ebhardt, T.M. Lowe, P. Liang, M. Riley, L. Hood, S. DasSarma. 2000. Genome sequence of Halobacterium species NRC-1. *Proc. Natl. Acad. Sci. USA* 97:1217612181.
- The HaloEd Project: A Web site dedicated to biotechnology education
<http://zdna2.umbi.umd.edu/~haloed/>
- http://www.carolina.com/life_science/halobacteria/index.asp

Basic Microbiology Skills Part 2

Introduction

For this exercise you will use a pure culture of *Halobacterium* sp. NRC-1. *Halobacterium* is a member of the **Archaea** and grows in extremely salty environments. Because it causes no known disease in humans, and because most other microbes will not survive the high salt concentration of its growing medium, *Halobacterium* is a good microbe for learning microbiology techniques. Using these microbes allows you to practice microbiology techniques without the concern that you will culture a pathogen or contaminate your workspace with the microbe you are using. However, part of learning good microbiology skills is to practice good sterile technique, so you should use the same care that you would use if you were working with a known pathogen.

In this laboratory activity, you will use a *Halobacterium* sp. NRC-1 culture to produce a microbial lawn on a petri plate. A **lawn** is a uniform growth of microbial cells evenly covering the surface of a medium. Lawns are often used in studies of the effect of various chemicals, such as antibiotics, on microbes. You will also learn how to make **serial dilutions** and to plate each dilution on agar as a means of determining the number of viable cells in a microbial culture. The dilutions serve to distribute a small enough number of microbes on at least one of the plates so that the resulting colonies can be accurately counted.

Generally, microbes divide by **binary fission**, the splitting of one parent cell into two daughter cells, and each doubling represents a new generation. Usually, after approximately 25 generations, a single cell will have produced a colony visible to the naked eye. In most cases, this single colony is composed of millions of clones of the original parental cell. The founding parental cell of a colony is sometimes referred to as a **colony-forming unit** or **cfu**. The term **cfu** is often used in quantifying the number of viable cells present in a given volume of microbial culture.

Sterile Technique

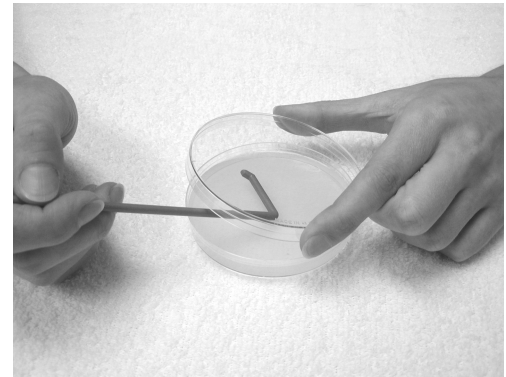
1. Before beginning, wipe your work area with a disinfectant such as alcohol or bleach and wash your hands with soap and water.
2. Light your burner. Openings (lips) of test tubes and flasks must be flamed during the transfer of media or cultures. Heating the container directs air convection currents upward and away from the opening, momentarily preventing airborne contaminants from entering. It is essential to perform the transfer quickly, before the opening cools. Hold test tubes at a 45° angle in your left hand if you are right-handed, or in your right hand if you are left-handed. Use your other hand to hold your transferring instrument (in this case a pipet) and to remove and hold the cap with your little finger. Flame the top of the tube. Take out the inoculum (culture you are transferring), reflare the opening, and replace the cap.
3. When working with petri plates, always hold the lid over the plate to prevent contaminants from landing on the surface of the agar. Always place the lid back on as soon as possible. Realize that the lid is designed to fit loosely to allow diffusion of air around the edges, while minimizing the possibility of contamination. When you lift the lid, hold it directly over the plate. Do not place the lid on the bench top.
4. When finished, turn off the burner and again wipe your work area with a disinfectant and wash your hands.

5. Dispose of microbial cultures in a safe manner. This usually involves either autoclaving all lab ware that comes in contact with cultures or soaking it in a 10% bleach solution before disposal.

Procedures

Establishing a Lawn of *Halobacterium* sp NRC-1

1. Review sterile technique, as described above. Since live microbes will be used, it is vital that you follow good laboratory technique.
2. Start with a clean, organized, and disinfected work surface. Wipe down the laboratory benchtop with a disinfectant such as bleach or ethanol.
3. Label your plate on the bottom of the smaller half of the petri dish. Make sure the date, type of organism plated, and your initials are clearly written along the edge, so as not to impede viewing the culture as it grows.
4. Remove the cap of the starter culture and flame the lip. Aspirate several drops of culture into a sterile disposable pipet.
5. Uncover the petri plate. Hold the lid over the plate while you work.
6. With the sterile pipet, place 5 drops (~250 μ L) of the starter culture onto the center of the agar plate.
7. With a sterile spreader, spread the drop evenly across the surface of the plate. Use a smooth, circular motion.
8. Replace the cover on the petri plate.
9. Let the covered plate sit for 30 minutes to allow the cells to adhere to the agar.
10. Now, turn the petri plate upside-down to prevent any condensation from dripping onto the agar surface.
11. Your inverted plate along with those of your classmates will now be placed together in airtight plastic boxes or bags and incubated. (Incubation time will range from 3 days to 2 weeks or longer, depending on the temperature at which the plates are incubated.)
12. After overnight incubation, check the plates for dryness. If there is condensation, leave the plates out of the airtight container until the condensation has dried.
13. After incubation, the surface of the entire plate should appear pink. This uniform microbial growth is called a lawn.



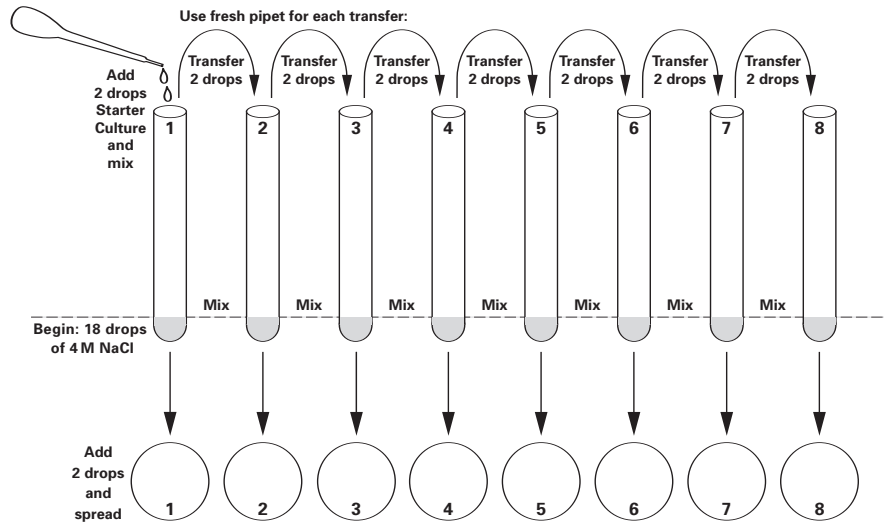
Spreading the drop with a sterile spreader.

Using Serial Dilution to Estimate the Number of Viable Cells Per mL of Culture

There are enough plates remaining so that as a group you can perform serial dilutions to estimate the number of cfu/mL within the original liquid culture of microbes. (Alternatively, this activity may be demonstrated by your teacher.)

1. Make a 4 M solution of NaCl by measuring 2.5 g NaCl and adding sterile water to a final volume of 10 mL.
2. Number eight tubes, from 1 to 8. You will create a series of eight different dilutions as shown in the diagram and described in step 3.

- 1—1:10
- 2—1:100
- 3—1:1000
- 4—1:10,000
- 5—1:100,000
- 6—1:10⁶
- 7—1:10⁷
- 8—1:10⁸



3. Make the dilutions:

A. With a pipet, add 18 drops of the 4 M NaCl solution to each labeled tube.

From this point forward, use a fresh, sterile pipet for each dilution so that you do not cross-contaminate your dilutions.

B. Add 2 drops of starter culture to tube 1 and mix well.

C. With a fresh pipet, add 2 drops of the 1:10 dilution from tube 1 to tube 2 and mix well. Now, tube 2 contains a 1:100 dilution.

D. With a fresh pipet, add 2 drops of the 1:100 dilution from tube 2 to tube 3 and mix well. Now, tube 3 contains a 1:1000 dilution.

E. With a fresh pipet, add 2 drops from tube 3 to tube 4 and mix well.

F. With a fresh pipet, add 2 drops from tube 4 to tube 5 and mix well.

Continue this process through tube 8, which will have a dilution of 1:10⁸.

4. Number eight petri plates, from 1 to 8. Using a fresh pipet and spreader for each plate, spread 2 drops (about 100 μ L) of each dilution on its corresponding plate. Follow the spreading directions you used previously in making lawns.
5. Incubate the plates until colonies form. This may take 1–2 weeks or longer, depending upon your

incubation conditions.

Analysis and Follow-up

Date: _____

Name: _____

1. Count the number of colonies on the plates that were plated using the serial dilutions. Some of the plates will have too many colonies to count accurately. Other plates may have too few colonies to be statistically accurate. Calculate the colony-forming units/mL in the starter culture using numbers from plates with between 50 and 150 colonies on them. As you are calculating the number of cfu, remember that each drop is approximately 50 μL .
2. Think of an experimental question that you could investigate using the materials you have used in this activity along with common household items and/or substances.

Write your hypothesis:

List the materials that you would use:

Write out the methods you would use, step by step. Remember to include controls.

3. Use a hand lens or a dissecting scope to observe individual colonies on plates that have well-separated colonies. Do all of the colonies look alike? Describe the appearance of the colonies you see.

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