DILUTIONS

The number of microorganisms in an undiluted broth culture after a 24-hour incubation period is usually in the **millions**. In order to determine the actual number of organisms in the tube or culture flask, it is necessary to dilute the culture to a point where there are a few hundred organisms per milliliter, or to a point where the number of organisms plated onto (or into) an agar medium will be statistically valid.

Both spread plates and pour plates are utilized to obtain bacterial counts. A spread plate is made by careful, aseptic pipetting of a known volume of sample (usually 0.1 or 1.0 ml) onto an appropriate culture medium and spreading the liquid with a sterile glass spreader. A pour plate is made by adding a known amount of either the original or diluted sample to a tube of melted agar, mixing the tube and pouring the mixture into a sterile petri plate. When bacteria are plated on a medium that supports growth, only **viable** cells will grow, multiply and form colonies. Each of the colonies is presumed to have arisen from only one cell, although this may not be true if pairs, chains or groups of cells are not completely broken apart before plating. For this reason, the number of viable cells on either spread plates or pour plates are usually given as **colony-forming units** (CFU)**/ml**, not as cells/ml. If the number of viable cells on a plate is too great, the colonies will merge and be impossible to count, or some cells will have insufficient room to form colonies, and the count will be erroneous. At the same time, it is important that the number of cells or colonies not be too few. For statistical validity, it is recommended that **only plates with between 30 and 300 colonies be counted**. Normally, in order to obtain this number, the original culture must be diluted several fold.

Dilutions are usually made ten-fold, hundred-fold or multiples thereof; that is, the most common dilutions are 1/10, 1/100, and 1/1000. As an example, if a ten-fold dilution is to be made, it is feasible to use 0.5 ml of sample in 4.5 ml of diluent or 1.0 ml of sample in 9.0 ml of diluent. The latter gives the fraction:

$$\frac{\text{1 ml of sample}}{\text{1 ml of sample + 9 ml of diluent}} = \frac{\text{1 ml}}{\text{1 ml + 9 ml}} = \frac{1}{10} = 10^{-1}$$

If 100-fold dilutions are to be made, one can use 0.05 ml of sample in 4.95 ml of diluent, or 0.1 ml in 9.9 ml of diluent, or 1.0 ml in 99 ml of diluent.

Generally, a dilution of 1/1,000,000 (10^{-6}) is sufficient to decrease the number of viable cells to the point that an appropriate number of colonies will develop on solid medium. A 10^{-6} dilution can be achieved by making three 1:100 dilutions, or six 1:10 dilutions, or a combination of 100-fold and 10-fold dilutions.

When following either of the above procedures, it must be remembered that in order to achieve a 10^{-6} dilution, **1.0 ml** of sample from the last dilution must be plated. If you plate only **0.1** ml of sample, you have diluted the sample another 10-fold, and the dilution would be 10^{-7}.

The liquid used as the diluent must be one that does not cause harm to the microorganisms. Tap water, distilled water or deionized water may produce hypotonic conditions and cause microorganisms to swell and lyse. It is preferable to use the same type of medium in which the organisms are growing, but to avoid this rather expensive procedure, the diluent may be made of phosphate buffers or saline. The diluent **must** always be sterile and should be at room temperature.

FIRST PERIOD

Material:

1. 1-ml pipets
2. Seven 9-ml dilution tubes of sterile saline
3. Two melted agar talls (50°C)
4. Two sterile petri plates
5. Culture of *Serratia marcescens*

Procedure: (work in pairs)

1. From the tube marked *Serratia marcescens*, take 1.0 ml of culture and transfer it into a 9-ml dilution tube marked 10^{-1}. Mix the solution completely with a vortex mixer or by rolling the tube between your hands, being careful not to spill any of the contents.

2. After changing your pipet, now take 1.0 ml from the 10^{-1} dilution and transfer it into the dilution tube marked 10^{-2}. Mix well in the same manner as described above.

3. Change your pipet again. Why keep changing pipets? Because any fluid left in the pipet from the previous dilution will contain many more cells per milliliter than any successive dilution and, if used, will grossly confuse the final results. Now, transfer 1.0 ml from the 10^{-2} dilution, transfer it to the tube marked 10^{-3}, and mix well.

4. Changing your pipet between each tube, continue your dilution series through 10^{-7}.

5. Remove two agar talls from the water bath or insulated container.

6. To one of the agar talls, add 1.0 ml from the tube marked 10^{-6}, mix thoroughly, and carefully pour the contents into a sterile petri dish.

7. To the other agar tall, add 1.0 ml from the tube marked 10^{-7}, mix thoroughly, and pour the contents into the other sterile petri dish. Do **not** move these petri dishes until the agar has hardened.

8. Mark the petri dishes with your name and the appropriate dilution.

9. After the agar has hardened, invert the plates and incubate them for 2 days at 25°C.

SECOND PERIOD

Material:

1. Colony counter

Procedure:

1. Count the number of colonies on each plate and record.

Results:

PLATE	Number of Colonies
10^{-6}	
10^{-7}	

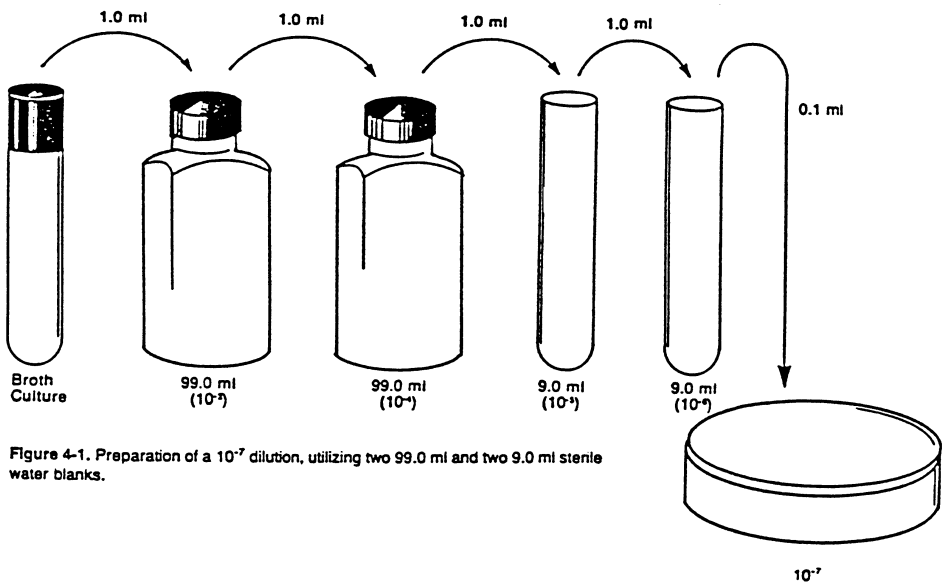


Figure 4-1. Preparation of a 10^{-7} dilution, utilizing two 99.0 ml and two 9.0 ml sterile water blanks.

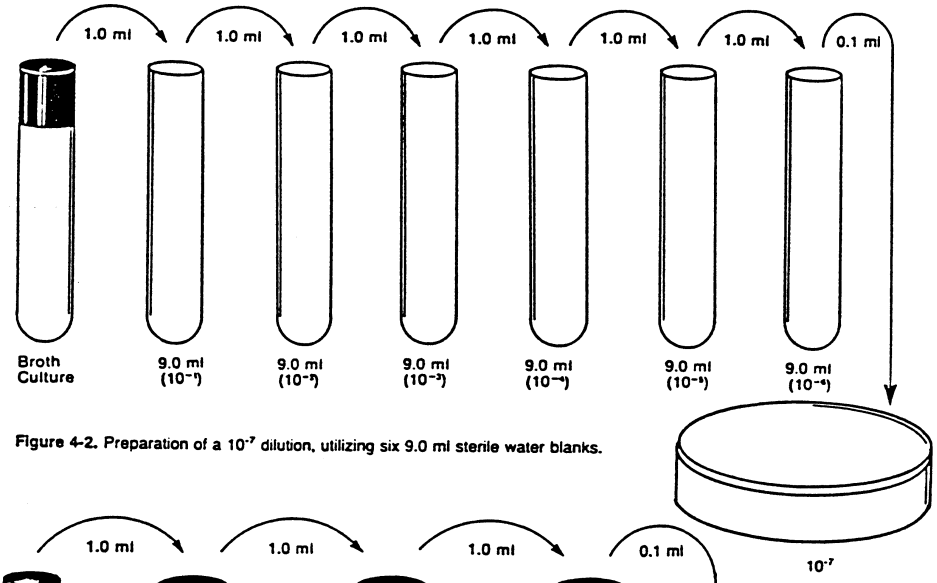


Figure 4-2. Preparation of a 10^{-7} dilution, utilizing six 9.0 ml sterile water blanks.

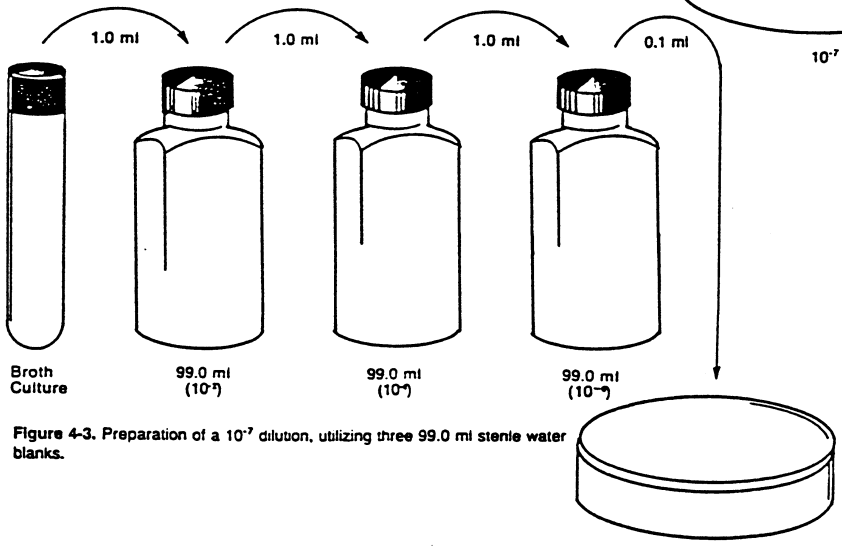


Figure 4-3. Preparation of a 10^{-7} dilution, utilizing three 99.0 ml sterile water blanks.

PREPARATION OF CULTURE MEDIA

A culture medium provides the basic nutritional requirements of bacteria. The essential requirements of all microorganisms include water, four elements, an energy source, several minerals, and vitamins or vitamin-like compounds. The four elements (carbon, nitrogen, phosphorus and sulfur) may be provided by organic or inorganic compounds. Similarly, the energy sources for the growth of bacteria may be either organic or inorganic substances. Minerals (or metallic elements) such as sodium, potassium, calcium, magnesium, manganese, iron, zinc, copper and cobalt are required in low concentrations.

Media used for the cultivation of bacteria, as well as for the cultivation of other microorganisms, may be liquid, solid or semi-solid. The ingredients may range from highly purified chemical compounds (**defined media**) to complex materials such as milk, diluted blood, or digests of plant and animal tissues (**complex media**). The most common ingredients of media used in routine microbiological work are beef extract and peptone for liquid media, and beef extract, peptone and agar for solid media.

Liquid media, such as nutrient broth and tryptic soy broth, are used for the propagation of large numbers of microorganisms. Solid media, such as nutrient agar and tryptic soy agar, are used for surface growth of microorganisms; this aids in pure culture isolations and observation of colonial morphology. Agar or gelatin may be used as solidifying agents. Gelatin is less suitable because it is a protein which melts at temperatures slightly above 25°C; it is also digested by many organisms. Agar is a complex polysaccharide extracted from marine algae. Agar is not digested by most microorganisms and, after solidification, will remain solid at temperatures up to 100°C. Semi-solid media often contain both gelatin and agar in lower concentrations (usually less than 1%) than found in solid media. Semi-solid media may be used for motility and fermentation tests.

In order to perform work in a microbiology laboratory, the culture media and glassware must be sterilized. **Sterilization** is a chemical or physical process by which all living microorganisms are eliminated from a material. The most common method of sterilizing media is **autoclaving**, whereby moist heat is applied under pressure (see diagram of autoclave). The pressure allows

temperatures in excess of 100°C to be obtained without the occurrence of boiling. As the pressure is increased, the temperature increases. The heat irreversibly modifies cellular enzymes and other proteins, thus killing the organisms. Since proteins are affected, viruses are also destroyed in the process. Even bacterial endospores, the most heat-resistant component of some cells, are killed by extreme temperatures. The moisture conducts heat better than dry air and aids in the denaturation of proteins by disrupting intramolecular peptide bonds. The conditions for autoclaving are generally **15 pounds per square inch (PSI) of pressure at 121ºC for 15 to 20 minutes**.

Sterilization by dry heat, filtration or gases can also be employed. **Dry heat** is used for objects such as glass petri plates and pipets which are not damaged by high heat. Because no moisture is present, there is a slower penetration of porous materials and less denaturation of proteins. For example, to kill endospores by dry heat, a temperature of 165-170°C must be maintained for 2 hours.

Filtration and exposure to gases are used on solutions and objects that could be damaged by heat. **Filtration** is used to sterilize **heat-labile** liquids such as antibiotics or vitamins. Filters vary in diameter, pore size and composition. The most common membrane filters are made of cellulose acetate or cellulose nitrate. The most commonly used pore size for sterilization of liquids is 0.45 μm, although filters with 0.2 μm pores may be used in certain situations. It is important to note that filter sterilization does **not** remove viruses. Viruses, however, are rarely a problem in media preparation. Prior to use, the filter, filter holder and receiving container must all be sterilized (usually by autoclaving). However, pre-sterilized, disposable filter units are now available from a large number of vendors of microbiological equipment and supplies. **Gases** such as ethylene oxide are used under pressure to sterilize plastic ware, delicate instruments, etc. The action of ethylene oxide is much slower than that of moist or dry heat. The gas is also highly explosive when mixed with air and so must be mixed with 90% CO_2 instead.

Today there are only a few microbiological media that must be prepared from separate raw ingredients. Almost all referenced media are available in a dehydrated form, which requires only the addition of water, adjustment of pH and sterilization. During this exercise, each group of students will prepare one of the basic kinds of media (nutrient broth, nutrient agar slants and nutrient agar plates) used for isolation and growth of microorganisms.

GENERAL INFORMATION ABOUT MEDIA PREPARATION

1. Unless otherwise noted, use distilled water.

2. Add powdered media mixture to the water, and be sure that it is fully dissolved.

3. If the medium contains agar and must be dispensed before sterilizing, heat the medium, with constant stirring to boiling on a hot plate. It generally requires about 20 minutes to completely dissolve 15 grams of agar per liter of medium in a one-liter quantity. Allow more time for larger volumes. Be sure to keep stirring from the bottom with a glass stirring rod so that the medium does not char on the bottom of the container. Do not heat the medium longer than necessary, and mix the melted agar medium well before dispensing.

4. If the medium contains agar and does not need to be dispensed before sterilizing, it can be placed directly in an autoclave. For volumes up to 2 liters, the agar will dissolved during the sterilizing process. After autoclaving, it is important to swirl the flask containing the medium in order to mix the layer of dissolved agar on the bottom of the flask. Try to avoid swirling so vigorously that bubbles are formed, since they will be transferred to the petri plates.

5. Since liquid expands when heated, the volume of liquid in any container to be sterilized in an autoclave should never exceed 2/3 or 3/4 of the container volume. For example, do not put more than 750 ml of liquid in a 1-liter flask. If specially designed media bottles are used, screw caps down tight to prevent boil-over due to minimal headspace.

6. Dispensing media into tubes is easier if an automatic pipetter is used. However, when hot liquids are being dispensed, the operator should be extra careful as splashing often occurs.

7. Tubes, bottles and flasks should be covered with self-venting closures, which allow for release of pressure during sterilization in an autoclave. Examples of self-venting closures are cotton or Styrofoam plugs, plastic caps, screw caps left slightly loose, and foil. This is not necessary when using media bottles, where heat and pressure are transferred across reinforced glass and cap.

GENERAL STEPS FOR AUTOCLAVING

1. Place material to be sterilized in the autoclave. Be sure all containers are heat- and pressure-resistant and that all closures are self-venting. Containers with liquids should not be more than 75% full. To allow maximum heat exchange, allow enough space between each container for steam to contact all sides.

2. Close the autoclave door firmly. If the door is closed properly, no steam or liquid should escape during the actual autoclaving.

3. Read and follow the directions carefully for use of your particular autoclave. If the autoclave has automatic controls, set the timer for the length of time appropriate for the volume of material to be autoclaved (but **not** for less than 15 minutes) and set the exhaust routine for "slow exhaust" for loads containing liquids or "fast exhaust" for dry loads such as empty tubes or flasks. Slow exhaust is required for liquids because during sterilization the liquid is at 121°C, and is therefore approximately 21°C above its boiling point at atmospheric pressure. Slow exhaust allows the liquid to cool below its boiling point as the pressure decreases. The super-heated liquid will boil over if you use a “fast exhaust” routine.

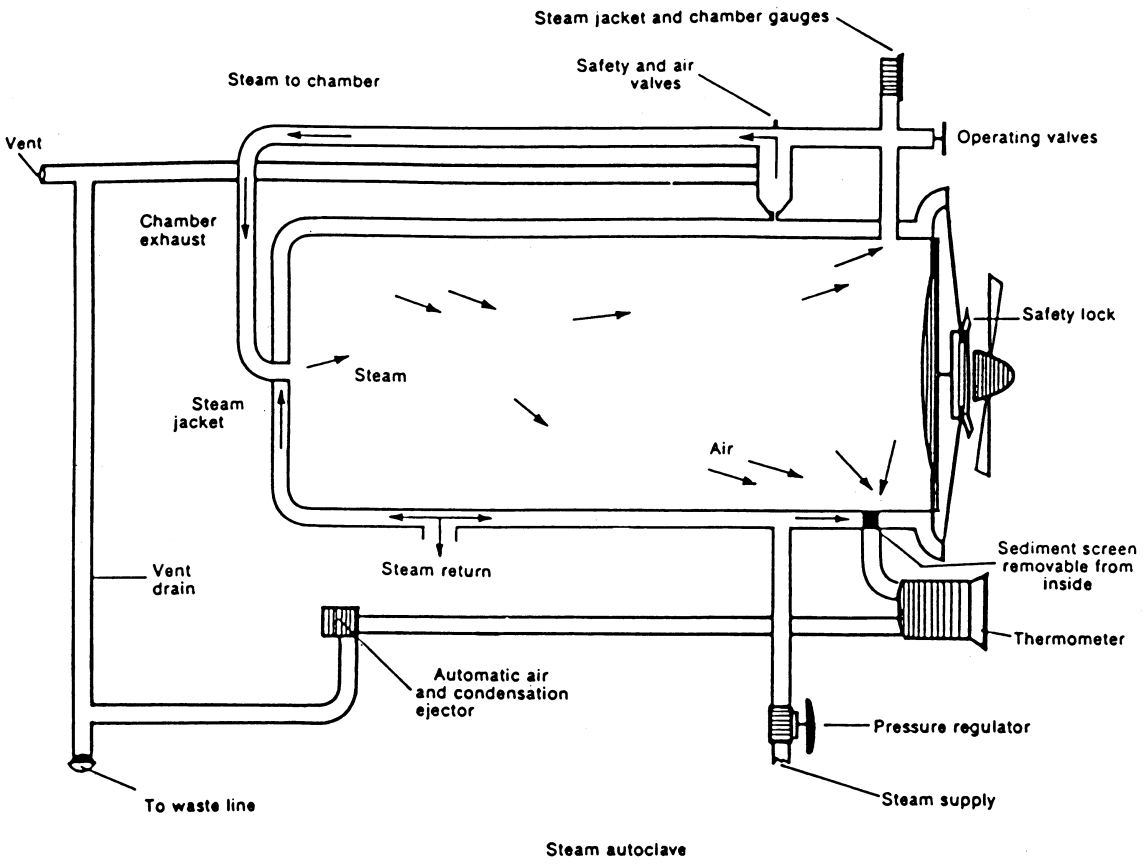
4. Turn on the steam (or if automatic, set to start). Begin timing when the temperature reaches 121°C.

5. For non-automatic autoclaves: after the appropriate time, turn off the steam. For slow exhaust, do not open the exhaust vent; for fast exhaust, open it.

6. Allow the autoclave to exhaust properly (indicated by a return to 0 on the pressure gauge on non-automatic autoclaves or by a signal on automatic ones). Open the autoclave carefully, keeping your arms and face away from the door to avoid being burned by any steam that may leave the autoclave as the door is opened.

7. Using properly insulated gloves, remove the material from the autoclave. Do not remove any containers while the liquid is still boiling, as the liquid in the containers may boil out and cause a burn.

Tubes which contain media for agar slants should be tilted in baskets or racks for cooling. Flasks containing media for plates should be placed in a 50-55°C water bath until cool enough for pouring.



Steam autoclave

ISOLATION OF PURE CULTURES

A **pure culture** theoretically contains a single bacterial species. There are a number of procedures available for the isolation of pure cultures from mixed populations. A pure culture may be isolated by the use of special media with specific chemical or physical agents that allow the enrichment or selection of one organism over another. The *differential* and *selective* procedures will be utilized later in this course. Simpler methods for isolation of a pure culture include: (i) spread plating on solid agar medium with a glass spreader and (ii) streak plating with a loop. The purpose of spread plating and streak plating is to **isolate** individual bacterial cells (colony-forming units) on a nutrient medium.

Both procedures (spread plating and streak plating) require understanding of the aseptic technique. **Asepsis** can be defined as the absence of infectious microorganisms. However, the term is usually applied to any technique designed to keep unwanted microorganisms from contaminating sterile materials.

FIRST PERIOD

Material:

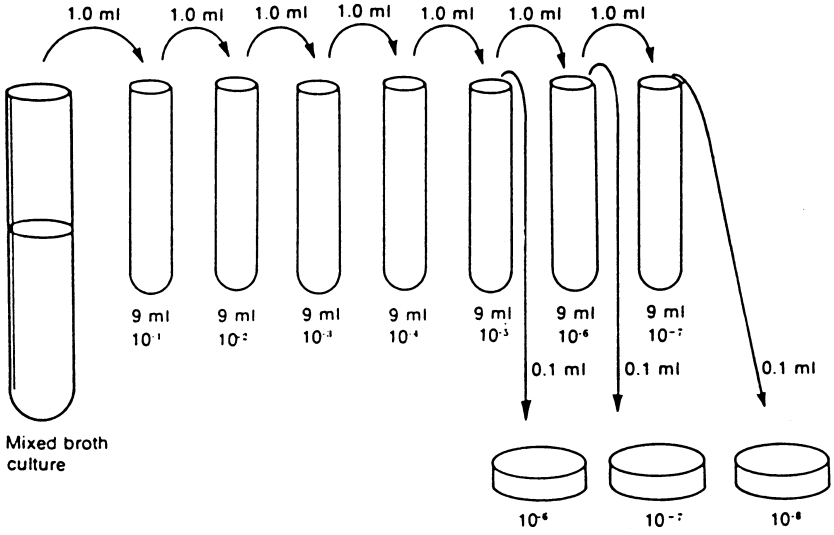
1. Seven 9-ml dilution tubes of sterile saline
2. Seven nutrient agar plates
3. 1.0 ml and 0.1 ml pipets
4. Glass spreader aka “hockey stick”
5. 95% ethyl alcohol in glass beaker (**WARNING**: Keep alcohol away from flame!!)
6. Mixed overnight broth culture of *Staphylococcus aureus* and *Serratia marcescens*

Procedure: (work in pairs)

A. Spread Plate Technique

In this technique, the number of bacteria per unit volume of sample is reduced by serial dilution *before* the sample is spread on the surface of an agar plate.

1. Prepare serial dilutions of the broth culture as shown below. Be sure to mix the nutrient broth tubes before each serial transfer. Transfer **0.1 ml** of the final three dilutions (10^{-5}, 10^{-6}, 10^{-7}) to each of three nutrient agar plates, and label the plates.



Preparation of 10^{-6} to 10^{-8} dilutions utilizing seven 9 ml sterile nutrient broth blanks.

2. Position the beaker of alcohol containing the glass spreader **away from the flame**. Remove the spreader and very carefully pass it over the flame just once (lab instructor will demonstrate). This will ignite the excess alcohol on the spreader and effectively sterilize it.

3. Spread the 0.1 ml inoculum evenly over the entire surface of one of the nutrient agar plates until the medium no longer appears moist. Return the spreader to the alcohol.

4. Repeat the flaming and spreading for each of the remaining two plates.

5. Invert the three plates and incubate at room temperature until the next lab period.

B. Streak Plate Technique

The streak plating technique isolates individual bacterial cells (colony-forming units) on the surface of an agar plate using a wire loop. The streaking patterns shown in the figure below result in continuous dilution of the inoculum to give well separated surface colonies. Once again, the idea is to obtain isolated colonies after incubation of the plate.

1. Label two nutrient agar plates No. 1 and No. 2.

2. Prepare two streak plates by following two of the 3 streaking patterns shown in the figure below. Use the $\mathbf{10^{-1}}$ dilution as inoculum.

3. Invert the plates and incubate at room temperature until the next lab period.

Various methods of preparing streak plates for the individual organisms.

C. Exposure Plates

Exposure of sterile media to the environment will demonstrate the importance of aseptic technique.

1. Label two nutrient agar plates as "Exposure I" and "Exposure II."

2. Uncover the plate marked "Exposure I" and allow it to remain exposed in the lab for about 5 minutes.

3. Expose the plate marked "Exposure II" to a source of possible contaminants. Use your imagination: cough or sneeze, place your fingers on the surface of the agar, etc.

4. Invert the plates and incubate at room temperature until the next lab period.

SECOND PERIOD

Material:

1. Colony counter

Procedure:

A. Spread Plate Technique

1. Count the number of colonies on each plate and record.

DILUTION	Red Colonies	White Colonies	Total Number
10^{-6}			
10^{-7}			
10^{-8}			

B. Streak Plate Technique

1. Observe plates. Did you obtain isolated colonies on the agar plates which were streaked with *Serratia marcescens*? Which streaking technique do you prefer? If you did not obtain isolated colonies, what changes should you make in your technique to ensure isolated colonies?

C. Exposure Plates

1. Observe plates. Describe the morphology, size and color of representative colonies.

COLONIAL MORPHOLOGY

Both colonial and cellular morphology are characteristic of each species of bacteria and are sometimes useful in the identification of an unknown microorganism. When a bacterium grows on a solid agar surface, the number of cells increases until a visible mass of cells, called a colony, appears. It is usually inferred that each colony arises from the division of a single cell. The most useful culture characteristics are morphology, size and pigmentation of the colony. The figures presented below illustrate some of the morphological characteristics of bacterial colonies and provide helpful terminology for the description of colony morphology.