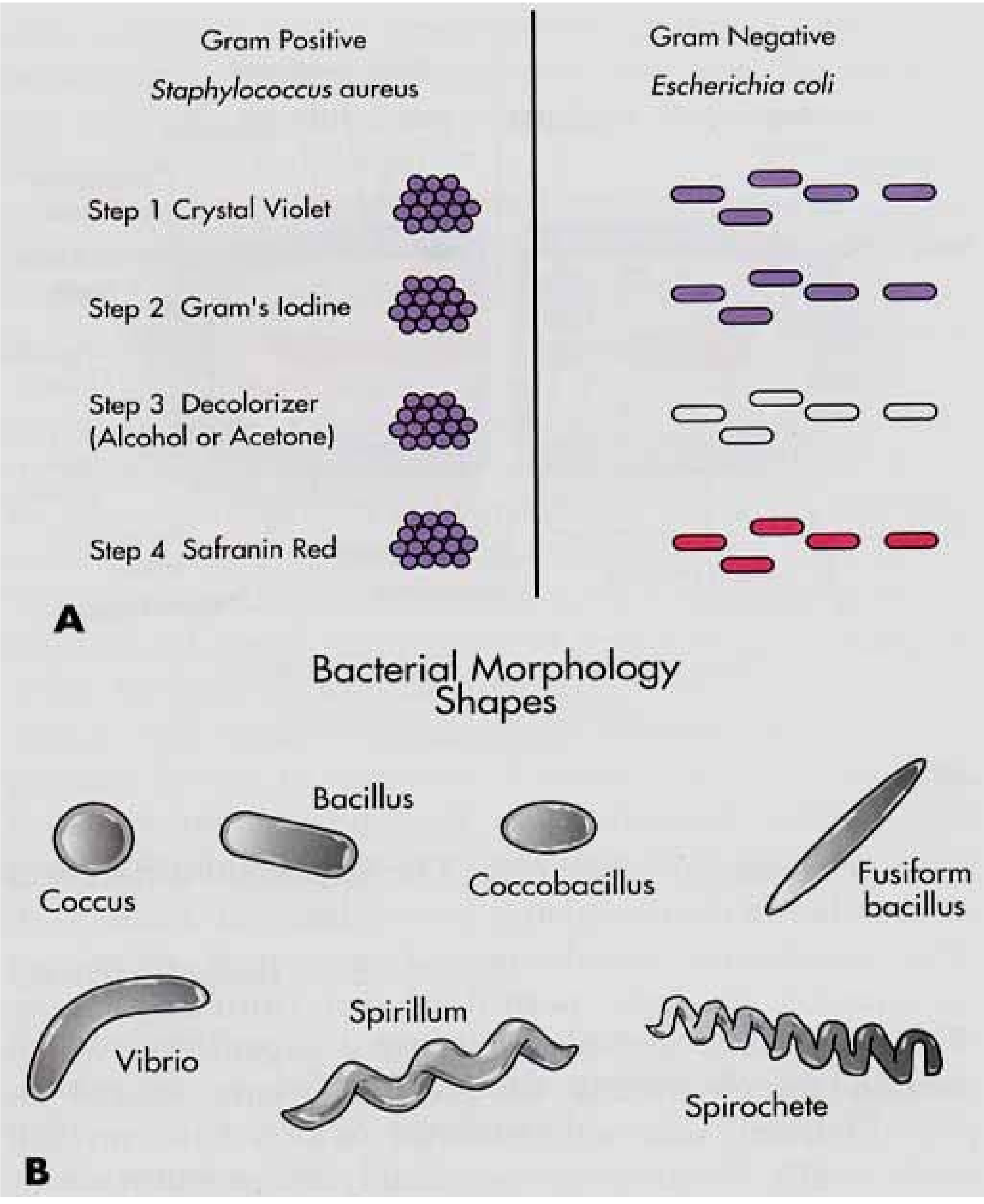
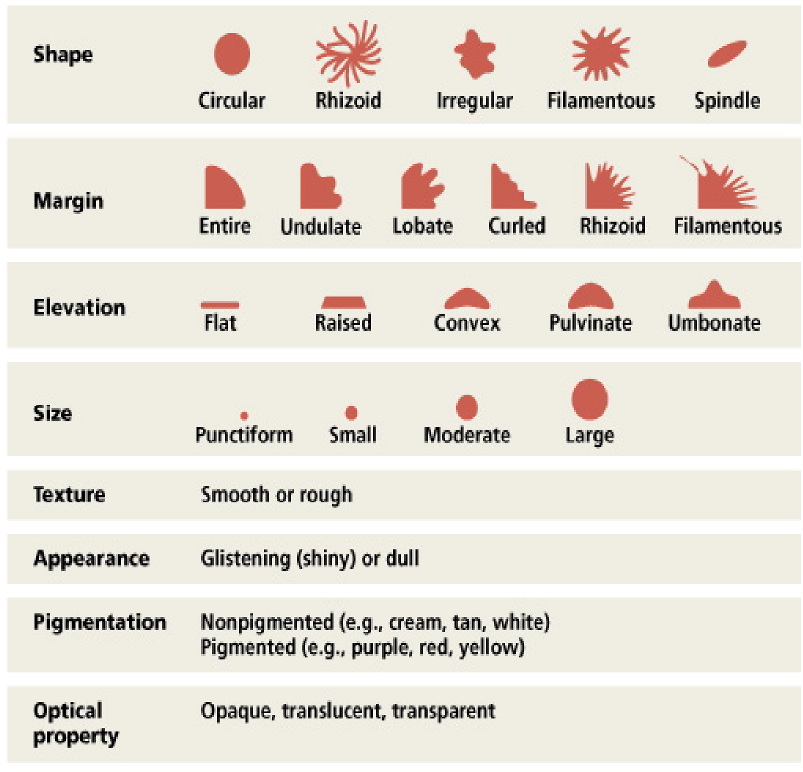
APPENDIX I

Cellular and colonial morphology



Colony morphology



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APPENDIX II

USE OF THE SPECTRONIC 20

The Bausch and Lomb Spectronic 20 is a single beam spectrophotometer. All reliable measurements made on this instrument should be measured against a blank. A blank contains all the components (water, buffer, etc.) less the material whose absorbency or less the cells whose turbidity is being determined. For most uses a water blank is sufficient. The blank is kept ready during the experiment to adjust (re-zero) the instrument to 0.000 OD (Absorbancy scale) just prior to making an experimental measurement. To make reliable measurements you will need two cuvettes: one for the water blank and the other for the experimental measurements. Even if the instrument simply sits between measurements, it can "drift" and needs readjustment. Also, when changing the wavelength, the instrument must be readjusted to zero OD

The Spectronic 20 contains two scales: **Transmittance** and **Absorbance**. Transmittance is given in % Transmittance; while this has some usefulness, %T is not directly proportional to concentration or turbidity. Absorbance is directly proportional to concentration or turbidity. Use the Absorbance scale.

Using the Spectronic 20.

1. Turn on the instrument and allow it to warm up for at least 10 to 15 minutes.

2. Select the wavelength you will use.

3. With **no cuvette** in place, adjust the left front knob so that the needle is aligned with an Absorbance of infinity (0 %T). This is the dark current adjustment.

4. **Mark a cuvette** with a vertical short line at the top with a wax marking pencil. Put this cuvette containing more than 2.5 ml of water into the Spect 20 so that the wax line is aligned with the alignment mark on the cuvette holder. Adjust the right adjustment knob so that the needle aligns with zero Absorbance.

5. Remove the cuvette, readjust the darkcurrent if necessary.

6. Place in **another marked cuvette** with water this will become the experimental sample cuvette. If the cuvettes are similar, you should get zero Absorbance. If you observe greater or less absorbance, the cuvettes are not matched and you should repeat this with another tube to find one that matches the water blank.

7. Add 2.5 ml or more of the experimental sample, place in the Spect 20 with marks aligned and observe the OD.

APPENDIX III

MEDIA INGREDIENTS

Ames Test Minimal Medium

Glucose	20 g
Agar	15 g
$MgSO_4 \cdot 7H_2O$	0.3 g
Citric Acid	3 g
K_2HPO_4	15 g
$Na(NH_4)HPO_4$	5.25 g
Distilled Water	1000 ml

Asparagine Minimal Broth

Asparagine	2 g
K_2HPO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Tap Water	20 ml
Distilled Water	980 ml

Asparagine Minimal Agar

Agar	15 g
Asn Min-Broth	1000 ml

Blood Agar

BHI Broth	37 g
NaCl	10 g
Agar	15 g
Blood*	50 ml
Distilled Water	1000 ml

* Human, Bovine, Sheep or Rabbit blood, sterile, is added after all other ingredients have been mixed, sterilized by autoclaving, and cooled to 45°C.

Brain Heart Infusion Agar

BHI Broth	37 g
Agar	15 g
Distilled Water	1000 ml

Brain Heart Infusion Broth

Calf Brain Infusion	200 g
Beef Heart Infusion	250 g
Proteose Peptone	10 g
Glucose	2 g
NaCl	5 g
Na_2HPO_4	2.5 g

BHI Starch Agar

BHI Broth	10 g
Starch	5 g
NaCl	15 g
Agar	15 g
Distilled Water	1000 ml

Decarboxylase Test Media

Peptone	5 g
Yeast Extract	3 g
Glucose	1 g
Bromcresol Purple, 1.6% Solution	1 ml
Amino Acid*	5 g
Distilled Water	1000 ml

* Lysine, Arginine or Ornithine, after adding the amino acid, adjust to pH 6.8.
Dispense as 3 ml deeps in 13x100 mm capped tubes. Sterilize mineral oil to be added on top of inoculated media.

Dilution Fluid (Diluent)

Peptone	1 g
Distilled Water	1000 ml

Note: dispense as 9 ml or 9.9 ml in 16x150 mm loose cap tubes or as 99 ml in square milk dilution bottles.

Na_2SSO_3	0.3 g
Distilled Water	1000 ml

Eosin Methylene Blue Agar

Peptone	10 g
K_2HPO_4	2 g
Lactose	10 g
Eosin-Y	0.4 g
Methylene Blue	0.07 g
Agar	15 g
Distilled Water	1000 ml

Glycerol-HEPES-Broth

Peptone	5 g
Yeast Extract	3 g
Glycerol	3 ml
NaCl	20 g
HEPES	5.95 g
$MgSO_4$	3 g
NH_4Cl	1g
KCl	0.75 g
alpha glycerol PO_4	0.21 g

Adjust to pH 7.5 with 50% KOH.

Glycerol Marine Agar

Difco Marine Agar	55 g
Glycerol	3 ml
Distilled Water	1000 ml

Kligler's Iron Agar

Beef Extract	1 g
Peptone	15 g
Yeast Extract	3 g
Lactose	10 g
Glucose	1 g
NaCl	5 g
Agar	15 g
Phenol Red	0.025g
Ferrous sulfate	0.2 g

Lactate Minimal Agar

K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
$(NH_4)_2SO_4$	1 g
$Na_3Citrate \cdot 2H_2O$	0.5 g
Agar	15 g
Distilled Water	990 ml

Sterilize by autoclave and then add aseptically when cool to 45°C:

a. 1 ml 20% $MgSO_4 \cdot 7H_2O$
b. 10 ml 20% Na-lactate
c. 0.5 ml 1% Thiamine HCL

Lauryl-SO_4 Lactose Broth

Tryptose	20 g
Lactose	5 g
K_2HPO_4	2.75 g
KH_2PO_4	2.75 g
NaCl	5 g
Na-Lauryl-SO_4	0.1 g
Distilled Water	1000 ml

Litmus Milk

Skim milk powder	100 g
Litmus..enough to give a light lavender color.	
Distilled Water	1000 ml

Note: fill 13x100 mm tubes with 2 ml and use a short, 10 minute autoclave time.

Mannitol Salt Agar

Peptone	10 g
NaCl	75 g
Mannitol	10 g
Agar	15 g
Phenol Red	0.025 g
Distilled Water	1000 ml

Marine Dilution Fluid

(Marine Dilutent)

Peptone	1 g
NaCl	20 g
$MgSO_4$	6 g
KCl	0.75 g
Distilled Water	1000 ml

Note: dispense as 9 or 9.9 ml in 16x150 mm loose cap tubes or 99 ml in square milk diltuion bottles.

MRVP Medium

Peptone	10 g
Glucose	5 g
K_2HPO_4	5 g
Distilled Water	1000 ml

Note: dispense as 4 ml per 13x100 mm capped tube. After incubation this medium is split in half for each test: Methyl Red and Voges-Proskauer.

Nutrient Agar

Beef Extract	3 g
Peptone	5 g
Agar	15 g
Distilled Water	1000 ml

Nutrient Gelatin

Beef Extract	3 g
Peptone	5 g
Gelatin	120 g
Distilled Water	1000 ml

O-F Medium (Hugh-Liefson)

Glucose	10 g
Peptone	2 g
NaCl	5 g
K_2PO_4	0.3 g
Agar	3 g
Bromthymol Blue	0.003 g
Distilled Water	1000 ml

Phenol Red Broth Base - Sugar Fermentation Broth

Peptone	10 g
NaCl	5 g
Phenol Red	0.018 g
Sugar*	5 or 10g

* Adjust to pH 7 if necessary after adding the sugar. The medium should be red after autoclaving. Add Durham tube.

Potato Dextrose Agar

Potato Infusion	200 g
Glucose	20 g
Agar	15 g
Distilled Water	1000 ml

Purple Non Sulfur Bacteria Broth

Na-acetate	0.2 g
Na-malate	0.2 g
Yeast Extract	1 g
Distilled Water	1000 ml

Note: adjust pH to 7, fill tubes to the top, screw cap tight before autoclaving.

Purple Sulfur Bacteria Broth

$Na_2S_2O_3$	1 g
KH_2PO_4	0.1 g
NH_4Cl	0.1 g
$MgCl_2 \cdot 6H_2O$	0.05 g
NaCl	1 g
$NaHCO_3$	4 g
$MnCl_2$	0.01 g
Yeast Extract	0.5 g
Distilled Water	1000 ml

Note: adjust to pH 7, fill 13x100 mm screw cap tubes to the top, screw cap tight before

autoclaving.

Sabourauds's Dextrose Agar

Peptone	10 g
Glucose	40 g
Agar	15 g
Distilled Water	1000 ml

Adjust pH to 5.6.

Simmons Citrate Agar

Na-citrate	2 g
K_2HPO_4	1 g
$(NH_4)H_2PO_4$	1 g
$MgSO_4$	0.2 g
NaCl	5 g
Bromthymol Blue	0.08 g
Agar	15 g
Distilled Water	1000 ml

Soft Agar (Top Agar)

Beef Extract	3 g
Peptone	5 g
Agar	7.5 g
Distilled Water	1000 ml

Starch Agar

Beef Extract	3 g
Peptone	5 g
Soluble Starch	2 g
Agar	15 g
Distilled Water	1000 ml

Sucrose Agar

Beef Extract	3 g
Peptone	5 g
Sucrose	50 g
Agar	15 g
Distilled Water	1000ml

Thioglycollate Medium

Peptone	10 g
Tryptone	5 g
Yeast Extract	5 g
Glucose	10 g
NaCl	5 g
Agar	20 g
Na-thioglycollate	2 g
Resazurin	0.002 g
Na-formaldehyde sulfoxylate	1 g
Distilled Water	1000 ml

Note: dispense this medium so each tube is **more than** 3/4 full.

Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS)

Yeast Extract	5 g
Proteose Peptone	10 g
Sucrose	20 g
Na_2SSO_3	10 g
Na-citrate$\cdot H_2O$	10 g
Na-cholate	3 g
Oxgall	5 g
NaCl	10 g
Fe-citrate	1 g
Bromthymol Blue	0.04 g
Thymol Blue	0.04 g
Agar	15 g
Distilled Water	1000 ml

Trypticase Soy Agar

Trypticase Peptone	15 g
Phytone Peptone	5 g
NaCl	5 g
Agar	15 g
Distilled Water	1000 ml

Tryptone Broth

Tryptone	10 g
Distilled Water	1000 ml

Tryptone Glucose Yeast Extract Agar (TGY)

Tryptone	10 g
Yeast Extract	5 g
Glucose	5 g
K_2HPO_4	3 g
Agar	15 g
Distilled Water	1000 ml

Urea Agar

Urea solution: 20 g urea in 100 ml of distilled water, sterilize by membrane filtration.

Basal Medium:

Peptone	1 g
Glucose	1 g
NaCl	5 g
KH_2PO_4	2 g
Phenol red	0.012 g
Agar	20 g
Distilled Water	1000 ml

Sterilize Basal Medium in the autoclave, after autoclaving, cool to 45°C, add **warmed**, sterile urea solution, mix and dispense aseptically to plates or tubes, 3 ml in 13x100 mm capped tube.

APPENDIX IV

STAINS and REAGENTS

Acid Fast Stain
Ziehl's Carbol Fuchsin

Solution A

Basic fuschsin	0.3 g
95% Ethanol	10 ml

Solution B

Phenol	5 ml
Distilled Water	95 ml

Mix A and B when complete

Acid-Alcohol

95% Ethanol	97 ml
HCl	3 ml

Methylene Blue (Loeffler's)

Solution A

Methylene Blue	0.3 g
95% Ethanol	30 ml

Solution B

KOH	0.01 g
Distilled Water	100 ml

Mix A and B when complete.

Gram Stain Reagents
Crystal Violet (Hucker's)

Solution A

Cyrstal Violet	2 g
Ethanol	10 ml

Solution B

Ammonium oxalate	0.8 g
Distilled Water	80 ml

Mix A and B when complete.

Gram's Iodine (Lugol's)

Iodine	1 g
KI	2 g
Distilled Water	300 ml

Safranin

Safranin	0.25 g
Ethanol	10 ml
dissolve dye in ethanol	
Distilled Water	90 ml

Indole Test Reagent

p-Dimethylamino-benzaldehyde	5 g
95% Ethanol	75 ml
dissolve, then add	
Conc HCL	25 ml

Lactophenol Cotton Blue Stain
(Mounting Solution)

Phenol	20 g
Lactic acid	20 ml
Glycerol	40 ml
Distilled Water	20 ml
dissolve by heating, then add	
Cotton Blue*	0.05 g

* Poirrier's Blue

Liefson's Flagella Stain

Solution A

NaCl	1.5 g
Distilled Water	100 ml

Solution B

Tannic acid	3 g
Distilled Water	100 ml

Solution C

Basic Fuchsin	1.2 g
Ethanol	100 ml

When solutions are complete, mix all three **in order**. Dispense in dropping bottles and store at 0°C. Stable for only a short time (1-2 weeks).

Methyl Red

Methyl Red	0.1 g
95% Ethanol	300 ml
dissolve, then add	
Distilled Water	200 ml

Note: Methyl red should be orange, not red or yellow.

Nigrosin

Nigrosin	10 g
Distilled Water	100 ml
Formalin (37%)	0.5 ml

Nitrite Test Reagents

Reagent I

Sulfanilic acid	0.5 g
Glacial Acetic acid	30 ml
Distilled Water	100 ml

Reagent II

alpha-naphthylamine	0.5 g
Distilled Water	100 ml

dissolve is water, boil if necessary, then add.

Glacial Acetic Acid	30 ml

Oxidase Reagent (Kovac's)

Ascorbic acid	0.05 g
Distilled Water	100 ml
dissolve, then add	
Tetramethyl-para-phenylene-diamine HCl	1 g

Note: Keep refrigerated, protected from light. Discard if blue.

Ryu Flagella Stain

Solution A

Phenol	0.5 g
Distilled Water	10 ml
dissolve, then add	
Tannic Acid	2 g
Aluminum Potassium sulfate-12 hydrate, sat'd soln	10 ml

Solution B

Crystal Violet	12 g
Ethanol	100 ml
(this is a sat'd soln)	

Mix 10 parts Solution A with one part Solution B. Dispense through a 0.22 micrometer porous membrane filter.

Spore Stains

Malachite Green

Malachite Green	0.5 g
Distilled Water	100 ml

Safranin...see Gram Stains

Voges-Proskauer Test Reagents

VP Reagent I

alpha-naphthol	5 g
Absolute ethanol	100 ml

VP Reagent II

KOH	40 g
Distilled Water	100 ml

Note: VP Reagent I is only stable for a few days at Room Temperature and should be made fresh each week.

APPENDIX V

MOST PROBABLE NUMBER DETERMINATION
from
MULTIPLE TUBE ANALYSIS

Number of Tubes Giving Positive Results from sets of three inoculated from ten fold differences in inoculum level (by volume or 1.0 ml aliquots by dilution).

10	1	0.1	MPN*
0	0	1	3
0	1	0	3
1	0	0	4
1	0	1	7
1	1	0	7
1	1	1	11
1	2	1	11
2	0	0	9
2	0	1	14
2	1	0	15
2	1	1	20
2	2	0	21
2	2	1	28
3	0	0	23
3	0	1	39
3	0	2	64
3	1	0	43
3	1	1	75
3	1	2	120
3	2	0	93
3	2	1	150
3	2	2	210
3	3	0	240
3	3	1	460
3	3	2	1100

* MPN per 100 ml of the middle (1.0 ml) dilution. If the sample has been diluted, take the MPN times the dilution factor for the MPN titer of the undiluted sample. Adapted from: Standard Methods for Examination of Water and Wastewater. 12th Edition. The American Public Health Association, Inc. New York.

APPENDIX VI

DILUTIONS AND DILUTION PROBLEMS

Dilutions are used extensively throughout microbiology and other areas of biology. The same principles apply whether you are diluting molar solutions or suspensions of cells. Dilutions can be expressed in several ways, but no matter what way it is expressed, all **dilutions are a simple ratio** of the amount of sample that is being diluted to the total (sample + diluent):

Dilution = sample / (sample + diluent)

The dilution is always less than one and like other ratios has no units. A simple dilution would be to take one ml of lake water and aseptically add that to 9 ml of sterile diluent. The total volume is now 10 ml (1 ml of lake water + 9 ml of diluent) and this is a 1/10 dilution. This can also be written as a 1:10 or a 10^{-1} dilution. Thus, the concentration of bacteria in the dilution is now 10 times less than in the lake water itself.

A slightly more complex dilution would be to place 3.5 ml of a dye reagent into 31.5 ml of distilled water. This is also a 1:10 dilution because 3.5 ml was diluted to a total of 35 ml. Dilutions do not necessarily have to come out even. For example, if you had put 5 grams of hamburger in a sterile blender jar and blended this with 95 ml of sterile diluent, you would have made a 1:20 dilution. This 1/20 dilution could also be expressed as 5×10^{-2}. Dilutions are much easier to work with when expressed in their most mathematically reduced form: usually 1: # .

Immunologist often make doubling dilutions...that is 1:2 dilutions. To do this one part of the undiluted solution is mixed with one part of saline or buffer. This is 1/(1+1) or a 1:2 dilution. How much sterile diluent would you have to add to one ml to get a 1:3 dilution? (Ans: 2 ml). And, suppose you had 0.5 ml and had to dilute it 1:15, how much sterile diluent should you add? (Ans: 7 ml). What would be the dilution if instead 4 ml was added to the 0.5 ml to be diluted? (Ans: 1:9).

In the microbiology lab, samples are often diluted to get "countable" plates: that is dilutions are plated on to various nutrient media so that between 30 and 300 colonies will grow. Because we don't know the number of bacteria in advance, we will be making **serial dilutions** and plating aliquots from each dilution tube. The **final dilution** is the product of all the dilutions. Thus if you had made an initial dilution by taking 0.1 ml of a sample and aseptically placed it into 99.9 ml of sterile diluent, you would have made a 1:1,000. If you then make a second dilution by taking 1 ml of the diluted sample and aseptically placing that into 99 ml of sterile diluent, you would have made a 1:100 dilution as a second dilution. Now, from the original sample, the cells (and molecules) have been diluted:

$$\frac{1}{1,000} \times \frac{1}{100} = \frac{1}{100,000}$$

This means in the final dilution tube, the concentration is 100,000 times less than in the original. Thus if this was used to plate 0.1 ml onto Nutrient Agar, and after incubation 34 colonies grew, the resulting titer would be 34 bacteria/0.1ml in the last dilution: 340 bacteria/ml. But this dilution was 100,000 times less concentrated than the original. Thus to calculate the number in the original, one simply multiplies by the **dilution factor** which in this case is 10^5.

$$340 \text{ bacteria/ml} \times 10^5 = 3.4 \times 10^7 \text{ bacteria/ml}$$

Note that the dilution factor is the reciprocal of the dilution: in this case the dilution was 1:100,000 or $1{:}10^5$ or 10^{-5} and the dilution factor was 100,000 or 10^5. So,

Dilution Factor = 1 / Dilution

Like the dilution, the dilution factor has no units and is always greater than one.

Calculation of Titer is done using dilutions, dilution factors and number of colonies per plate or cells counted in a Petroff Hauser counter. Titer is the number of particles (cells) per unit volume. The milliliter is normally used and for most work a gram is considered to be an ml. When counting colonies, we assume that one cell gave rise to a colony, but in most instances can not be really sure about that. Therefore, the most appropriate term is the **C**olony **F**orming **U**nit or CFU. The actual number of bacteria has to be more than the CFU because two or more cells could have likely given rise to a colony. In the example above, we simply used bacteria/ml, but it would have been more accurate to have used CFU/ml.

For the standard 9 cm diameter petri plate, we usually plate multiple dilutions so that one set will yield statistically significant numbers that can be multiplied by the dilution factor to find the titer. It is preferable to adhere to the **30-300 Rule**. That is statistical counts on less than 30 individuals are counts that can have unacceptable levels of sampling error. Thus we have greater reliability in counts above 30. At the other end, crowding can lead to depletion of nutrients and some colonies will not grow. Hence, we tend to rely on counts below 300 per plate.

Suppose a dilution series were performed from a sample of industrial juice. We will assume that excellent technique was used throughout and that all the counts were accurate. In this example three plates were spread inoculated (0.1 ml each) from each dilution. The following data was obtained:

Dilution	Number of Colonies/Plate
10^{-3}	TNTC*
10^{-4}	234, 244, 212

10^{-5}	31, 18, 25
10^{-6}	1, 0, 8

From this data we can discard the counts from all dilutions except the one that gave the **most reliable** results: the 10^{-4} dilution. (The 10^{-5} dilution has a countable plate, but the numbers are at the very bottom, statistically less significant portion of the **30-300 Rule**. First we must get the **average CFU/plate**. This is:

$$(234 + 244 + 212)/3 = 230 \text{ CFU/plate.}$$

One tenth of a ml was plated, therefore this dilution had:

$$230 \text{ CFU/0.1 ml} = 2.3 \times 10^3 \text{ CFU/ml.}$$

The dilution factor is 10^4, thus the titer is:

$$2.3 \times 10^3 \times 10^4 = 2.3 \times 10^7 \text{ CFU/ml.}$$

If 0.05 ml were plated instead of 0.1 ml per plate, then the titer would be different: 4.6×10^7 CFU/ml.

Added Note:

The photographic industry has made life difficult by doing dilutions that are not mathematically correct. You will find in the dilution of photographic chemicals that you are asked to dilute some chemical 1:1. In reality you can't as this would be **undiluted!** Photographic companies that make such specifications mean to make a dilution by mixing one part + one part. (They mistakenly equate ":" as "+").