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Faculty of Science

Laboratory Manual

Basic Microbiology

Bachelor of Biotechnology (Hons.)

Edited By: : Dr. Farzana Yasmin

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LINCOLN UNIVERSITY COLLEGE
Wisma Lincoln, No. 12,14,16 & 18,
Jalan SS 6/12, Off Jalan Perbandaran
47301 Petaling, Jaya,
Selangor Darul Ehsan, Malaysia
Tel.: +603-7806 3478
Fax: +603-7806 3479
Toll Free: 1-300-880-111
E-mail: lucp@lincoln.edu.my
info@lincoln.edu.my
Web: www.lucp.net
www.lincoln.edu.my

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Basic Microbiology

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LINCOLN UNIVERSITY COLLEGE
FACULTY OF SCIENCE (DEPARTMENT OF BIOTECHNOLOGY)
LABORATORY SAFETY RULES

The following rules must be obeyed by all students in the science laboratory of the faculty. Wilful or repeated in advertent non-compliance may result in dismissal or suspension from the laboratories

- No entry without permission:
  - Outsiders are not allowed to enter the laboratory without permission.
  - No student is allowed to enter the laboratory unless permission has been given by a laboratory assistant or a lecturer.

- At work in the laboratory:
  - No experiment may be attempted without the knowledge and permission of a lecturer.
  - Students must wear shoes in the laboratory. Students wearing slippers or sandals are not allowed to work in the laboratory.
  - Lab coat must be worn at all times during practical work in the laboratory.
  - Do not mouth pipette chemicals.
  - Do not eat or smoke in the laboratory.
  - Do not taste any chemicals, including dilute solutions. If any acid or alkali accidentally enters your eyes or mouth, wash immediately with plenty of tap water. Inform your lecturer, and seek medical attention if necessary.
  - Paper should be used to light up the Bunsen burners.
  - Used match sticks, filter papers, and other solid waste must never be thrown into the sinks. They must be thrown into the dustbins provided. Lighted match sticks and smoldering materials must be extinguished with tap water before thrown in to the dustbins.
  - Any equipment broken or damaged must be reported to the laboratory assistant.

- Before leaving the laboratory:
  - All the equipment and benches must be cleaned at the end of each practical session.
  - Wash hands and arms with soap and water before leaving the laboratory.
  - No student is allowed to take away any chemicals, equipment or other property of the laboratory.

INTRODUCTION

1. The Scientific Method

- Making observations
- Generating hypotheses
- Making predictions
- Designing and carrying out experiments
- Constructing scientific models

2. Practical Exercises

To get the most out of the practical exercises, you need to follow carefully the instructions given. These instructions have been designed to provide you with the experience in the following skills:

- Following instructors
- Handling apparatus
- Having due regard for safely
- Making accurate observations
- Recording results in an appropriate form
- Presenting quantitative results
- Drawing conclusions

3. Following Instructions

Instructions are provided in the order in which you need to carry them out. We would advise that before carrying out the instructions, you read through the entire exercise. This will help you to remember what you have learned.

Each practical exercise in the book begins with a few lines describing its purpose in most cases the following headings are also used:

- Procedure-numbered steps that need to be carried out.
- For consideration -some questions to help you think carefully about the results you have obtained.
- Materials-a list of the apparatus, chemicals and biological materials you need.

4. Handling apparatus

Biologists need to able to use many different types of apparatus, for example, photometers (to measure water uptake by plants), respirometers (to measure oxygen uptake or carbon dioxide production), Petri dishes (for plating out bacteria and other microorganisms) and the light microscope (to magnify specimens). Many of the practical exercises are designed to help you derive the maximum benefit from a piece of apparatus.

5. Having Due Regard for Safety

Surveys have been shown that science laboratories are among the safest places to be. Nevertheless, this is no cause for complacency.

- Always move slowly and carefully in a laboratory.
- Never put your fingers in your mouth or eyes after using chemicals or touching biological specimens until you have washed your hands thoroughly with soap and warm water, and dried them.
- Make sure glass objects (e.g, thermometers, beakers) cannot roll off tables or be knocked onto the floor.
- Wear safely goggles whenever there is a risk of damage to the eyes.

<u>Situations of risk include:</u>

- Heating anything with a Bunsen burner (even heating water has its dangers')
- Handling many liquids, particularly those identified as corrosive, irritant, toxic or harmful

- Handling corrosive or irritant solids
- Some dissection work
- Allow Bunsen burners, tripods, gauzez and beakers to cool down before handling them.
- Never allow your own body fluids (especially blood and saliva) to come into contact with someone else, or theirs into contact with you.
- Keep long hair tied back and do not wear dangly earrings.
- Do not allow electrical equipment to come into contact with water.
- If you are unsure how to carry out a scientific procedure, ask.
- Make sure you understand why you are going to do something before you do it.
- Wear a lab coat when using chemicals or handling any biological specimens.
- Follow exactly agreed procedures with regard to cuts, burns, electric shocks and other accidents (e.g. with chemicals).
- Follow exactly all specific safely instructions given in this book or provided by your teacher for particular practical exercises (e.g. use of gloves, disinfection)

With practice, these procedures should become second nature to you. They will enable you to carry out practical work in safety.

6. Making Accurate Observations

In most cases the practical exercise will make it clear what you need to observe, e.g. the time taken for a certain volume of gas to be evolved or the width of a sample cells. Ensure that you know how to use any necessary equipment before starting practical. Think carefully about the precision with which you will make your observations.

7. Recording Results in an Appropriate Form

Results can be recorded in various ways. Often it is helpful to record raw data in a table. Most data will be in the form of numbers, i.e. they will be quantitative data (also known as numerical data). However, some data, e.g. flower colour, will be qualitative data.

One form in which some biological findings can be recorded is a drawing. You don't need to be professional artist to make worthwhile biological drawings. If you follow the following guidelines, a drawing can be of considerable biological value:

- Ensure that your completed drawing will cover at least a third of A4 page.
- Plan your drawing so that the various parts are is proportion and will not be drawn too small. Small marks to indicate the length and breadth of the drawing are a great help in planning and a faint outline can be rapidly drawn to show the relative positions of the parts.
- The final drawing should be made with clean, firm lines using a sharp HB pencil and, if needed, a good quality eraser (not a fluid).If important details are too small to be shown in proportion, they can be put in an enlarged drawing at the side of the main drawing.
- Avoid shading and the use of colour unless you are an excellent artist and they really help, for example when drawing soil profiles.
- When drawing structures seen with the naked eye or hand lens, use two lines to delineate such things as blood vessels and petioles. This will help you to indicate the relative widths of such structures.
- When drawing low power plan drawings from the light microscope, do not attempt to draw individual cells-just different tissues.
- When drawing plant cells at high power under the light microscope, use two lines to indicate the width of cell walls, but a single line to indicate a membrane.
- Always put a scale on each drawing.

8. Presenting Quantitative Results

Presentation of data is all about using graphs or other visual means to make it easier to see what your results tell you. The following four ways of presenting data are the most frequently used in biology: line graphs, bar charts, histograms and scatter graphs (Figure 1).

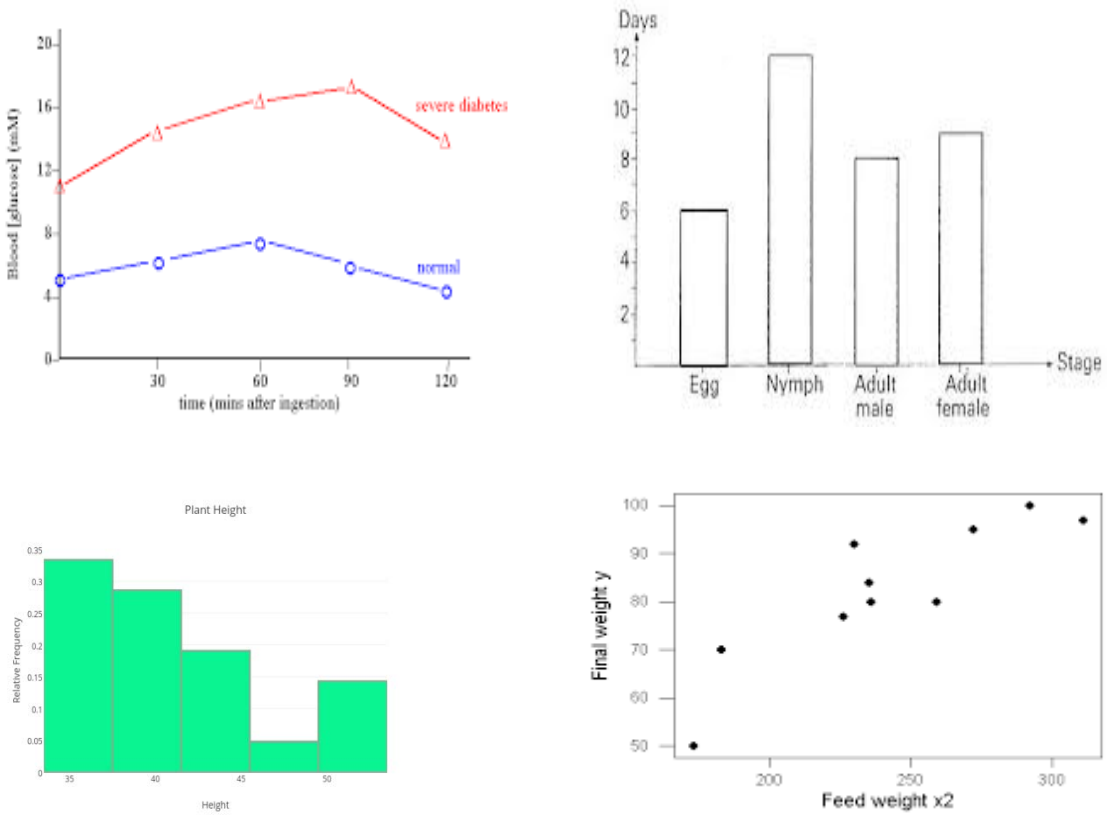


Figure 1: Line graphs, bar charts, histograms and scatter graphs

9. Drawing Conclusions

Finally, you will need to draw conclusions. If your practical exercise has involved the testing of a hypothesis, for example that the enzyme pepsin works better at low pH than in neutral or alkaline conditions, your conclusion should indicate whether the hypothesis has been refuted (i.e. shown not to be the case) or supported. Of course, even if your hypothesis has been supported, it doesn't mean that it has been confirmed with 100% certainty- in other words it isn't proved. Science proceeds more by showing that certain ideas are wrong than by showing that others are right (think about that!). Your conclusion might therefore include further ways of testing the original hypothesis, or might raise new possibilities to be investigated.

Often you will only be able to arrive at your conclusions after statistically analysing your data.

10. Writing a Scientific Lab Report

Title

- Communicate the subject investigated in the paper.

Introduction

- State the hypothesis.
- Give well-defined reasons for making the hypothesis.
- Explain the biological basis of the experiment.
- Cite sources to substantiate background information.

- Explain how the method used will produce information relevant to your hypothesis.
- State a prediction based on your hypothesis. (If the hypotheis is supported, then the results will be.)

Materials and Methods

- Use the appropriate style.
- Give enough detail so the reader could duplicate your experiment
- State the control treatment, replication and standardized variables that were used.

Results

- Summarize the data (do not include raw data).
- Present the data in an appropriate format (table or graph).
- Present tables and figures neatly so they are easily read.
- Label the axes of each graph completely.
- Give units of measurement where appropriate.
- Write a descriptive caption for each table and figure.
- Include a short paragraph pointing out important results but do not interpret the data.

Discussion

- State whether the hypothesis was supported or proven false by the results, or else state that the results were inconclusive.
- Cite specific results that support your conclusions.
- Give the reasoning for your conclusions.
- Demonstrate that you understand the biological meaning of your results.
- Compare the results, with your predictions and explain any unexpected results.
- Compare the results to other research or information available to you.
- Discuss any weaknesses in your experimental design or problems with the execution of the experiment.
- Discuss how you might extend or improve your experiment.

Conclusion

- Restate your conclusion.
- Restate important results.

Literature Cited

- Use the proper citation form in the text.
- Use proper citation form in the Literature Cited section.
- Refer in the text to any source listed in this section.

Acknowledgement

- State any appropriate acknowledgement that you think is necessary.

Practical 1
Title: Aseptic techniques

Objective:

After completing the practical, you will be able:
1. To learn the aseptic techniques

Introduction:

Microorganisms include bacteria, fungi, and molds, and they are everywhere in the air, in water, and on every kind of surface imaginable. Their widespread presence means that microbiologists have to take certain precautions when working with bacteria to avoid contamination. When studying bacteria, it is important that the bacterial culture be pure, meaning that it contains only cells of that bacterial species. Otherwise, the results of any tests or procedures are meaningless. From a clinical standpoint, it is imperative to avoid cross-contamination of samples, lest the wrong diagnosis be made.

In this lab we will learn methods for transferring and working with bacterial cultures aseptically, so that the cultures do not get contaminated. You will learn to transfer broth culture to broth, slant culture to broth, broth culture to slant, and slant culture to plate culture.

Materials:

Test tube rack labeled with your name (share half with a partner)

1. 1.5 ml nutrient broth culture of bacteria
2. 1 nutrient agar slant culture of bacteria
3. 2.5 ml tubes of sterile nutrient broth
4. 1 nutrient agar plate
5. Inoculating loop

Procedures:

A. Transfer of bacteria from broth to broth

1. Using your Sharpie pen, label your plates and tubes with your first name (and last initial if necessary). When labeling plates, always label the BOTTOM of the plate rather than the lid.
2. Light your Bunsen burner.
3. Sterilize your loop by holding it in the flame of the Bunsen burner until it glows red. It works best to hold the loop at a slant so that 1 to 1 1/2" of the loop is in the flame. Avoid heating the handle of the loop.
4. Let the loop cool thoroughly. You may set the loop down on the bench, but make sure the sterilized end does not touch anything. Failure to let the loop cool thoroughly can kill the bacteria that you are trying to transfer with the loop. You know it's too hot if the broth sizzles when you put the loop into it, or if the agar melts when you touch the loop to an agar plate.
5. With the loop in your dominant hand, pick up the broth culture in the other hand and shake it gently to make sure it's mixed.
6. With the little finger of the hand holding the loop, grasp the cap of the tube and pull it off.
7. Immediately flame the mouth of the tube, but do not hold it in the flame too long or the tube may crack. Remove the tube from the flame.
8. Insert the loop into the culture, making sure you don't go too deep and risk exposing the culture to an inflamed portion of your loop.

9. Remove the loop, flame the mouth of the culture, and replace the cap. Set the tube down in the rack.
10. Pick up the new tube of sterile broth, remove the cap as before, and flame the mouth of the tube.
11. Without flaming the loop, insert the loop containing some of the original culture into the new tube. Remove the loop and flame the mouth of the tube before replacing the cap.
12. Flame the loop to sterilize it.

B. Transfer of bacteria from agar to broth

1. With a loop sterilized and cooled as described in Part 1 in one hand, pick up your agar slant culture of bacteria in the other. Remove the cap as before, and flame the mouth of the tube.
2. Insert the loop and pick up a small amount of bacterial growth. Remove the loop, flame the mouth of the tube, and replace the cap. Put the culture back in your rack.
3. Pick up a fresh tube of nutrient broth, remove the cap, and flame the mouth of the tube.
4. Insert the loop containing the bacteria from the slant culture and stir gently to dislodge the bacteria into the broth.
5. Remove the loop, flame the mouth of the tube, replace the cap, and set it in your rack.
6. Sterilize your loop.

C. Transfer of bacteria from broth to agar

1. With a loop sterilized and cooled as described above, pick up your broth culture of bacteria. Remove the cap as before, and flame the mouth of the tube.
2. Insert the loop to get a drop of culture. Remove the loop, flame the mouth of the tube, and replace the cap. Put the culture back in your rack.
3. Carefully lift the lid of your agar plate with the hand that is not holding the loop. Do NOT set the lid down anywhere at any time, and try to avoid breathing on the plate while the lid is off.
4. Carefully streak the loop back and forth over the surface of the plate.
5. Put the lid back down and flame your loop.
6. Note: this type of streak plate is designed for maintenance of cultures that are already PURE. A different method of streaking is used when trying to purify different species of bacteria from a common source (e.g. from a throat swab or from an environmental sample
7. When you are finished with this part of the lab, give your labeled test tube rack and plates to your T.A. They will be incubated at 37 °C for 48 hours and then refrigerated until next lab period. Place your used cultures in the designated disposal area.

Results:

Questions:

1. Did something grow in all of your transfers? If not, what could have been the problem?
2. Do any of your agar plates appear to be contaminated?
3. How would you know if your broth cultures were contaminated with a microbe other than the one you wanted to grow?

Practical 2

Title: Media preparation

Objective:

After completing the practical, you will be able:

1. To learn how to prepare different types of media and sterilize each one using autoclave

Introduction:

The media are important for growth of microorganisms.

Nutrient Broth is a basic media composed of a simple peptone and a beef extract. Peptone contributes organic nitrogen in the form of amino acids and long-chained fatty acids. Beef Extract provides additional vitamins, carbohydrates, salts and other organic nitrogen compounds. If needed other enrichments may be added during the preparation of N8530 or to prepared media.

Nutrient agar is a general purpose medium supporting growth of a wide range of non-fastidious organisms. It typically contains (mass/volume):

- 0.5% Peptone - this provides organic nitrogen
- 0.3% beef extract/yeast extract - the water-soluble content of these contribute vitamins, carbohydrates, nitrogen, and salts
- 1.5% agar - this gives the mixture solidity
- 0.5% Sodium Chloride - this gives the mixture proportions similar to those found in the cytoplasm of most organisms
- distilled water - water serves as a transport medium for the agar's various substances

pH adjusted to neutral (6.8) at 25 °C (77 °F).

These ingredients are combined and boiled for approximately one minute to ensure they are mixed and then sterilized by autoclaving, typically at 121 °C (250 °F) for 15 minutes. Then they are cooled to around 50 °C (122 °F) and poured into Petri dishes which are covered immediately. Once the dishes hold solidified agar, they are stored upside down and are often refrigerated until used. Inoculation takes place on warm dishes rather than cool ones: if refrigerated for storage, the dishes must be rewarmed to room temperature prior to inoculation.

MacConkey Agar (MAC) is a selective and differential medium designed to isolate and differentiate enterics based on their ability to ferment lactose. Bile salts and crystal violet inhibit the growth of Gram positive organisms. Lactose provides a source of fermentable carbohydrate, allowing for differentiation. Neutral red is a pH indicator that turns red at a pH below 6.8 and is colorless at any pH greater than 6.8.

Organisms that ferment lactose and thereby produce an acidic environment will appear pink because of the neutral red turning red. Bile salts may also precipitate out of the media surrounding the growth of fermenters because of the change in pH. Non-fermenters will produce normally-colored or colorless colonies.

Materials:

1. 250 ml Erlenmeyer flasks
2. Sterile petri dishes
3. Sterile test tubes or universal bottles
4. Cotton wool/aluminum foil
5. Distilled water
6. Balance
7. Bunsen burner
8. Autoclave

Procedures:

A. Nutrient Broth

This is a general-purpose medium for the cultivation of microorganisms that are not exacting in the nutrient requirements. The formula is specified by the American Public Health Association in Standard Methods for the Examination of Water and Sewage and Standard Methods for the Examination of dairy products.

The formula contains:

Beef extract	3g
Peptone	5g

(Final pH 6.8 + 0.2 at 25^{o} C)

To prepare 100 ml of medium:

1. Dissolve 0.8g in 100 ml distilled water.
2. Dispense 10ml into tubes (Universal bottles).
3. Sterilize in the autoclave at 15 lb. /in^2,122 ^{o}C for 30 min.

B. Nutrient Agar

This is a common complex medium containing beef extract and peptone for culturing microorganisms. Since such dehydrated products usually have a pre-adjusted pH of about 7, further adjustment should not be necessary.

Nutrient broth powder	0.8g
Agar	2.0g

To prepare 100 ml of medium:

1. Weigh 0.8 g of dehydrated nutrient broth powder and place it in the Erlenmeyer flask. Slowly add 100 ml of distilled water while swirling the flask gently to dissolve the powder. The solution should be clear.
2. Add 2 g of agar. Melt the agar in the microwave oven.
3. Dispense 10 ml into universal bottles for making universal slants.
5. Cover the remainder in the flask with cotton wool/aluminium foil.
6. Autoclave at 15 lb. /in^2, 122 ^{o}C for 20 min.
7. After sterilizing, slant the universal bottles.

8. Cool media in the flask to about 45^{o}C and aseptically pour into 3 sterile plates (approximately 15ml per plate). If bubbles appear on the surface of agar, remove the lid, and quickly flame the surface with a Bunsen burner to remove the bubbles.
9. Labeled plates and keep in refrigerator. Check for contamination and to use them in the next practical session.

C. Mac Conkey Medium

This is a differential plating medium recommended for use in the isolation and differentiation of lactose-fermenting organisms from lactose non-fermenting gram negative enteric bacteria. The inhibitory action of crystal violet on the growth of gram-positive organisms allows for the isolation of gram-negative bacteria. The incorporation of lactose, bile salts and the pH indicator, neutral red permits differentiation of enteric bacteria. Bacteria capable of fermenting lactose produce a localized pH drop which, followed by absorption of the neutral red, gives a red colour to the colony. Colonies which do not ferment lactose remain colourless and translucent.

Mac Conkey agar formula contains:

Peptone	17.0g
Proteose peptone	3.0g
Lactose	10.0g
Bile salts	1.5g
Sodium chloride	5.0g
Agar	13.5g
Neutral red	0.03g
Crystal violet	0.001g

(Final pH 7.1 + 0.2 at 25^{o} C)

To prepare 100 ml of medium:

1. Put 5 g of Mac Conkey powder in the Erlenmeyer flask. Please refer to the Instruction written on the bottle for the amount of powder to be used.
2. Slowly add 100 ml of distilled water while swirling the flask gently to dissolve the powder.
3. Cover the flask with the flask cotton wool/ aluminum foil and autoclave 15 lb/in^{2}, 122 ^{o} C for 30 min.
4. After sterilizing, swirls it gently to disperse the agar evenly and cool to about 45^{o}C.
5. Aseptically, pour six plates, approximately 15 ml per plate. If bubbles appear on the surface of the agar, remove the lid, and quickly flame the surface with a Bunsen burner to remove the bubbles.
6. Labelled plates and keep in refrigerator. Check for contamination and to use them in the next practical session.

D. Potato Dextrose Agar

Potato Dextrose Agar (PDA) is used for the cultivation of fungi. Potato Dextrose Agar (PDA) is a general-purpose medium for yeasts and molds that can be supplemented with acid or antibiotics to inhibit bacterial growth. It is recommended for plate count methods for foods, dairy products and testing cosmetics. PDA can be used for growing clinically significant yeast and molds.

Commercial PDA Powder	39 g
Distilled water	1 liter

To prepare 100 ml of medium:

1. Add 39 g of Commercial PDA Powder to 1 liter of distilled water.
2. Boil while mixing to dissolve.
3. Autoclave 15 min at 121 ^{o} C.
4. Labeled plates and keep in refrigerator. Check for contamination and to use them in the next practical session.

Results:

Questions:

1. Did your agar plates solidify? If not, what could have been the problem?
2. Do any of your agar plates appear to be contaminated?
3. If agar plates contaminated, what could have been the problem?

Practical 3
Title: Staining techniques

Objective:

After completing the exercise, you will be able to:

1. To learn how to prepare different types of media and sterilize each one using autoclave

Introduction:

Gram staining is a differential staining technique that differentiates bacteria into two groups: gram-positives and gram-negatives. The procedure is based on the ability of microorganisms to retain color of the stains used during the gram stain reaction. Gram- negative bacteria are decolorized by the alcohol, losing the color of the primary stain, purple. Gram-positive bacteria are not decolorized by alcohol and will remain as purple. After de-colorization step, a counterstain is used to impart a pink color to the decolorized gram-negative organisms. To observe the colony morphology and microscopic examination of Gram-stained smears.

Cultures of bacteria:

1. You are provided with one gram-positive and one gram negative organism on nutrient agar.
2. Observe the colony morphology of the organisms provided.
3. Make direct smears of each organism provided.
4. Perform gram stain on each bacterial smear.
5. Observe the gram-stained smear under the microscope using objective 100X.
6. Record your observations in the table 1.

Procedure:

A. Preparation of bacterial smear

1. Place a drop of saline onto a slide.
2. Touch a colony with a sterilized loop mix the organism with the saline until there is a uniform, thin film of the organism on the slide.
3. Air dry and heat fix the slide by passing over the flame of a Bunsen burner. Allow the slide to cool.

B. Components of Gram stain

1. Primary dye: Crystal violet
2. Mordant: Gram's iodine (Lugol's iodine)
3. Counterstain: Safranin
4. Gram's de-colorizer: 25% acetone, 75% isopropyl alcohol

C. Gram-staining procedure

1. Stain with crystal violet for 1 minute. Rinse the water.
2. Apply Iodine solution (Lugol's iodine) for 1 minute. Rinse with water.
3. Decolorize with Gram stain decolorizer (acetone) for 30 seconds then IMMEDIATELY rinse with water.
4. Counterstain with Safranin for 1 minute. Rinse with water.
5. Dry the slide by putting the stained slide in between the filter paper.

D. Microscopic observation of a Gram-stained smear

A good approach to observing any bacterial smear is to examine it under low power (40x) in bright field to become oriented. After focusing, one then work up to 100x, and finally under oil immersion (100x). The immersion oil is placed directly on the smear.

Results:

Questions:

1. Write down the importance of gram stain.

2. Please fill up the below table.

Microbes	Staining Reaction	Morphology
Staphylococcus aureus		
Bacillus cereus		
Escherichia coli		

Practical 4
Title: Bacteria enumeration

Objectives:

After completing the exercise, you will be able:

1. To determine the number of bacterial cells in a broth culture or liquid medium
2. To determine the number of viable cells

Introduction:

In the study of microbiology, there are numerous occasions when it is necessary to either estimate or determine the number of bacterial cells in a broth culture or liquid medium. Determination of cell numbers can be accomplished by a number of direct or indirect methods. The methods include standard plate counts, turbidimetric measurements, visual comparison of turbidity with a known standard, direct microscopic counts, cell mass determination, and measurement of cellular activity.

Standard Plate Count (Viable Counts)

A viable cell is defined as a cell which is able to divide and form a population (or colony). A viable cell count is usually done by diluting the original sample, plating aliquots of the dilutions onto an appropriate culture medium, then incubating the plates under proper conditions so that colonies are formed. After incubation, the colonies are counted and, from knowledge of the dilution used, the original number of viable cells can be calculated. For accurate determination of the total number of viable cells, it is critical that each colony comes from only one cell, so chains and clumps of cells must be broken apart. However, since one is never sure that all such groups have been broken apart, the total number of viable cells is usually reported as colony-forming units (CFUs) rather than cell numbers. This method of enumeration is relatively easy to perform and is much more sensitive than turbidimetric measurement. A major disadvantage, however, is the time necessary for dilutions, platings and incubations, as well as the time needed for media preparation.

Turbidimetric Measurement

A quick and efficient method of estimating the number of bacteria in a liquid medium is to measure the turbidity or cloudiness of a culture and translate this measurement into cell numbers. This method of enumeration is fast and is usually preferred when a large number of cultures are to be counted. Although measuring turbidity is much faster than the standard plate count, the measurements must be correlated initially with cell number. This is achieved by determining the turbidity of different concentrations of a given species of microorganism in a particular medium and then utilizing the standard plate count to determine the number of viable organisms per milliliter of sample. A standard curve can then be drawn (e.g., this lab protocol section), in which a specific turbidity or optical density reading is matched to a specific number of viable organisms. Subsequently, only turbidity needs to be measured. The number of viable organisms may be read directly from the standard curve, without necessitating time-consuming standard counts. Turbidity can be measured by an instrument such as a colorimeter or spectrophotometer. These instruments contain a light source and a light detector (photocell) separated by the sample compartment. Turbid solutions such as cell cultures interfere with light passage through the sample, so that less light hits the photocell than would if the cells were not there. Turbidimetric methods can be used as long as each individual cell blocks or intercepts light; as soon as the mass of cells becomes so large that some cells effectively shield other cells from the light, the measurement is no longer accurate. Before turbidimetric measurements can be made, the

spectrophotometer must be adjusted to 100% transmittance (0% absorbance). This is done using a sample of uninoculated medium. Percent transmittance of various dilutions of the bacterial culture is then measured and the values converted to optical density, based on the formula: Absorbance (O.D.) = 2 - log % Transmittance. A wave length of 420 nm is used when the solution is clear, 540 nm when the solution is light yellow, and 600-625 nm is used for yellow to brown solutions.

Direct Microscopic Count

Petroff-Hausser counting chambers can be used as a direct method to determine the number of bacterial cells in a culture or liquid medium. In this procedure, the number of cells in a given volume of culture liquid is counted directly in 10-20 microscope fields. The average number of cells per field is calculated and the number of bacterial cells ml^{-1} of original sample can then be computed. A major advantage of direct counts is the speed at which results are obtained. However, since it is often not possible to distinguish living from dead cells, the direct microscopic count method is not very useful for determining the number of viable cells in a culture.

A. Dilution and spread plate

Materials:

1. Six 9 ml dilution tubes of Nutrient broth
2. Six nutrient agar plates
3. Sterile pipettes
4. Glass spreader/Hockey stick
5. 95% ethyl alcohol in glass beaker
6. Overnight broth culture of *Escherichia coli*
7. Bunsen burner

Procedures:

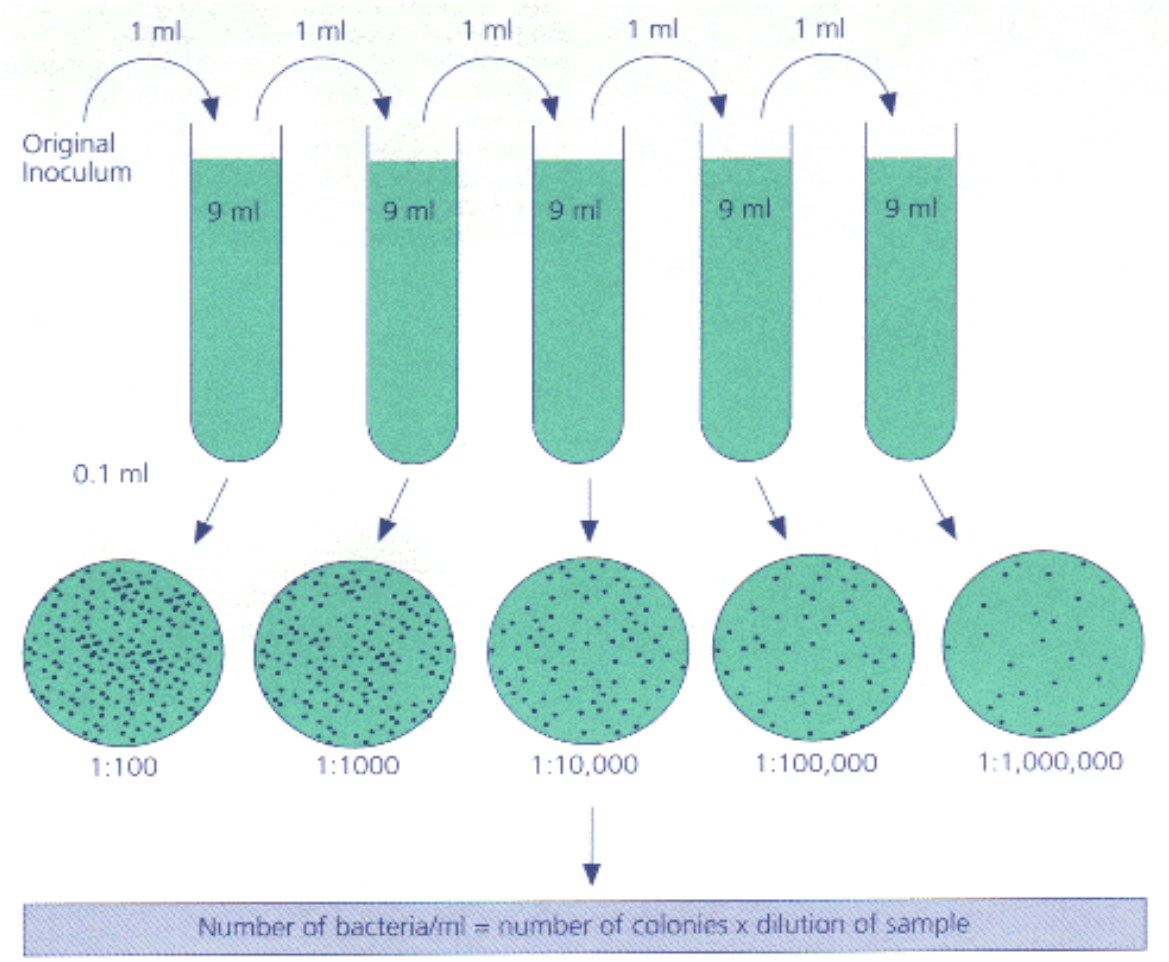
1. Place six sterile tubes in a rack and label them with number 1-6.
2. Add 9.0 ml of sterile broth to each test tube.
3. Transfer 1.0 ml from bacteria culture into tube 1.Mix the content well.
4. With a fresh pipette, Transfer 1.0 ml of the mix contents from tube 1 to 2.
5. Continue the dilution process until tube number 6.
6. NOTES: Mix all the tube well.
7. Place the tubes in the incubator for 24 hours.
8. After 24 hours incubation, observed the turbidity of the test tubes.
9. Transfer 1.0 ml of bacteria suspension from each test tube using a sterile pipette into the NA plate.
10. Spread the suspension on the NA plate using a sterile hockey stick.
11. 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.
12. Repeat the flaming and spreading for each of the remaining five plates.
13. Incubate the plates for 24 hours and count the colony form on the plates.

NOTE:

i. Do not mix the tube for too long.

ii. All of the equipment must be sterile and the experiment must be conducted by using a proper aseptic technique.

Dilution method



Results:

Dilution method result picture:

Most Probable number:

Test Tube	Dilution	Colony Count			Most probable number (MPN)= No of colonies X amount of sample plated/dilution factor
		R1	R2	Average	
1	10^{-1}	40	38	39	$3.9X10^{-1}$ CFU/ml
2	10^{-2}				
3	10^{-3}				
4	10^{-4}				
5	10^{-5}				
6	10^{-6}				
7	Control				

If your plate has more than 300 colonies record as "TNTC" for Too numerous to count

Questions

1. Why it is important to have the control test tube?
2. Why we need to use the fresh pipette each time?

B. Colony counting

Materials:

1. Colony Counter

Procedure:

1. Remember to pull plates and refrigerate after 48 hours max. either then or next lab period, count the number of colonies on each plate, calculates an average and record results (plates that have 30 – 300 colonies)

Results:

	10^{-6}	10^{-7}	10^{-8}
Plate #1			
Plate #2			
Average			

Number of colony-forming units per ml _____

LINCOLN UNIVERSITY COLLEGE

Wisma Lincoln, No. 12, 14, 16 & 18,
Jalan SS 6/12,47301 Petaling Jaya,
Selangor Darul Ehsan, Malaysia.
Tel.: +603-7806 3478 Fax: +603-7806 3479
Toll Free: 1-300-880-111
E-mail: lucp@lincoln.edu.my
Web.: www.lucp.net

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