Advance Microbiology Practical Manual

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Practical 1: Introduction of basic component-1 for microbiology laboratory

Facilities Required for Microbiology lab:

1. **Main Laboratory Room**
   - Most of the laboratory activities are performed in the main laboratory. It should have sufficient space inside. A concrete shelf with glazed tile or marble top should project from the wall for keeping the equipments and for performing routine works.
   - Few sun mica-top laboratory tables with shelves, sinks and gas connections should be kept in the lab.

2. **Instrument Room**
   - This room should be kept neat and clean, as most of the sophisticated instruments are kept in it. It should be air-conditioned to make it dust-free and to reduce humidity as well as to avoid high ambient temperatures. Otherwise, accumulation of dust particles on and inside the instruments as well as exposure to high room temperatures decreases the longevity of the instruments.
   - At the same time, high humid conditions lead to the rusting of their metallic components and fungal growth on optical parts, such as microscope lenses and phototubes of spectrophotometers. The instruments to be kept on the floor of the room include fridge, refrigerated centrifuge and ultracentrifuge.
   - Other equipments, such as single-pan precision balance, Quebec colony counter, electronic colony counter, particle counter, electrophoresis apparatus, UV-cum-visible double beam spectrophotometer, computer, Tran illuminator, gas chromatography (GC), high performance liquid chromatography (HPLC), pH meter, trin-ocular research microscope with photomicrography attachment, projection microscope, fluorescence microscope, dark-field microscope, phase-contrast microscope and PCR thermo cycler should be arranged on a continuous concrete shelf projecting from the wall.

3. **Chemical Storage Room**
   - The store room should be closed from all sides with entrance into the main lab. There should be no window; otherwise the chemicals may get spoiled. There should be a number of concrete shelves for storage of chemicals, reagents, glassware's and other such items. The room should not be opened unless needed. The chemicals should be arranged alphabetically on the shelves for easy location.
   - Principles of storage of chemicals such as Labelling, Compatibility, Maintain good stock, do not store chemicals under sinks, Store large breakable containers, particularly of liquids, below shoulder height.

- **Storage Facilities:** Shelving, Acid Cabinets, Flammable solvent cabinets, Fridges & Freezers and Cupboards.
- **Storage of Different Materials:** Acids, Alkalis, Flammable solvents, Chlorinated solvents, Noxious chemicals and Oxidizers.

4. Media Preparation Place

- Media Preparation area should be near to chemical storage room, So that we can access all chemicals very easily.
- Media preparation area should also have distilled water plant, weighing balance, and tap water.

5. Inoculation Room

- This room is meant for inoculation of bacteria i.e. Transfer of bacteria from one container to another. Sometimes, unwanted microbes, usually floating in air on dust particles, may enter into the containers and contaminate the pure stock culture as well as the inoculated ones. To overcome this, the room is kept extremely hygienic. The walls should be plastic-painted and the room should be air-conditioned.
- There should be a laminar flow chamber with gas connection, for inoculation of bacteria. A bottle of disinfectant solution, a sponge pad and a dispose jar should be kept beside the laminar flow chamber as in case of tables in the main lab.

6. Growth Chamber Room

- In routine microbiological analysis, very often it is required to isolate different types of bacteria in samples, to maintain the isolated bacteria as pure cultures and to identify them in subsequent days by performing several tests.
- Store all the cultures in BOD incubator for short time to grow and observe all cultures.

7. Wash Room

- Room with a toilet and a place to wash your hands in it.

Microbiology Lab Practices and Safety Rules:

- Wash your hands with disinfectants when you arrive at the lab and again before you leave.
- Wear laboratory coats in the lab. Students with long hair must put up the hair.
- At the start and end of each laboratory session, students should clean their assigned bench-top area with a disinfectant solution provided. That space should then be kept neat, clean, and uncluttered throughout each laboratory period.

- Eating or drinking in the laboratory is not permitted. No mouth pipetting.
- Label everything clearly. Sterilize equipment and materials.
- Avoid loose fitting items of clothing. Wear appropriate shoes in the laboratory.
- Report any breakage of equipment to the instructor.
- Report any personal accidents such as cuts to the instructor at once.
- Turn off Bunsen burner when not in use.
- Discard all cultures and used glassware into the container labeled CONTAMINATED. (This container will later be sterilized.) Plastic or other disposable items should be discarded separately from glassware in containers to be sterilized.
- Never place contaminated pipettes on the bench top.
- When you flame sterilize with alcohol, be sure that you do not have any papers under you.
- Before beginning your laboratory exercise, wash off the bench top with the disinfectant provided. When exercises are completed, wash off the bench top again. Always wash your hands with soap and water before leaving the laboratory.
- Before leaving the laboratory, see that all the equipments are in the proper location and gas and water turned off.
- Purchase a fine point, waterproof marker and small roll of masking tape. Use them to clearly label your cultures.
- If you should spill or drop a culture or if any type of accident occurs, call the instructor immediately. Place a paper towel over any spill and pour disinfectant over the towel. Let the disinfectant stand for 15 minutes and then clean the spill with fresh paper towels. Remember to discard the paper towels in the proper receptacle and wash your hands carefully.
- Disinfect work areas before and after use with 70% alcohol or fresh 10% bleach. Laboratory equipment and work surfaces should be decontaminated with an appropriate disinfectant on a routine basis and especially after spills, splashes or other contamination.
- Replace caps on reagents, solution bottles and bacterial cultures. Do not open Petri dishes in the lab unless absolutely necessary.
- Cultures are not to be removed from the laboratory unless the instructor gives permission.
- Always place culture tubes (broth and slants) in the upright position in a rack or basket for incubation or disposal.
- Dispose off all solid waste materials in a biohazard bag and autoclave it before discarding in the regular trash.
- Treat all cultures as potentially pathogenic, *i.e.*, flood areas with disinfectant if

cultures are spilled, wash hands after contact and notify your instructor at once.

- Read the instructions carefully before beginning an exercise. Also, make sure you have all the materials needed for the exercise at hand before you commence the experiment. Ask the instructor for clarification of any points about which you are in doubt.
- Flame the inoculating loop or needle immediately before and after use. If viscous material is present on the loop or needle, dry it at the side of the flame before placing it directly in the flame.
- Laboratory note books must be kept up-to-date. Illustrations should be done when requested.
- Make sure you consult the instructor to dispose of the cultures that are not needed any longer. Remove all labels and markings from the tubes before disposing of them; do not discard anything into the sinks.
- Please inform your instructor if you have any medical condition that could potentially affect your safety in the laboratory (eg: diabetes, epilepsy, immune suppression etc.). This information will help the instructor to deal with any emergency that would arise. The information will be treated confidentially and it will not affect their ability to participate in the laboratory activities.
- Be systematic and logical. Keep a faithful record of all the experiments and observations. Update it regularly and submit it for evaluation at the end of each exercise.
- Work either using laminar air flow chamber or light the burner at least five minutes prior to making any inoculations and work near the burner.

Handling Microbial Cultures:

- Before working with media and microbial cultures, wipe your work area with 70% ethanol or an appropriate disinfectant.
- Ensure you are wearing appropriate protective clothing. This will protect you from the culture as well as reduce accidental culture contamination.
- Only use sterile glassware, equipment, media, and reagents. Check media for contamination by observing for turbidity.
- Handle only one microbial culture at a time. The risk of cross contamination or misidentification increases when more than one strain is handled at a time.
- When working with test tube cultures, hold cultures at an angle after you remove the lid to avoid airborne particles from falling into the culture. Sterilize the outside of the culture tube using a Bunsen burner flame.
- When working with plated cultures, hold the Petri dish lid at an angle after you remove the lid to avoid airborne particles falling into the culture dish.
- When handling a microbial culture, work quickly and carefully in an environment that has minimal distractions. Do not leave the lid off your culture

for extended periods of time.

- Never take cultures outside of the laboratory.
- Notify the laboratory supervisor immediately of any spills.

It is mandatory to have clear and accurate records of all experiments conducted in the laboratory.

Practical 2: Introduction of basic component-2 for microbiology laboratory

Basic Requirement:

Basic requirements of a microbiology laboratory:

A microbiology laboratory requires well-built rooms equipped with Chemicals, glassware, tools and equipments. Some of the most important items of equipment are the following.

I) Common Glassware

The most important glassware used in a microbiological laboratory are test tubes, culture tubes, Petri dishes, Measuring cylinder, pipettes, glass spreader, Flasks, screw-capped glass bottles, haemocytometer etc.

2) **Test Tubes, Culture Tubes and Screw-Capped Tubes**

The culture tubes are used in microbiology and related sciences for handling and culturing of all kinds of live organisms, such as molds, bacteria, seedlings, plant cuttings, etc. The round bottom makes aeration of culture during laboratory condition.

3) **Petri dish**

Petri dishes are widely used in biology to cultivate microorganisms. It is most suited for organisms that thrive on a solid or semisolid surface. The culture medium is often an agar plate, a layer a few mm thick of agar containing whatever nutrients the organism requires (such as blood, salt, aminoacids, carbohydrates) and other desired ingredients (such as dyes, indicators and drugs). The agar and other ingredients are dissolved in warm water and poured into the dish and left to cool down. Once the medium solidifies, a sample of the organism is inoculated ("plated"). The dishes are then left undisturbed for hours or days while the organism grows, possibly in an incubator.

4) Pipettes and Micropipettes

A pipette (sometimes spelled pipet) is a laboratory tool commonly used to transport a measured volume of liquid, often as a accurate media dispenser.

5) Glass Spreader

A cell spreader or plate spreader is a hand tool used in biology and related fields to smoothly spread cells and bacteria on a culture plate, such as a Petri dish. Cell spreaders can be made from glass, plastic, or metal, and come in various shapes.

6) **Haemocytometer**

A hemocytometer consists of a thick glass microscope slide with a grid of perpendicular lines etched in the middle. The grid has specified dimensions so that the area covered by the lines is known, which makes it possible to count the number of cells in a specific volume of solution

7) **Conical flasks and measuring cylinder**.

Erlenmeyer flasks are used in microbiology for the preparation of microbial cultures. It enhances gas exchange during incubation and shaking. The oxygen transfer rate in Erlenmeyer flasks depends on the agitation speed, the liquid volume, and the shake-flask design. Measuring cylinder are used to measure the accurate volume for microbial culture.

Tools and Equipments in Microbiology Laboratory:

The most common equipment are inoculation needle, inoculation/transfer loop, Bunsen burner, autoclave (or pressure cooker), incubator, hot air oven, refrigerator, centrifuge, spectrophotometer, magnetic stirrer, orbital shaker, hot plate, Distillation water still, UV- lamp, water-bath, carbon dioxide cylinder, single-pan balance with weights (for rough use), chemical balance, pH meter, colony counter, laminar air flow, electrophoretic apparatus, microscopes etc.

1) Inoculation Needle & Inoculation Loop

An inoculation needle is laboratory equipment used in the field of microbiology to transfer and inoculate living microorganisms. A standard reusable inoculation needle is made from nichrome or platinum wire affixed to a metallic handle. A disposable inoculation needle is often made from plastic resin.

2) **Bunsen Burner**

A **Bunsen burner** is a kind of gas **burner** used as laboratory equipment; it produces a single open gas flame, and is used for heating, sterilization, and combustion. The gas can be natural gas (which is mainly methane) or a liquefied petroleum gas, such as propane, butane, or a mixture.

3) Water Bath

A water bath is laboratory equipment made from a container filled with heated water. It is used to incubate samples in water at a constant temperature over a long period of time.

1) Autoclave

An autoclave is a machine used to perform sterilization of biological sample, culture medium. Many autoclaves are used to sterilize equipment and supplies by subjecting them to pressurized saturated steam at 121 °C (250 °F) for around 15–20 minutes depending on the size of the load and the contents.

2) Laminar Air Flow Chamber

A laminar flow cabinet or tissue culture hood is a carefully enclosed bench designed to prevent contamination of biological samples, or any particle sensitive materials. Air is drawn through a HEPA filter and blown in a very smooth, laminar flow towards the bottom. It prevents microbial contamination during the experiments.

3) Incubator

An incubator is a device used to grow and maintain microbiological cultures or cell cultures. The incubator maintains optimal temperature, humidity and other conditions such as the CO_2 and oxygen content of the atmosphere inside. The different optimum condition can be fixed according to need of experiments.

4) Colony Counter

Colony counters are used to estimate the viable microbial colonies over the agar solid plates. It has more accuracy so minimize the technical error during the analysis of results. It is typically used in Ames testing, bacterial mutation assays, and E. coli bacterial colonies.

Practical 3: Preparation of nutritional media for cultivation of bacteria.

The LB broth is a nutrient rich broth requires for growth of bacteria (*Escherichia coli*) in laboratory condition.

Materials and Reagents

- 5g Bacto-tryptone
- 2.5g yeast extract
- 5g NaCl
- 7.5g agar (Only necessary if making LB agar plates)
- 500mL of dH2O (distilled water)

Equipment

- 1L Pyrex bottle
- 1L graduated cylinder
- Filter paper and scoopula
- Stack of sterile plates (this protocol makes approximately 25)
- Bunsen burner/ethanol burner
- 70% EtOH wash bottle
- Paper towels/wipes

Procedure

Part 1: Making the LB broth

This part can be carried out at a regular lab bench.

1. Obtain a clean 1L pyrex bottle
2. Obtain a graduated cylinder with 500mL of dH2O and add to the bottle. Record the amount added.
3. Using filter paper, separately measure out 5g of NaCl, 5g of Tryptone, and 2.5g of yeast extract on a scale and add them to the bottle. Swirl the bottle in a circular motion to mix. Remember to re-calibrate your scales in between measurements.
4. If you are making LB agar plates, weigh and add 7.5g of agar and swirl to mix. Record the amount added.

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Part 2: Autoclaving

1. Lightly seal the top of the beaker with aluminium foil, and label the beaker with autoclave tape stating LB (agar)–Marked the bottle properly.
2. Use appropriate transportation protocols to bring the LB bottle into the autoclave room.

Remember to store the beaker in an autoclavable basin, in case of spills.

3. Check the water level on the autoclave, if necessary. Autoclave on the liquid setting for

approximately 20 min.
4. The contents of the beaker will be hot after autoclaving, therefore take the necessary measures to prevent burns.
5. After autoclaving, allow the LB media to cool to 45°C before handling.
6. The LB broth can be stored in sterile conditions at room temperature, and should be good for 1-2 months. Flame the lip of the bottle each time the LB is used. If the LB contains antibiotics, store in a -4°C freezer.
However, it is not recommended to store longer time, LB with antibiotics as the antibiotics will degrade over time

Part 3: Pouring the plates (for LB agar)
While pouring the plates, it is crucial to maintain a sterile environment. This should be done in room with a sterile environment provided by a lit Bunsen burner.
Note: steps 1-3, in addition to the clean up from Part 1, can be done while waiting for autoclave.
1. Sterilize the workspace with 70% EtOH before depositing your materials. Light the Bunsen burner.
2. Obtain a stack/roll of empty plates. The plates should still be in their plastic sleeve/wrapping, as they should be sterile. Don't throw out the wrapping as it can be used to store the plates. It is essential that you minimize any chance of contaminating the plates. Make sure that you open the package at the top and expose the plates as minimally as possible.
3. Once you take the plates out, store them upside down on your lab bench. Label the plates properly. Once labeled, you may stack the plates to free up workspace.
4. Allow the LB media to cool before pouring. The LB will start to settle at ~30°C.
5. If you are preparing selective media, add antibiotic to the mixture. Swirl the flask in a circular motion to mix.
7. Take an empty plate and open it slightly. You do not need to open it all the way to pour the agar.
8. Pour agar until 2/3 of the plate has been covered, or approximately half of the plate has been filled when viewed from the side. Pour the agar slowly to prevent the formation of bubbles. Swirl the plate in a circular motion to distribute the media evenly on the plate.
9. If you pour too much LB, you will not be able to produce 25 plates. If you don't pour enough media, it may minimize bacterial growth.
10. After pouring, set the plates to cool in stacks of 4-5 to save space and flip the plates to prevent condensation forming on the agar. Don't stack plates too high - we want to minimize the risk of spills. Allow the plates to cool for at least 20 minutes until the agar has solidified.
11. Rinse the Pyrex bottle with water before the remnants solidify and become hard to

remove.

12. The plates can then be stacked and stored in plastic bags (ideally, reuse the plastic bags.

13. Store LB agar plates in a 4°C freezer. They should be good for 1-2 months.

Practical 4: Isolation of bacteria in pure culture from mixed culture by streak plate method.

The main objective of the streak plate method is to produce well separated colonies of bacteria from concentrated suspensions of cells. A sterilized inoculating needle with a loop made up of either platinum or nichrome wire is used for streaking.

Matarial and reagents:

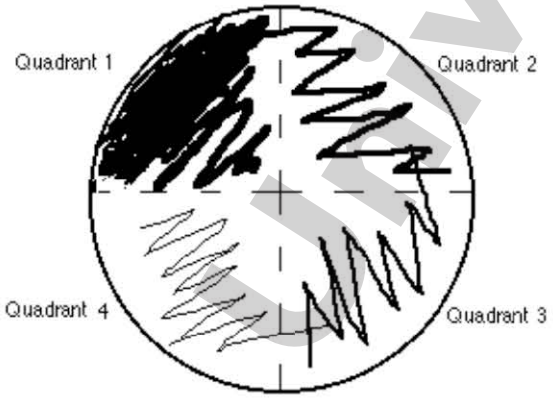
- Dilution tubes -10
- Nutrient agar plates
- ml and 0.1 ml pipets
- Glass spreader/ steaker
- 95% ethyl alcohol in glass beaker (Keep alcohol away from flame)
- Mixed broth culture of microbes
- Sterile PBS solution

PROCEDURE

Streaking for Isolation by the Quadrant Method

1. Obtain one suitable agar plates Turn these culture media dishes bottom side up and label the perimeter of the dishes with your initials, date, section number and table number, temperature of incubation, type of medium and specimen.
2. Draw two perpendicular lines with a marker on bottom of the plate to divide the circle into 4 quadrants.
3. Holding an inoculating loop between your thumb and index finger, insert the wire portion into the Bunsen burner flame, heating the entire length of the wire until it is red and glowing. Allow the wire to cool before doing the next step.
4. Using your free hand, pick up the tube containing the mixed culture and gently shake it to disperse the culture. Remove the tube cap or plug with free fingers of the hand holding the sterile inoculating loop and carefully flame the lip of the tube in the Bunsen burner flame.
5. Tilt the tube to bring the broth culture within 1 inch from the lip of the tube. Insert the sterile loop and remove a small amount of growth; a loopful is usually sufficient. Try not to touch the sides of the tube with the loop.
6. Flame the tube lip again, carefully replace the tube cap or plug, and return the culture tube to the test tube rack.

7. Expose the agar surface of each plate for inoculation by raising the lid at an angle over the agar, thus keeping the plate surface protected from aerial contamination.
8. Apply the mixed culture on the loop onto the first quadrant by sweeping the area of this quadrant. Spread the specimen out well.
9. Flame the loop and allow it to cool. You may cool the loop in an uninoculated area of the medium.
10. Now streak the inoculums from quadrant 1 into quadrant 2. Use smooth, nonoverlapping strokes. Utilize the entire quadrant 2 as shown in the figure below. Flame the loop when done.



11. Let the loop cool. Now streak the inoculum from quadrant 2 into quadrant 3 by smooth, non-overlooking strokes again.
12. Flame your loop one more time and let it cool. Now bring some inoculum from quadrant 3 into quadrant 4 in the same manner as for other previous quadrants.
13. Flame your loop and cool.
14. Invert the plates and incubate plate at 37°C or according the requirement
The reason the plate is inverted is the fact that the air space between the dish lid and the agar surface is saturated with moisture; during incubation the moisture condenses on the upper lid as droplets. As these droplets collect into a large drop, the water drips onto the agar surface causing the spread and mixing of colonies. Inversion of the plate eliminates this problem.
15. When plates from the mixed culture, sufficiently incubated, look for growth and the number of different colonies appear in quadrants 3 and 4. The colonies in quardent 1 and 2 overlap (confluent) and may look different because of competitive phenomena.
16. The separated colony can be considered as pure and used for stock preparation with proper labelling.
17. Write characterestics of each colony like shape, texture, color etc. in table for further analysis.

Practical 5: Importance of aseptic technique in microbial culture.

In the microbiology lab we use aseptic technique to: Prevent contamination of the specific microorganism we are working with. Prevent contamination of the room and personnel with the microorganism we are working with.

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

Material and Reagents:

- Nutrient agar plates
- ml and 0.1 ml pipets
- Glass spreader aka ihockey stickî
- 95% ethyl alcohol in glass beaker

Procedure:

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. Control plates without any environmental exposer.
4. Invert the plates and incubate at room temperature/37C for 12hr.
5. Observe the grown colony over the plate.
6. Count colony observe in expose plate and control plate
7. Compared and analyzed results accordingly

Practical 6: Enumeration of bacteria by standard plate count.

It is used to 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.

Material and Reagents

- Dilution tubes of PBS sterile solution
- Nutrient agar plates
- ml and 0.1 ml pipets
- Glass spreader
- 95% ethyl alcohol in glass beaker
- Overnight grown broth culture of microbes

Procedure:

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. Be sure to mix the nutrient broth tubes before each serial transfer. Transfer 0.1 ml of the final three dilutions to each of three nutrient agar plates, and label the plates.
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. This will ignite the excess alcohol on the spreader and effectively sterilize it.
3. Spread the 0.1 ml inoculums 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.
6. Observe the bacterial colony appear over the plate
7. Count the colonies and used them for further analysis.

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.

Practica 7: Enumeration of bacteria by Direct Microscopic Counts.

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.

Matarials and Reagents:

- Petroff-Hausser counting chamber
- Cover slips
- Sterile diluent (nutrient broth or sterile PBS)
- Pasteur pipets
- Microscope
- Culture Stock

Procedure:

Be extremely careful handling Petroff-Hausser counting chambers!

1. Clean P-H counting chamber with 70% alcohol an let air dry.
2. Mix culture well and apply a single drop to counting chamber with Pasteur pipet. Examine the counting chamber using high power, oil immersion objective.
3. Make a preliminary estimation of the concentration of cells from the overnight culture of bacteria using the following formula:

$$\frac{\text{Total cells counted} \times 2.0\times10^{7} \times \text{dilution factor}}{\text{\# small squares counted}} = \text{cells/ml}$$

4. You may have to adjust downward using one of your initial serial dilutions so that the counts per small square are in the 5 to 15 cell range.
5. Once this is done, make sure to allow time for cells to settle and move focus through the suspension (i.e., up and down) so as to count all cells within the small square "box". Most cells will have attached to the bottom and/or top glass interface. You can also check the depth, which is 20 μm. The small square should also be 50 by 50 μm.
6. Count the number of bacterial cells in at least 10 small squares. Variability should be less than +/- 10%.
7. Remember to pull plates and refrigerate after 48 hours max. Either then or next lab period, count the number of colonies on each plate, calculate an average and record results.
8. Compare results from the standard plate counts with P-H direct microscopic counts.

Practical 8: Preparation of bacterial smear and Gram's staining.

The performance of the Gram Stain on any sample requires four basic steps that include applying a primary stain (crystal violet) to a heat-fixed smear, followed by the addition of a mordant (Gram's Iodine), rapid decolorization with alcohol, acetone, or a mixture of alcohol and acetone.

Materials and Reagents:

- Clean glass slides
- Inoculating loop
- Bunsen burner
- Microscope
- Lens paper and lens cleaner
- Immersion oil
- Distilled water
- 18 to 24 hour cultures of organisms
- Primary Stain - Crystal Violet
- Mordant - Grams Iodine
- Decolourizer - Ethyl Alcohol
- Secondary Stain - Safranin

Procedure:

1. Wipe the slide with sprit or alcohol and keep it for drying.
2. Labeling of the slides properly.
3. Place one needle of solid bacterial growth or two loops of liquid bacterial growth in the center of a clean slide.
4. If working from a solid medium, add one drop (and only one drop) of water to your specimen with a water bottle. If using a broth medium, do not add the water.

5. Now, with your inoculating loop, mix the specimen with the water completely and spread the mixture out to cover about half of the total slide area.
6. Place the slide on a slide warmer and wait for it to dry. The smear is now ready for the staining procedure.
7. Place slide with heat fixed smear on staining tray.
8. Gently flood smear with crystal violet and let stand for 1 minute.
9. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
10. Gently flood the smear with Gram's iodine and let stand for 1 minute.
11. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
12. Decolorize using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Be careful not to over-decolorize.
13. Immediately rinse with water.
14. Gently flood with safranin to counter-stain and let stand for 45 seconds.
15. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
16. Blot dry the slide with tissue paper.
17. View the smear using a light-microscope under oil-immersion.

Practical 9: Antimicrobial sensitivity test and demonstration of drug resistance.

The performance of antimicrobial susceptibility testing by the clinical microbiology laboratory is important to confirm susceptibility to chosen empirical antimicrobial agents, or to detect resistance in individual bacterial isolates.

Materials and Reagents

- Petriplate containing microbial culture (For example, Escherichia coli)
- Inoculation loop
- Bunsen burner
- Saline solution
- Cotton swab
- Antibiotic disks (Streptomycin, Ciprofloxacin , Chloramphenicol,
- Tooth pick
- Incubator
- Ruler

Procedure:

1) Select a pure culture plate of one of the organisms to be tested.

2) Aseptically emulsify a colony from the plate in the sterile saline solution. Mix it thoroughly to ensure that no solid material from the colony is visible in the saline solution.
3) Repeat until the turbidity of the saline solution visually match that of the standard turbidity.
4) Take a sterile swab and dip it into the broth culture of organism.
5) Gently squeeze the swab against the inside of the tube in order to remove excess fluid in the swab.
6) Take a sterile LB agar plate.
7) Use the swab with the test organism to streak agar plate for a lawn of growth.
8) After the streaking is complete, allow the plate to dry for 5 minutes.
9) Antibiotic discs can be placed on the surface of the agar using sterilized forceps.
10) Gently press the discs onto the surface of the agar using flame sterilized forceps or inoculation loop.
11) Carefully invert the inoculated plates and incubate for 12 hours at 37° C.
12) After incubation, use a metric ruler to measure the diameter of the zone of inhibition for each antibiotic used.
13) Compare the measurement obtained from the individual antibiotics with the standard table to determine the sensitivity zone.
14) Compare the measurement obtained from the individual antibiotics to the standard table to determine whether the tested bacterial species is sensitive or resistant to the tested antibiotic.