Practical 1

General safety guidelines in laboratory

Microbiology laboratory is a place for culturing and dealing with different types of microorganisms to be examined and diagnosed in the laboratory. To ensure a positive result that must be obtained from our work, avoiding contamination which could be occurred, and also for our personal safety in the same time, it is important to have a Good Microbiological Laboratory Practice (GMLP) that aimed to develop our proficiency to contain uncontrolled microbes spreading. It is important to make our work under aseptic techniques, for that general guidelines and precautions must be followed in the Laboratory represented by:

1. Reading the notices that are related to laboratory before you entering.
2. Leave bags, books and unnecessary items outside.
3. Avoiding eat, drink and smoke inside the laboratory.
4. Wearing a clean and knee length coat for laboratory with arm covering to the wrist.
5. Follow the laboratory protocols before working and kept it away from the specimens, also for labeling the specimens, separate marker pens should be used.
6. Avoid touching eyes, nose and mouth during the work.
7. Avoid pipetted material by mouth.
8. Using permanent labeling marker pen to label test tubes, plates or other glass material you working with to avoid losing information you need.
9. Wearing gloves as you are handled and cultured the clinical specimens.
10. After finishing your work, the gloves should be removed by pulling one glove toward the fingers to turn the glove inside out and hold it in the gloved hand. The other glove is removed by putting finger or two of ungloved hand under the glove and turning the inside out with containing the first one, and then discarded the gloves into contaminated waste container (figure 1.1).

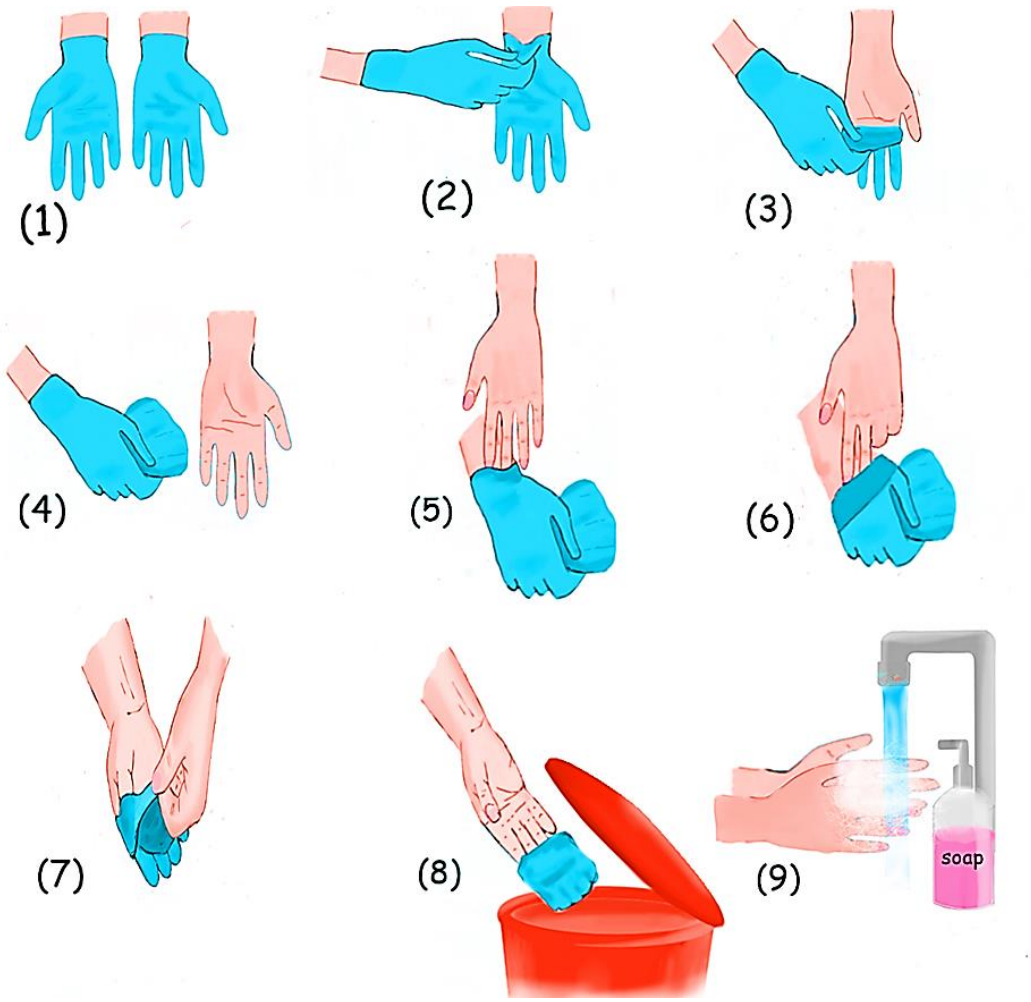


Figure 1.1: Removing laboratory gloves after finishing work.

11. When the procedure you work on is aerosols it is important to do this procedure inside a bio-safety cabinet wearing goggles and mask for protection.
12. Using the disinfectant to clean and sterilize the work area of bench before and after your laboratory working.
13. Spillage of bacterial specimens, cultures or any contaminated materials should be reported immediately to the attending technician and do not remove without permission.

14. The spillages materials should be removed by wearing gloves firstly and secondly covering the area with layers of bleach-soaked cloth and leaving about 15 to 30 minutes. After that the spillages materials have been swept and removed with any contaminated and surrounded debris likes glass wears or cotton plugs to a proper container with lid (figure 1.2), (figure 1.3).

Figure 1.2: Biohazard waste container.

Figure 1.3: Biohazard container for sharps and needle disposal.

15. Removing gloves before handling with any records of the laboratory.
16. Washing hands after finishing your work that have been contaminated specimen, bacterial cultures or any chemical materials.
17. The coat and gloves should be removed and not worn outside the laboratory.
18. Microorganisms are leveled to be handled in biological safety cabinet in to groups as shown in table (1.1):

Table 1.1: Levels of microorganisms.

Hazard	Risk level	Description	Aerosols	Treatment	Biological safety cabinet (BSC)
Group 1	1	Unlikely to cause diseases	Not aerosols	——	Clean bench hood (Figure 1.4)
Group 2	2	Rarely cause diseases to workers in laboratory	Not aerosols	Usually available	Class 1 bio-safety cabinet (Figure 1.5)
Group 3	3	May cause severe diseases and serious hazard to workers in laboratory	Aerosols	Available	Class 2 bio-safety cabinet (Figure 1.4,5,6,7,8 and 9) or Class 3 (Figure 1.10)
Group 4	4	Cause severe disease and pose a risk to workers in laboratory	Aerosols	Not available	Class 3 bio-safety cabinet (Figure 1.10)

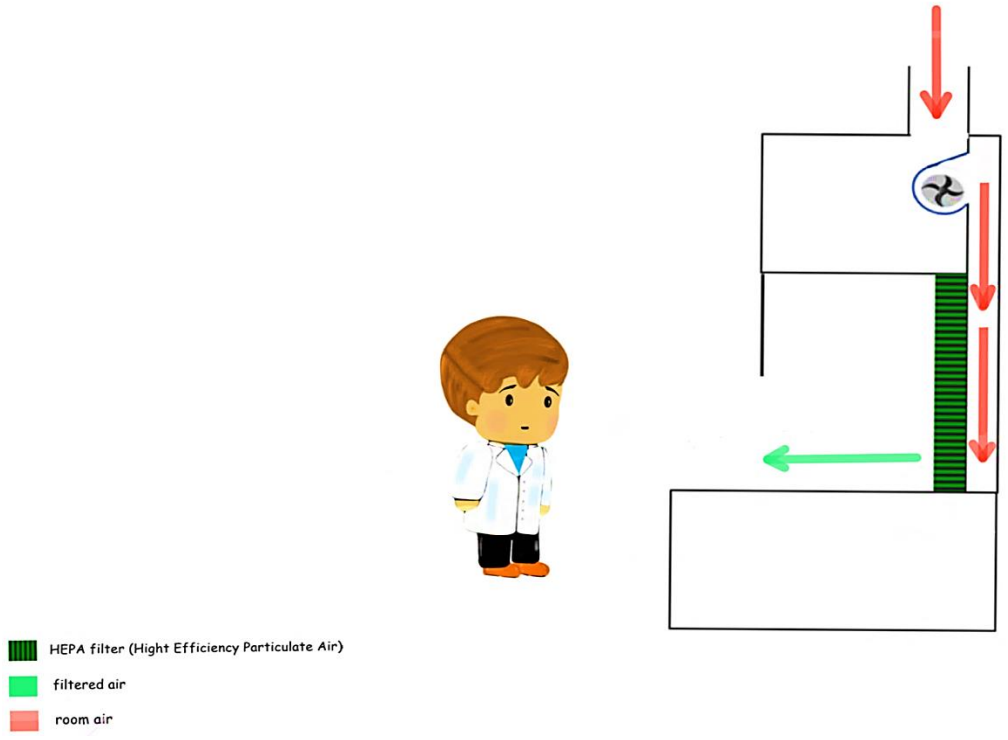


Figure 1.4: Clean bench suitable to deal with hazard group 1 and designed with 100% High Efficiency particulate Air (HEPA) supply air is filtered supply air for product protection and not suitable for biological agent or chemicals.

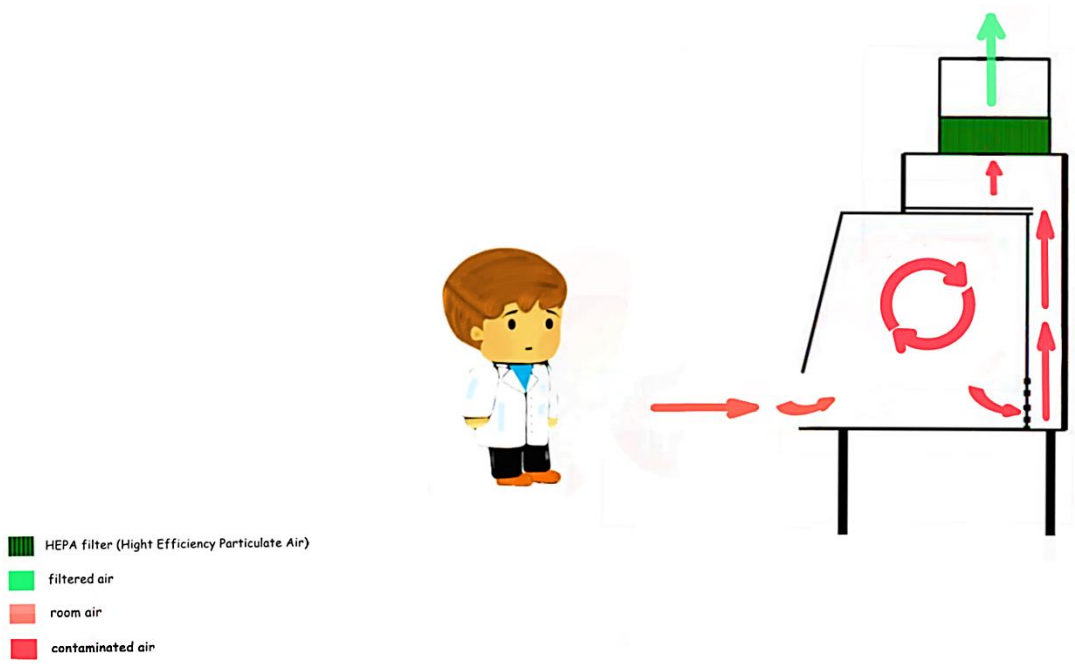


Figure 1.5: Class 1 BSC design to allow the air room inflow to the cabinet across the work area and exhausting filtered air to the environment by HEPA filter which provides protection for the person and environment but not for the biological material.

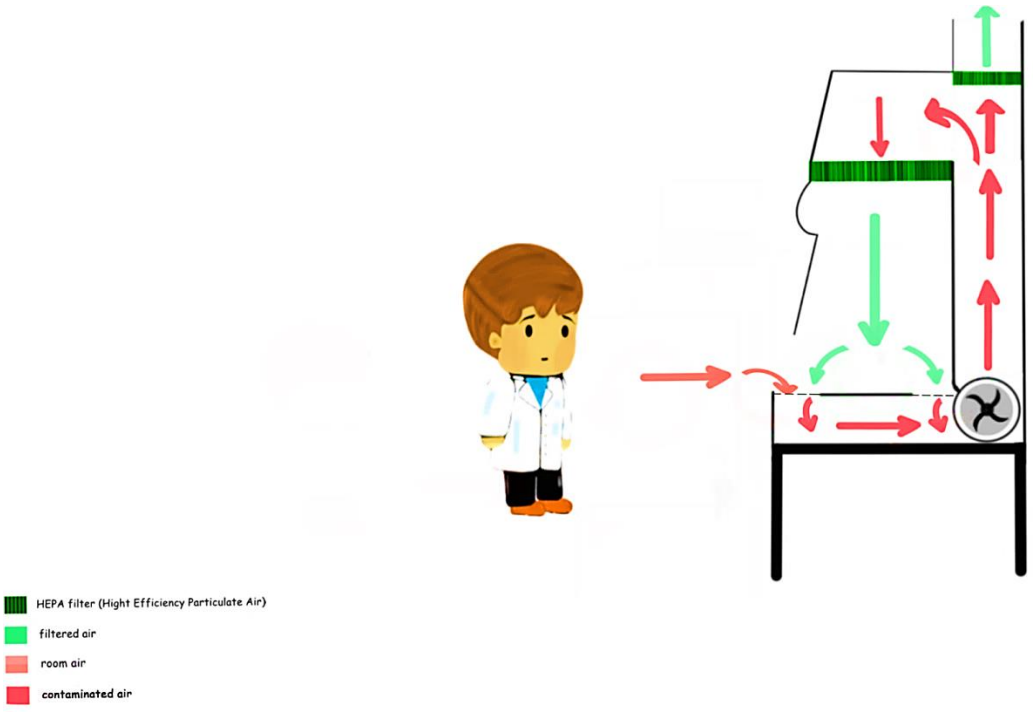


Figure 1.6: Class 2 type A1 BSC design to have an inward airflow and recirculate about 70% of the discharge air through the supply HEPA filter back to the work zone or may be exhaust about 30% outside using a canopy connection which provides protection for person, environment and biological material and suitable for work using biological agents without volatile toxic chemicals and volatile radionuclides.

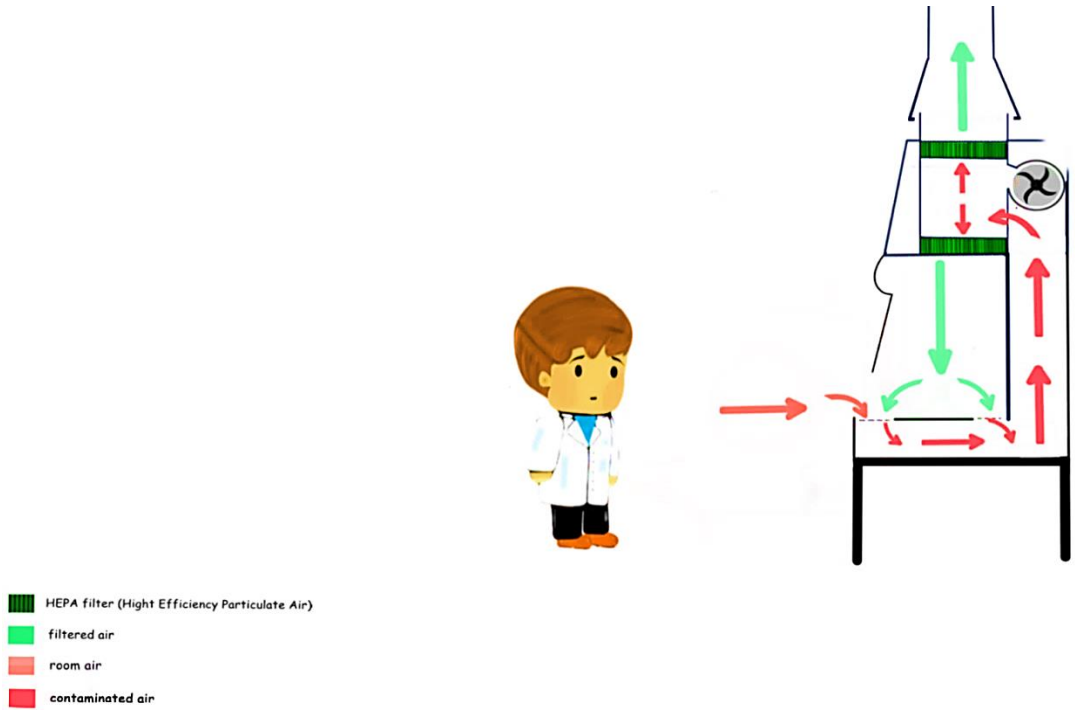


Figure 1.7: Class 2 type A2 BSC similar to A1 design to have an inward airflow and recirculate about 70% of the discharge air through the supply HEPA filter back to the work zone or may be exhaust about 30% outside using a canopy connection which provides protection for person, environment and biological material and suitable for work using biological agents without volatile toxic chemicals and volatile radionuclides.

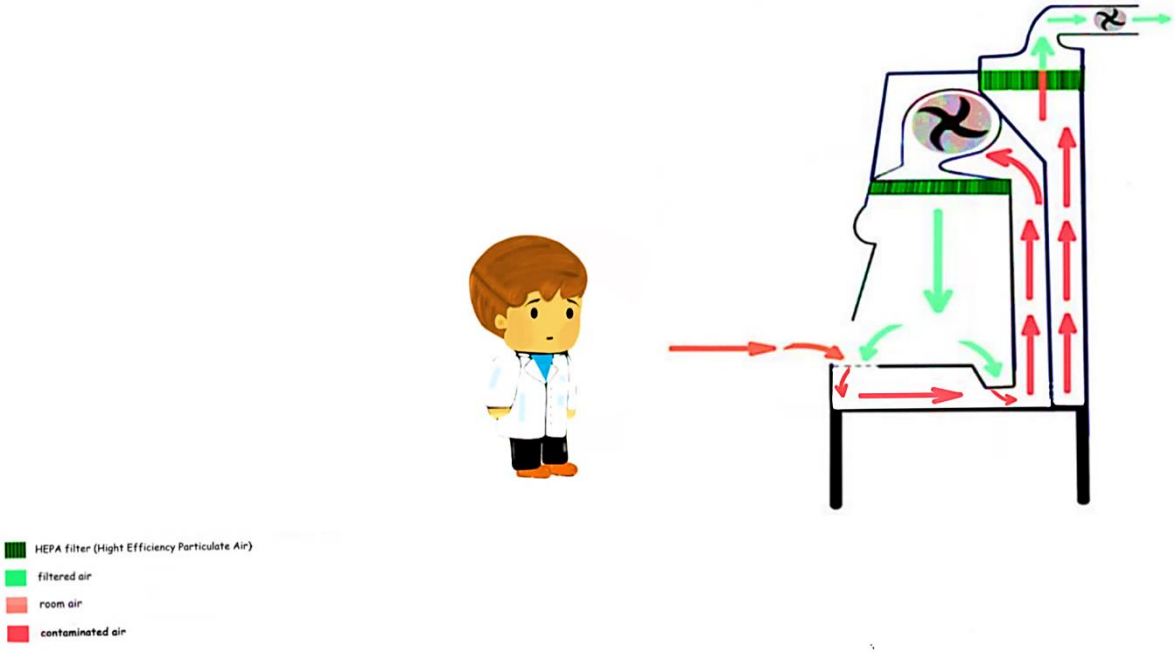


Figure 1.8: Class 2 type B1 BSC design to have an inward airflow and recirculate about 30% of the discharge air through the supply HEPA filter back to the work zone and exhaust about 70% outside using a duct system which provides protection for person, environment and biological material and suitable for work using biological agents with little quantities volatile toxic chemicals and volatile radionuclides.

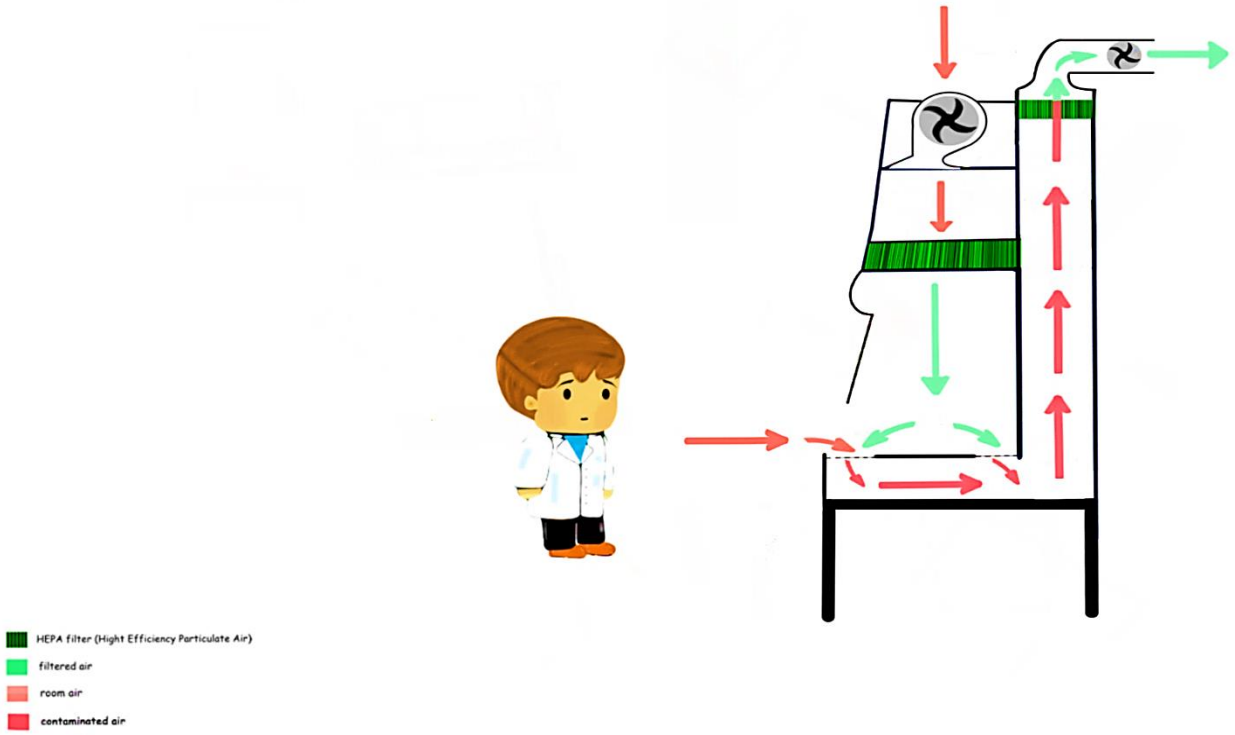


Figure 1.9: Class 2 type B2 BSC design to have an inward airflow and recirculate about 0% of the discharge air and exhaust about 100% outside using a duct system through the supply HEPA filter which provides protection for person, environment and biological material and suitable for work using biological agents with volatile toxic chemicals and volatile radionuclide.

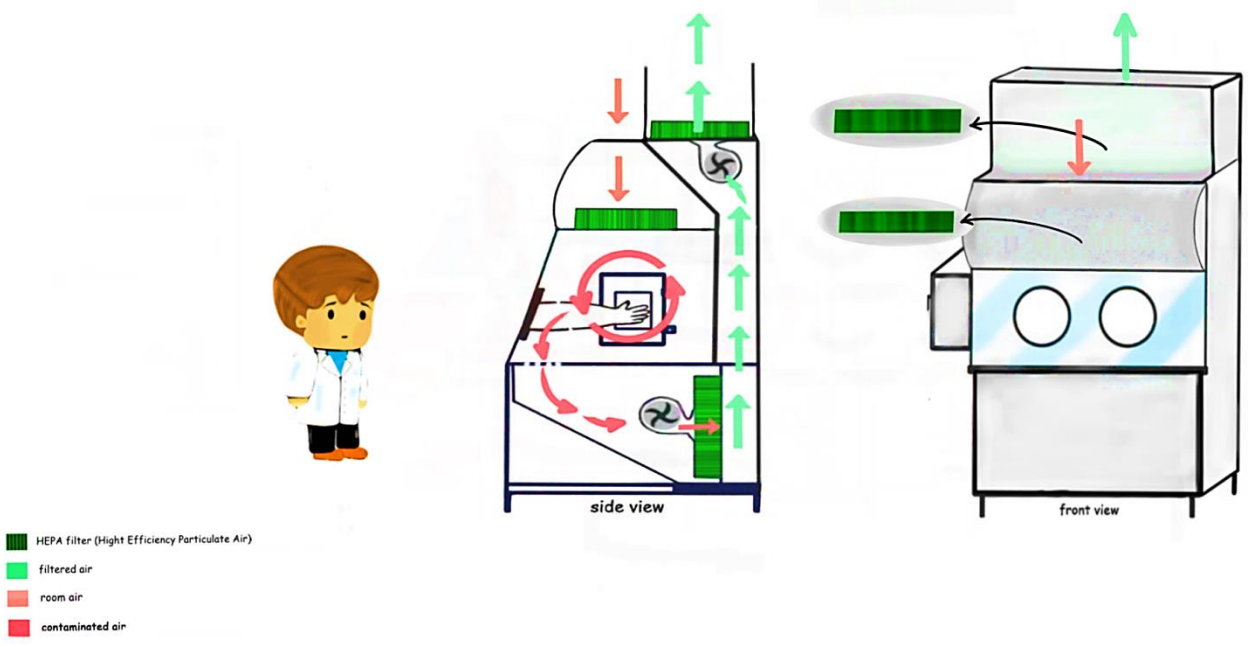
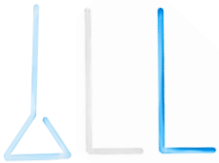


Figure 1.10: Class 3 BSC design to exhaust the air outside using a duct system through the double HEPA filter provides a maximum protection for person, environment and biological material and suitable for work using biological agents with volatile toxic chemicals and volatile radionuclide.

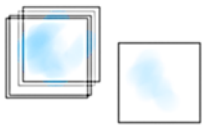
Equipment of laboratory:

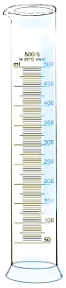
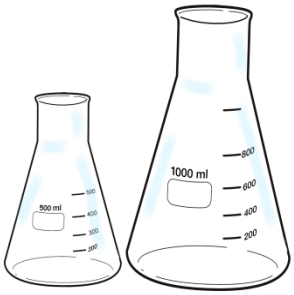
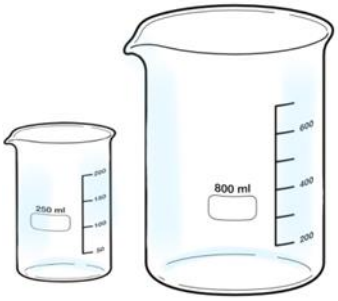
In microbiology laboratory equipment are needed to work and complete procedures. It is important to know what equipment is and how to use. The following table summarizes the most common laboratory equipment (table 1.2).

Table 1.2: Laboratory equipment

Equipment	Description
 Loop	**Platinum - nichrome / plastic material Transferring and spreading of an inoculum from culture media to another**
 Straight needle	**Platinum - nichrome / plastic material Picking small inoculum and transferring to liquid culture or for stabbing solid culture media**
 Spreader	**Glass /plastic material Spreading of an inoculum on the surface of solid culture media**
 Sterile swab stick	**Wooden stick material Spreading of an inoculum on the surface of solid culture mdia**
 Tips	**Polypropylene material Tip could be autoclaving, used with micropipette for transferring of specific volume of liquid culture or sterile solutions**

Equipment	Description
 Micropipette	Used with suitable tips for transferring of specific volume of liquid culture or sterile solutions
 Teat	Rubber material Using for filling and discharge the glass pasteur pipette
 Pipette	Glass material Transferring of specific volume of liquid culture or sterile solutions
 Pasteur pipette	Glass / plastic material Using for filling and transferring of specific volume of liquid culture or sterile solutions
 Test tube	Glass / plastic material Holding of prepared liquid, slant solid culture or sterile solutions

Equipment	Description
Petri plate	Glass / plastic material Holding prepared solid culture media for microorganism growth, glass plate could be used to sterile materials like filter paper in oven
 Slide	Glass material Microscopic examination for specimen smear
 Cover slide	High quality glass material Covering and keeping the examined specimen flat, without drying for better examination and kept from contamination
 Culture bottle	Glass material Storage of sterile culture media or solutions. The screw bottle cap allowing of a long duration of time of storage by preventing contamination or evaporation of media or solutions.
 Bijou bottle	Glass material Holding of prepared liquid culture or sterile solutions

Equipment	Description
 Measuring cylinder	Glass / plastic material Using for measuring liquids and solutions
 Conical flask	Glass / plastic material Commonly used is glass for preparing culture media and storage media for short periods
 Beaker	Glass / stainless steel / aluminum / plastic material Commonly used is glass for preparing and mixing solutions
 Spatule	Stainless steel material With round and flat ending, using for scraping and transferring powder or solid chemicals
 Forceps	Stainless steel material Picking up and transferring sterile material like filter paper disc or antibiotics disc

Practical 2

Decontamination, sterilization and disinfection

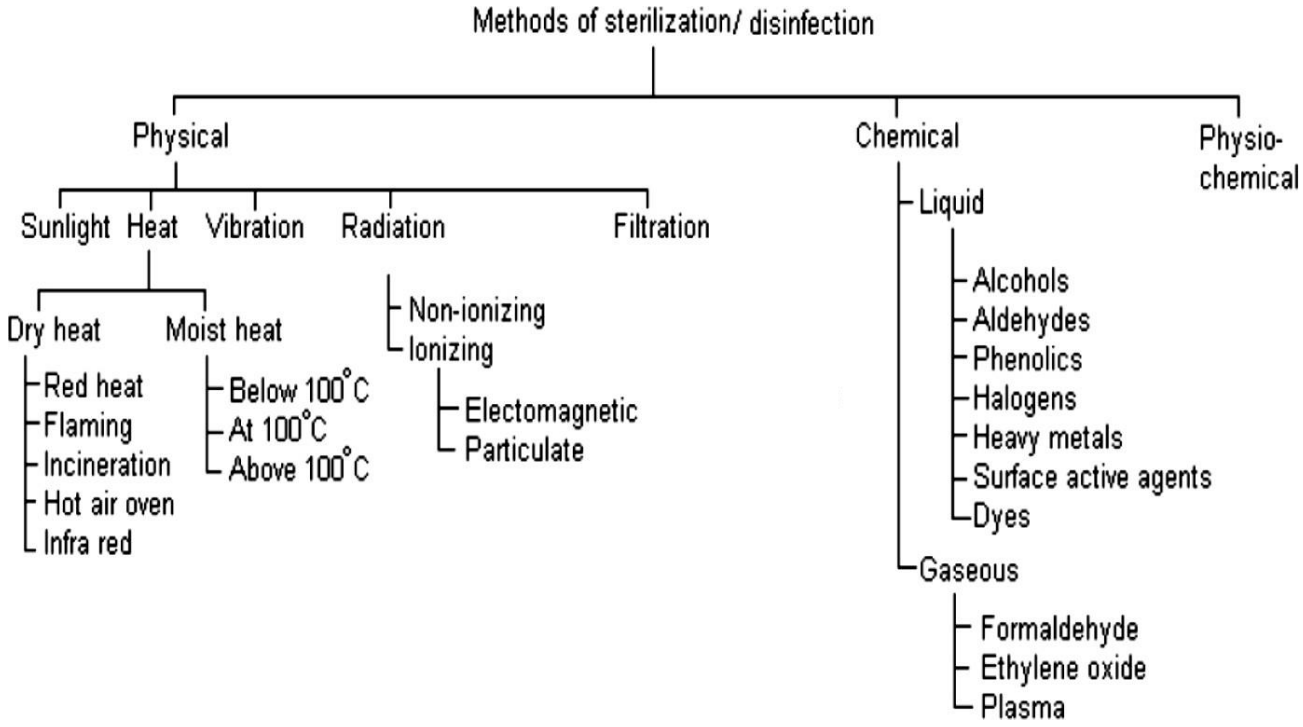
Sterilization is the process of destruction or elimination of all forms of microorganisms.

Disinfection is the process of destruction of pathogenic microorganisms by chemical means which called **disinfectant**.

Decontamination is the process of removal of contaminating pathogenic microorganisms, by using of physical or chemical means to remove, inactivate, or destroy living organisms.

Bacteriostasis is a condition where the multiplication of the bacteria is inhibited without killing them.

Bactericidal is that chemical that can kill or inactivate bacteria.



Methods of sterilization techniques

A. Physical method

1. **Sunlight:** The microbicidal activity of sunlight is mainly due to the presence of ultra violet rays in it. It is responsible for spontaneous sterilization in natural conditions. By killing bacteria suspended in water, sunlight provides natural method of disinfection of water bodies such as tanks and lakes. Sunlight is not sporicidal, hence it does not sterilize.

2. **Heat**

   ✓ **Dry heat**

   a) **Hot air oven:** The material to be sterilized is placed in an oven and different temperature-time relations for holding time are 60 minutes at 160^{o}C, 40 minutes at 170^{o}C and 20 minutes at 180^{o}C. Increasing temperature by 10 degrees shortens the sterilizing time by 50 percent. The sterilized material should not be removed until the oven is cold. This is important particularly with petri dishes, as cold air will be sucked in to them, causing contamination, if they are removed before the oven is cold. This is due to the contraction of hot air as it cools. This method is used only metallic instruments (like forceps, scalpels, scissors), glasswares (such as petri-dishes, pipettes, flasks, all-glass syringes), swabs, oils, grease, petroleum jelly and some pharmaceutical products. The hot air oven must not be opened until the temperature inside has fallen below 60^{o}C to prevent breakage of glasswares.

   b) **Flaming:** Metal spatula, glass slides, and cover slips may be sterilized by passing them through a Bunsen flame, without letting them become red hot.

   c) **Incineration:** This is a method of destroying contaminated material by burning them in incinerator, such as soiled dressings; animal carcasses, pathological material and bedding etc. should be subjected to incineration.

   d) **Red - hot:** Wire loops and tips of forceps may be sterilized by heating them in a Bunsen flame until they become red hot and allow the materials (instruments) to cool before using them.

   e) **Infra-red rays:** Infrared rays bring about sterilization by generation of heat. Instruments to be sterilized are placed in a moving conveyer belt and passed through a tunnel that is heated by infrared radiators to a temperature of 180^{o}C for 7.5 minutes. Instruments sterilized included metallic instruments and glassware.

i. Moist heat At temperature below 100°C:

Pasteurization: Currently this procedure is employed in food and dairy industry. There are two methods of pasteurization, the holder method (heated at 63°C for 30 minutes) and flash method (heated at 72°C for 15 seconds) followed by quickly cooling to 13°C. Other pasteurization methods include Ultra-High Temperature (UHT), 140°C for 15 sec and 149°C for 0.5 sec. This method is suitable to destroy most milk borne pathogens.

Vaccine bath: The contaminating bacteria in a vaccine preparation can be inactivated by heating in a water bath at 60°C for one hour. Only vegetative bacteria are killed and spores survive.

Serum bath: The contaminating bacteria in a serum preparation can be inactivated by heating in a water bath at 56°C for one hour on several successive days. Proteins in the serum will coagulate at higher temperature. Only vegetative bacteria are killed and spores survive.

Inspissation: This is a technique to solidify as well as disinfect egg and serum containing media. The medium containing serum or egg are placed in the slopes of an inspissator and heated at 80-85°C for 30 minutes on three days. On the first day, the vegetative bacteria would die and those spores that germinate by next day are then killed the following day. The process depends on germination of spores in between inspissation. If the spores fail to germinate then this technique cannot be considered sterilization.

Day	Temperature	Time	Purpose
1	85 °C	60 minutes	Drying of the medium and killing the organisms in their vegetative form
Time in between		overnight incubation	Growth of vegetative forms from spores
2	75 to 80 °C	20 minutes	killing the organisms in their vegetative form
Time in between		overnight incubation	Growth of vegetative forms from any spores remaining
3	75 to 85 °C	20 minutes	killing the organisms in their vegetative form as well as the leftover spores

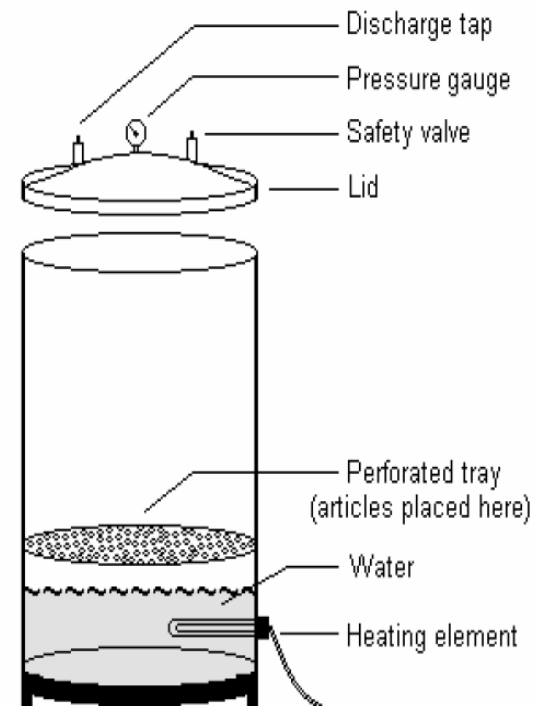
ii. At temperature 100°C:

Boiling water: Moist heat may be applied by boiling water or steam. Boiling water is generally used for sterilizing instruments and syringes. These are boiled for 10 minutes in a water bath. This will kill all non-sporing organisms but certain spore forming organisms can resist the temperature of boiling water for 1-2 hours. The addition of **2% sodium carbonate** increases the disinfecting power of boiling water for 1-2 hours. Spores, which resist boiling water for 10 hours, have been killed within 30 minutes by the addition of sodium carbonate. Sodium carbonate also prevents the rusting of metal instruments.

iii. At temperature above 100°C:

Autoclave (Steam under pressure): Autoclaving The principle of autoclave is that steam is kept at a pressure of 15 pound (lb.) per square inch to give a temperature of 121°C, which will kill spores within 15 minutes. At this particular temperature, pressure and time, all forms of lives are destroyed. Steam is more penetrating than hot air, and will give up its latent heat on striking a colder object; there by raising the temperature of the object rapidly. It is used to sterilize glasswares, culture media, etc.

Construction and operation of autoclave:



A simple autoclave has vertical or horizontal cylindrical body with a heating element, a perforated try to keep the articles, a lid that can be fastened by screw clamps, a pressure gauge, a safety valve and a discharge tap. The articles to be sterilized must not be tightly packed. The screw caps and cotton plugs must be loosely fitted. The lid is closed but the discharge tap is kept open and the water is heated. As the water starts boiling, the steam drives air out of the discharge tap. When all the air is displaced and steam start appearing through the discharge tap, the tap is closed. The pressure inside is allowed to rise up to 15 lbs per square inch. At this pressure the articles are held for 15 minutes, after which the heating is stopped and the autoclave is allowed to cool. Once the pressure gauge shows the pressure equal to atmospheric pressure, the discharge tap is opened to let the air in. The lid is then opened and articles removed. **It used to sterilize** culture media, dressings, certain equipment **etc.**

Precautions: Articles should not be tightly packed, the autoclave must not be overloaded, air discharge must be complete and caps of bottles and flasks should not be tight, autoclave must not be opened until the pressure has fallen or else the contents will boil over, articles must be wrapped in paper to prevent drenching, bottles must not be overfilled.

3. **Radiation:** Two types of radiation are used**:**
   a) **Non Ionizing rays:** Like UV rays. Microorganisms such as bacteria, viruses, yeast, etc. that are exposed to the effective UV radiation are inactivated within seconds. Since UV rays don't kill spores, they are considered to be of use in surface disinfection. UV rays are employed to disinfect hospital wards, operation theatres, virus laboratories, corridors, etc.**.**

   b) **Ionizing rays:** Ionizing rays are of two types, ***particulate and electromagnetic rays.***

   ✓ **Electron beams.** Employed to sterilize syringes, gloves, dressing packs, foods and pharmaceuticals. Sterilization is accomplished in few seconds.

   ✓ **Electromagnetic rays.** They have more penetrative power than electron beam but require longer time of exposure. These high-energy radiations damage the nucleic acid of the microorganism. It is used commercially to sterilize disposable petri dishes, plastic syringes, antibiotics, vitamins, hormones, glasswares and fabrics.

4. **Filtration:** Filtration does not kill microbes, it separates them out. Membrane filters with pore sizes between 0.2-0.45 μm are commonly used to remove particles from solutions that can't be autoclaved. It is used to remove microbes from heat labile liquids such as serum, antibiotic solutions, sugar solutions, urea solution.

B. Chemical methods: Generally, many chemicals are lethal to microorganisms.

1. *Liquid*

1. Alcohol: Ethanol and isopropanol, at 70 -80% concentration in water, are useful for skin, work surfaces of laboratory benches and biosafety cabinets, and to soak small surgical instruments. **A 70% aqueous solution is more effective at killing microbes than absolute alcohols**. They are active against vegetative bacteria, fungi and lipid containing viruses, but not against spores.
2. **Aldehydes**: include (Formaldehyde, Gluteraldehyde) probably damages nucleic acids. It kills all microorganisms, including spores, 40% Formaldehyde (formalin) is used for surface disinfection and fumigation of rooms, chambers, operation theatres, biological safety cabinets, wards, sick rooms etc.

3. **Phenolic compounds** are a broad group of agents, were among the earliest germicides. They are active against vegetative bacteria and lipid containing viruses. They are not active against spores and their activity against non-lipid viruses is variable. Many phenolic products are used for the decontamination of environmental surfaces, and some (example, triclosan and chloroxylene) are among the more commonly used antiseptics. They are used at 2 - 5%.
4. **Chlorine**, a fast - acting oxidant, is a widely available and broad-spectrum chemical germicide. It is normally sold as bleach, an aqueous solution of sodium hypo chloride, which can be diluted with water to provide various concentrations of available chlorine. They are effective against viruses as well as bacteria. Dilutions should not be kept for more than 24 hours so as to maintain its activity.

2. Casouse: for example **Ethylene oxide (EO),** it has good penetration and is well absorbed by porous material. It is used to sterilize heat labile articles such as bedding, textiles, rubber, plastics, syringes, disposable petri dishes, and complex apparatus like heart - lung machine, respiratory and dental equipment's.

Practical 3

Culture media and preparation of culture media

Culture media

A culture medium is an artificial prepared environment provides nutrients material including carbon, nitrogen, mineral salts and energy necessary for bacterial growth.

Many types of culture media are used for many purpose therefore we should specified the media type for our purpose, and for easy understanding these different types of media, it could be classified according to the **consistency**, **nutrition content** or **function usage** of these culture media as the following:

1. According to the consistency:

- **Liquid media**, or referred as broth (such as nutrient broth) used for bacteria growing in a large number quickly.
- **Solid media**, usually this type of media are solidified after preparing due to the addition of solidified agent called **(agar)** a purified carbohydrate from seaweeds red algae. Basically the agar is companied with culture media for:
    1. Bacteria cannot metabolize agar as a source of nutrient.
    2. Melting at 100°C and solidifying about 45°C.
- **Semi-solid media**, usually soft or gelatinous texture as the concentration of agar is reduced to 0.2% - 0.5%, often used to demonstrate the motility of bacteria.
- **Biphasic media**, both and solid media in the same bottle comprise biphasic media and used when the needing to subculture without opening the bottle to reduce the occurring of contamination of medium and kept for a long time

2. According to nutrition ingredients:

- **Simple media**, these types of media provides the nutrients for bacteria growth which could be minimum requirements of nutrient for non-fastidious bacteria.
- **Complex media**, that are required addition of special complicated nutrients supplement for fastidious bacterial growth (likes blood) in blood agar.
- **Synthetic media** are prepared for research with known component.

3. According functional usage:

- **Basal media** are basic simple media which supports the bacteria growth.
- **Enriched media** the addition nutrients like blood, sugars or other supplement to basal media form enriched media, such as blood agar, chocolate agar.
- **Enrichment media** these media are prepared specially for enhancement the bacterial growth in large number in compared with original number. This type of media usually broth.
- **Selective media** usually designed for supporting and isolating the growth of certain bacterial specie from many types of bacteria by addition an inhibitory agent (like dyes or antibiotics) without affecting on isolated bacteria.
- **Differential media** based on the colony color on media, different bacterial species can be distinguished.
- **Transport media** in case of collecting specimens away the laboratory, transport media are used to ensure the surviving of the clinical isolated specimens without overgrowth of the commensal or contaminating bacteria and avoiding the drying of specimen until transported to the laboratory.
- **Anaerobic media** provide low oxygen with high nutrient content for anaerobic bacteria.

- **Storage media** are broth or agar slant prepared media in screw bottles or test tubes provides moisture environment to keep specimen for long term studies. Screw bottles or test tubes are much preferred than agar plates for storage bacterial culture as the plates easily lose water by evaporation.

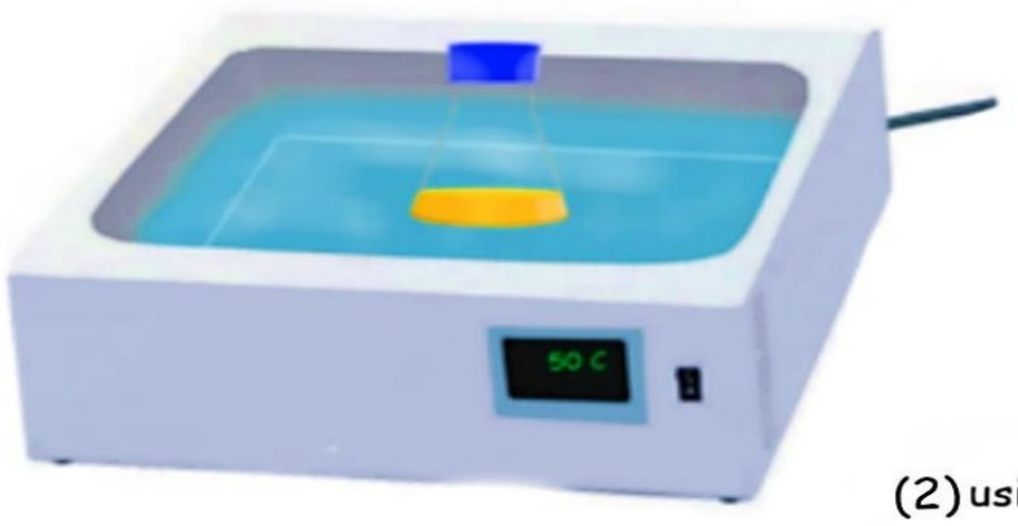
Preparation of culture media

Preparing of media:

To preparer cultures media the instruction of manufacturer should be followed, the ingredients of the powder media are completely dissolving in distilled water and in clean glassware beaker, test tubes or caped bottles using heat if necessary, it is important to prepare media as the culturing procedure need and allow for extra for mistaking and nearing future study, after that the prepared media should be sterilized by an autoclave.

A plate pouring:

1. After the autoclaving process the media cooled to 50°C in water bath on in the boiling water bath to prevent the media solidifying.
2. Using towel for wiping the beaker or bottle.
3. The mouth of the beaker or bottle should be flamed.
4. Slightly open the lid of petri plate and do not left it aside then poured the medium about 15-20 ml and then close the lid.
5. Avoiding the bubbles formation on the surface of the medium.
6. Flame the beaker or bottle before closing them.
7. Gently moving the plate in circular movement to ensure covering the pate with medium (figure 5.1).
8. Allow the solidifying of pouring plate at room temperature.
9. The plates should be used at the earliest possible time.
10. If the plates are not used they should be marked with the date of preparation and the media type and stored in sealed plastic bags at 2°C - 8°C to prevent the media from drying away.

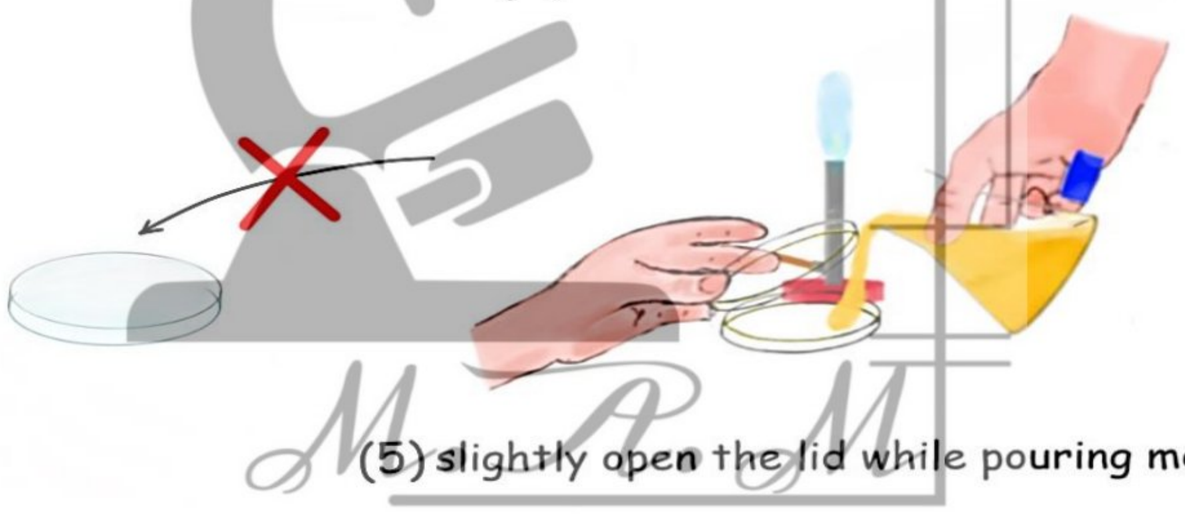


(1) after autocalving place media into water bath at 50°C

(2) using towel to wipe the beaker

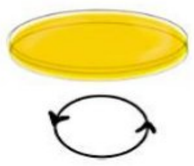
(3) using little finger to catch the cap while other hand hold the beaker

(4) flame the mouth of beaker



avoiding to place the lid a side

(5) slightly open the lid while pouring media

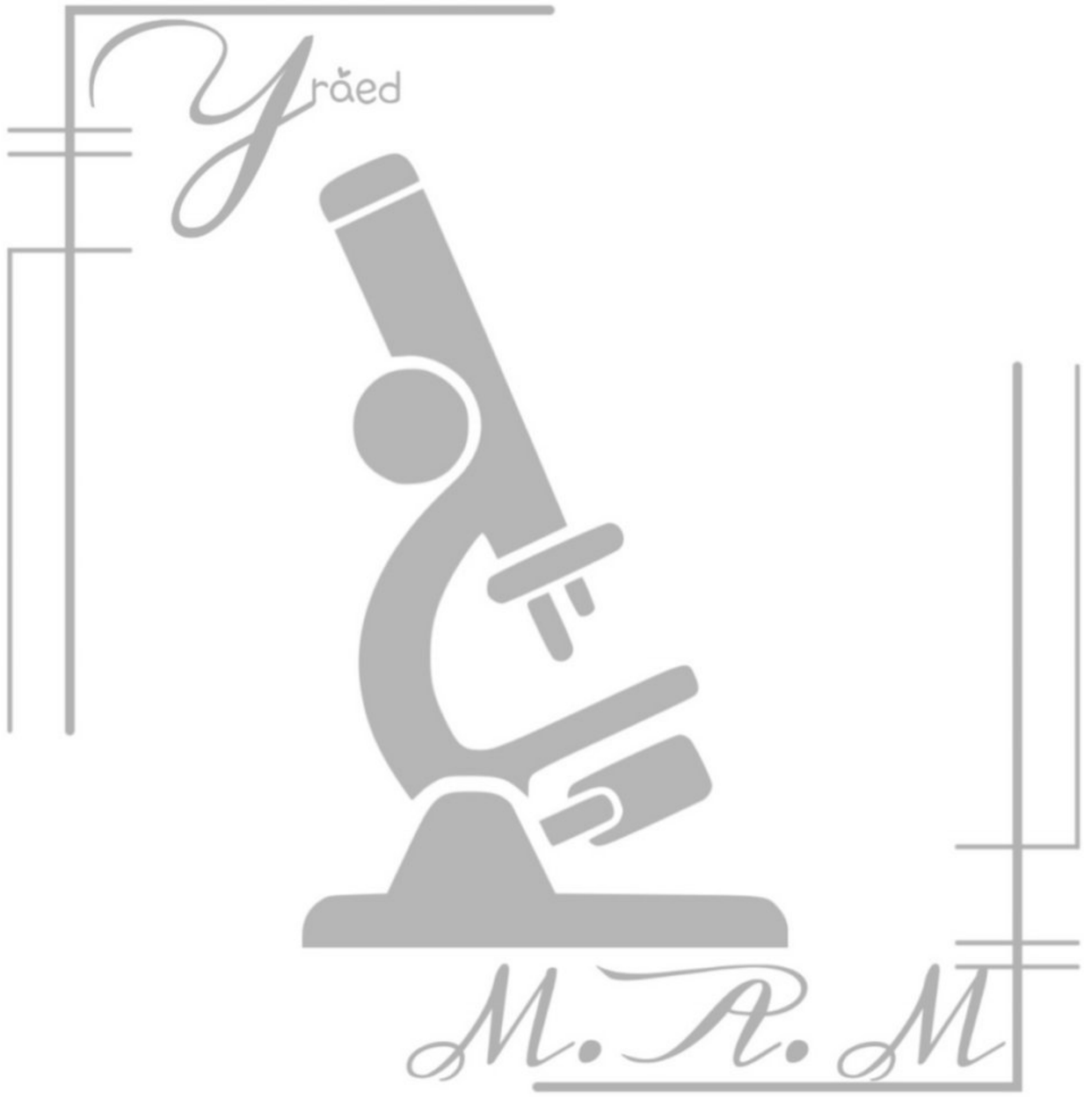


(6) moving the plate in cicular movment to cover the plate with media

Figure 5.1: Plate pouring method

Media storage:

The media prepared for future in beakers closed with plugs of cotton wool could be evaporated it should be kept in screw cap bottles. For using, the media should be re-melting either by using an autoclave, oven or boiling water bath, then kept in water bath at 50°C to prevent the media solidifying, then can be used again by pouring it in sterile plate for culturing or storing in sealed plastic bags. It is important to keep stored plates upside down to avoid condensation on a plate lid.



Practical 4

Aseptic technique

Aseptic technique refers to set a routine of procedure or practice to be taken in a sterile condition to prevent contamination of sterile media, cultures or equipments with undesired microorganisms during culturing or isolation procedures, it is also prevent the workplace and worker to be contaminated.
The sources of contamination could be from air, hands, hair, breath, clothes, equipment or the surface of working. Therefor it is important to begin with sterilization the working area with disinfectant before starting and after finishing the work.

Aseptic technique principles including:

A. Flaming:

This procedure is the basic to heat the end of the wire loop by Bunsen burner flame before start culturing and after the end of culturing procedure to ensure the removing of the remaining culture on the loop.

1. Always hold the loop as you holding the pen.
2. Place the end of the wire loop in the cone of the blue flame which is a cool flame area.
3. Slowly pull the rest of the wire up into the hottest part of the flame.
4. Hold on till it is hot red.
5. Ensure that the entire length of the wire loop is adequately heated.
6. Cooling the wire loop and use directly.
7. Avoiding shaking the loop around or putting it aside.
8. Re-flaming the loop after using directly (figure 6.1).

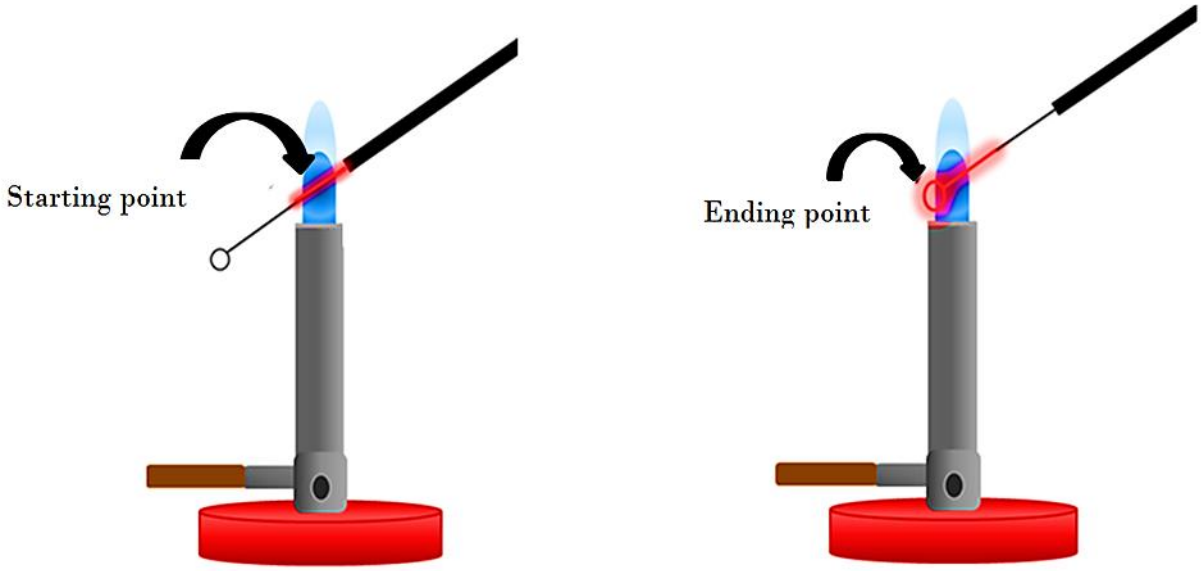


Figure 6.1: The flaming direction along the loop length until become red.

NOTE:

- **If the wire loop is using to transfer microorganism inoculum from a plate the loop could be cooled by touching the edge of the agar plate, while to transfer the inoculum from a broth (liquid culture) the loop will be cooled as it contact with the broth, therefore cooling it before transferring the inoculum as the hot wire loop may makes aerosols and causing briefly boiling broth culture which may causing the worker contamination.**
- **Note the parts of blue flame in compared with orange flame (Figure 6.2).**

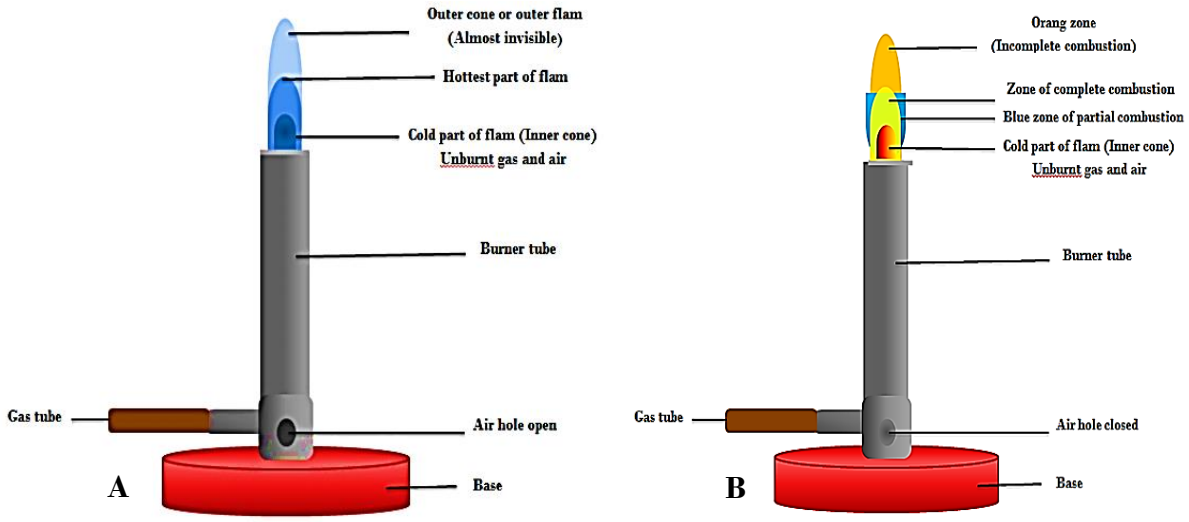


Figure 6.2: Parts of Bunsen burner flame; A) non luminous flame, B) luminous flame.

B. Pipetting:

Using Pasteur pipettes or graduated sterile pipettes are used for transferring cultures or sterile media and solutions.

1. To remove the pipette container or cover, hold it from its end only.
2. On the end of the pipette fitting the teat before completely removing it from the container or cover. (Avoiding touching the tip of pipette to prevent its contamination)
3. Hold the pipette as the way you hold the pen without pinch the teat.
4. In the same hand you hold the pipette, use the little finger to catch the cotton plug or cap of the test tube or bottle which is holed in the other hand. This would be helped in controlling the teat easily.
5. Flame the mouth of the test tube or bottle.
6. Before start pipetting, press on the teat genteelly and then pushing the pipette to the liquid of broth culture in the test tube or bottle and take the amount you need and return any excess. (Ensure that the tip of pipette is completely below the surface of the liquid to avoid the bubbles formation).

7. After finishing the use of the pipette putting it in a discard container containing disinfectant (figure 6.3). (Ensure to remove the teat only when the pipette is putting in discard container to avoid contamination of the working area with drops of liquid culture).

C. Flaming the mouth of test tubes, flasks or bottles:

1. The cap of test tube should be loosening, for easily removing.
2. Hold the test tube, flask or bottle in the left hand.
3. Removing the cap or cotton plug with the small finger of the right hand, noting to turn the bottle, but not the cap.
4. Avoiding putting down the cap or the bottle on the bench and keeping them with small finger of the right hand.
5. Flame the mouth of the test tubes, flasks or bottles by passing them through the flame of Bunsen burner.
6. After withdrawing the inoculum for culturing procedure, using the small finger to return the cap or cotton plug over the test tubes, flasks or bottles again, by turning the bottle, but not the cap. Not the steps 4,5,6,7 (figure 6.3).

NOTE:

- **The tubes should be labeled in a right position that would not remove by the worker hands, it is preferable to use permanent marker to label test tubes, bottles or even petri- dishes plate.**
- **The cotton plugs that are occasionally flamed should be covered with a piece of a dry cloth immediately, avoiding the blowing or using water.**

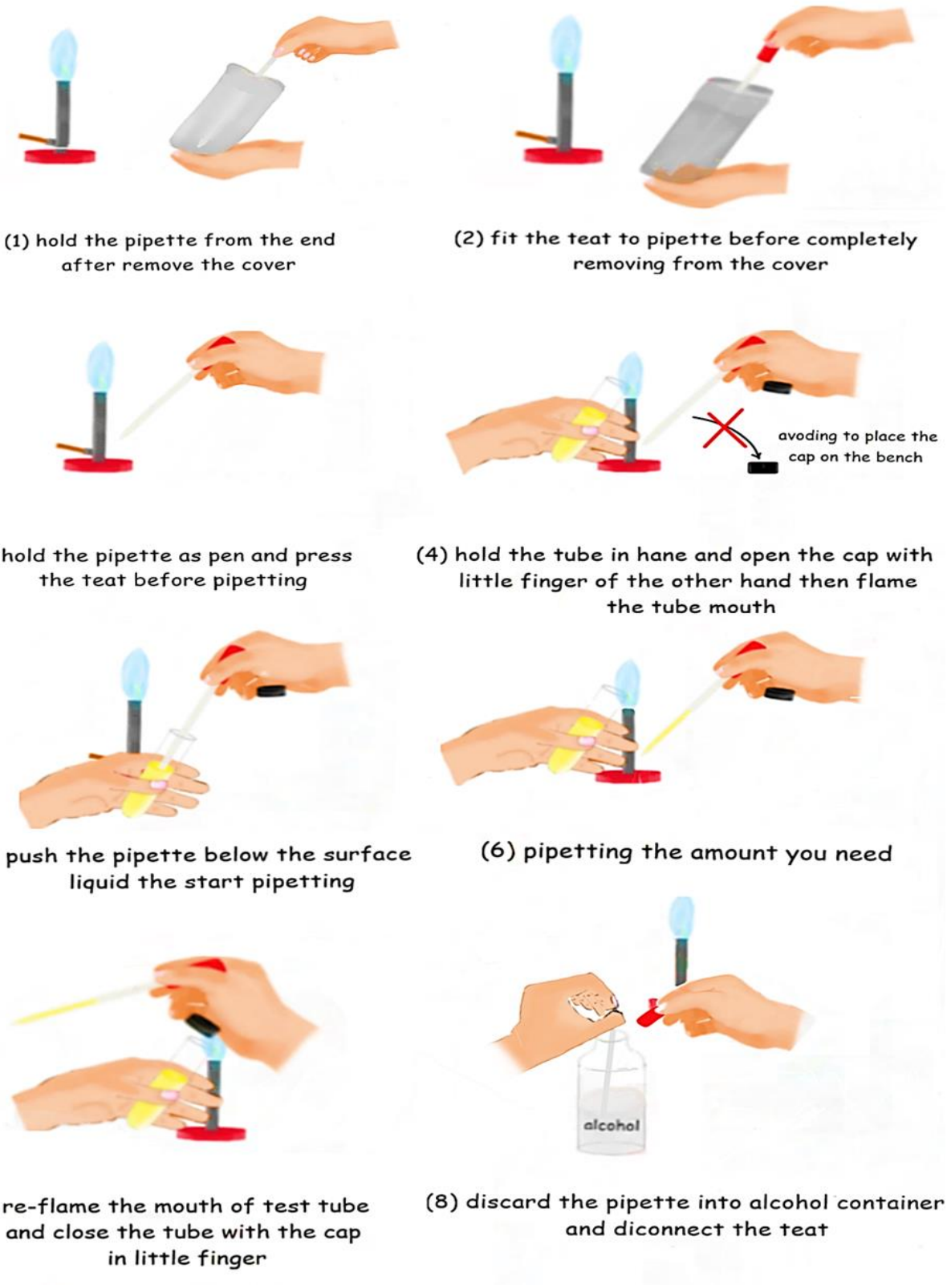


Figure 6.3: Aseptic techicque for pipetting and flaming mouthe of test.

Colony morphology

The growth of single bacterial cell on agar plate surface developing single colony. The bacterial colonies vary in morphology with the variation of bacterial strains. The colonies morphology considered as primary step for bacterial species identification but not finally.
Many characteristics important to gives signs for identification of bacterial species, therefor colonies characteristics could be category as the following:

1. **Size:** The colony size could be measured by millimeters. The varying of the colonies size based on the bacterial species, the agar medium type and the colonies number on the agar surface which often is smaller in large numbers.
   Some bacterial species are grows slow or under slow growing conditions which make these bacteria require more than 24 or 48 hours to measure their final size.
2. **Form:** The colonies shape refers to the general appearance of the colony over the agar surface which mostly oval or circular (figure 1.1).

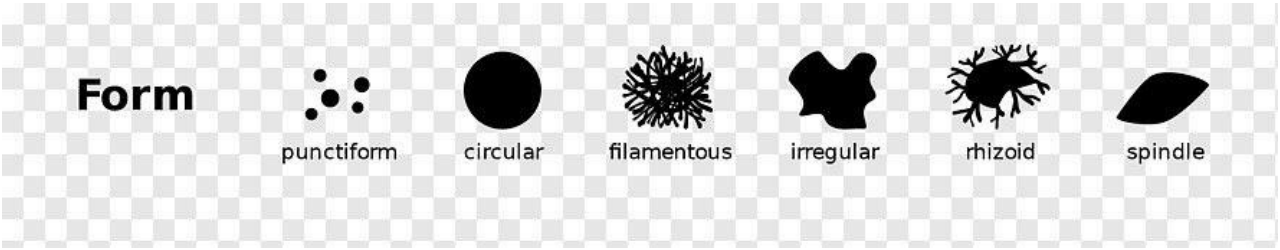


Figure 1.1: Types of colonies forms.

3. **Margin:** The outer edge of the colonies could either be entire, irregular (undulate), curled, lobate, erose, rhizoid or filamentous. The margin of the colony is often forming the general shape of the colony (figure 1.2).

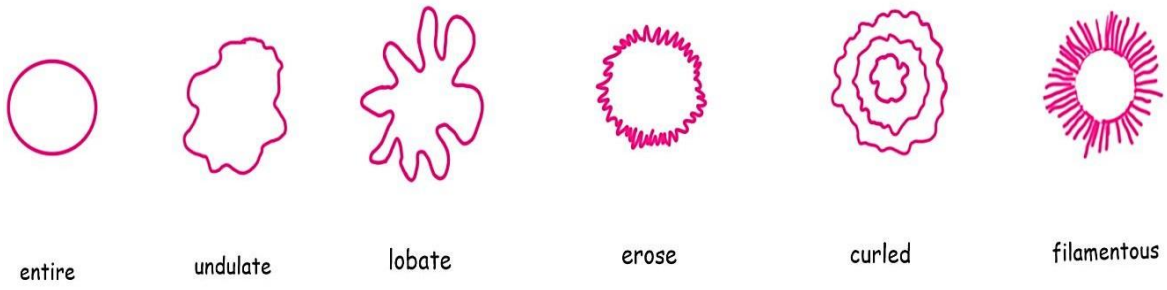


Figure 1.2: Types of colonies margin.

4. **Elevation:** The colony elevation could be flat, raised, convex, pulvinate or umbonate (figure 1.3).

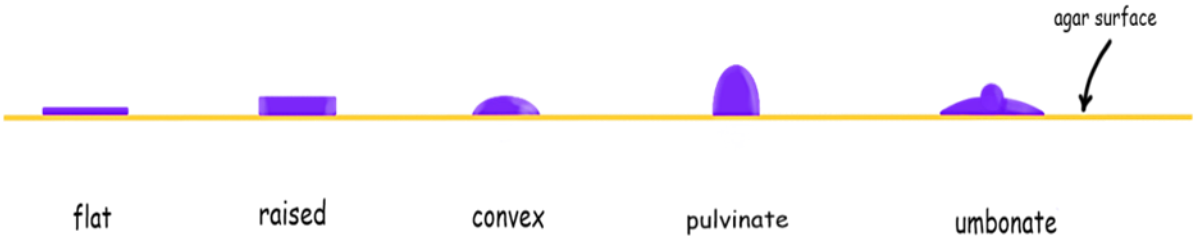


Figure 1.3: Types of colonies elevation.

5-Surface: The colony surface could be smooth or irregular.
6-Texture: The colony texture could be shiny or dull smooth, rough, wrinkle, granular or mucoid. The capsule formation gave the colonies smooth, shiny mucoid or non mucoid texture.

7-Colony transparency: This refers to the transmission of the light through the colony which could be complete transparency, intermediate or opaque.

8-Pigmentation: Pigmentation refers to the color the produced by some species of bacteria. These pigmentations are diffusible within agarmedia or non-diffusible.

Viable plates count

This method is usually used for bacterial cells counting for any sample under study. First the sample is diluting by sterile normal saline or phosphate buffer saline to serial dilutions, and then each dilution is plated on agar plate. The colonies that are formed on these final plats should be between 25 – 250 colonies. In case the colonies are overlie this range are not recommended because less than 25 or 30 colonies are represent as **too few to count (TFTC)** and are not statistically acceptable, while more than 250 colonies are represent as **too numerous to count (TNTC)** in this case the colonies are too closed to each other to be counted. Each single distinct colony distinguishes as **colony forming unites (CFUs).** The countable colonies on the agar surface which are CFUs gave the numbers of bacterial cells in milliliter for each dilution of serial dilutions.

Materials:

1. Broth culture or sample under study (water, milk, soil…etc.)
2. Sterile capped test tubes with 9 ml of sterile saline.
3. Sterile agar plates.
4. Alcohol ethanol.
5. Sterile pipettes.
6. Spreader.
7. Bunsen burne

Procedure:

1. Label the 6 sterile test tubes and sterile agar plates for dilutions of the sample.
2. In aseptic technique, the primer dilution is done by pipetting 1 ml from the broth or liquid sample or 1 gm from the solid sample to 9 ml sterile saline test tube. This make dilution 1/10 or 10^{-1} by followed the formula:

$$Dilution = \frac{\text{The sample volume}}{\text{The sample voulme} + \text{Diluent volume}}$$

3. The 10^{-1} tube is closed and mixed well several times by using a pipette.
4. Using a new pipette and transfer 1 ml from the 10^{-1} tube into the next tube. Mixed well this gave 10^{-2} tube of dilution.
5. Repeat this procedure by pipetting 1 ml from last tube you reached to next tube until you reached 10^{-6} tube of dilution. (In some procedure the dilution could be reached to 10^{-8}).
6. From each dilution test tube transfer 0.1 ml on the surface of the sterile agar plate that are labeled with the same dilution that was transferred `from. (Make duplicate for plates).
7. Spread the transferred volume by spreader. (This procedure could be done by pour plate technique).
8. Incubate the plates for 24 hours at 35ºC or for 48 hours at 20°C.
9. Count the colonies number on the agar surface (figure 9.1). (The plates with colonies numbers are out of the range 25-250 colonies are discarding).
10. Choosing the plate that is easily to count. (The counting process is done either by hand with using marker pen to avoid missing duplicating the colonies, or by using the colonies count.
11. Calculate the number of bacterial cells per milliliter by followed the formula.

$$CFU/ml = \frac{\text{The colonies number}}{\text{The dilution factor}}$$

In case you have two counts from one dilution, cfu/ml is counted by calculate the average of the colonies number divided on the dilution factor belongs to.

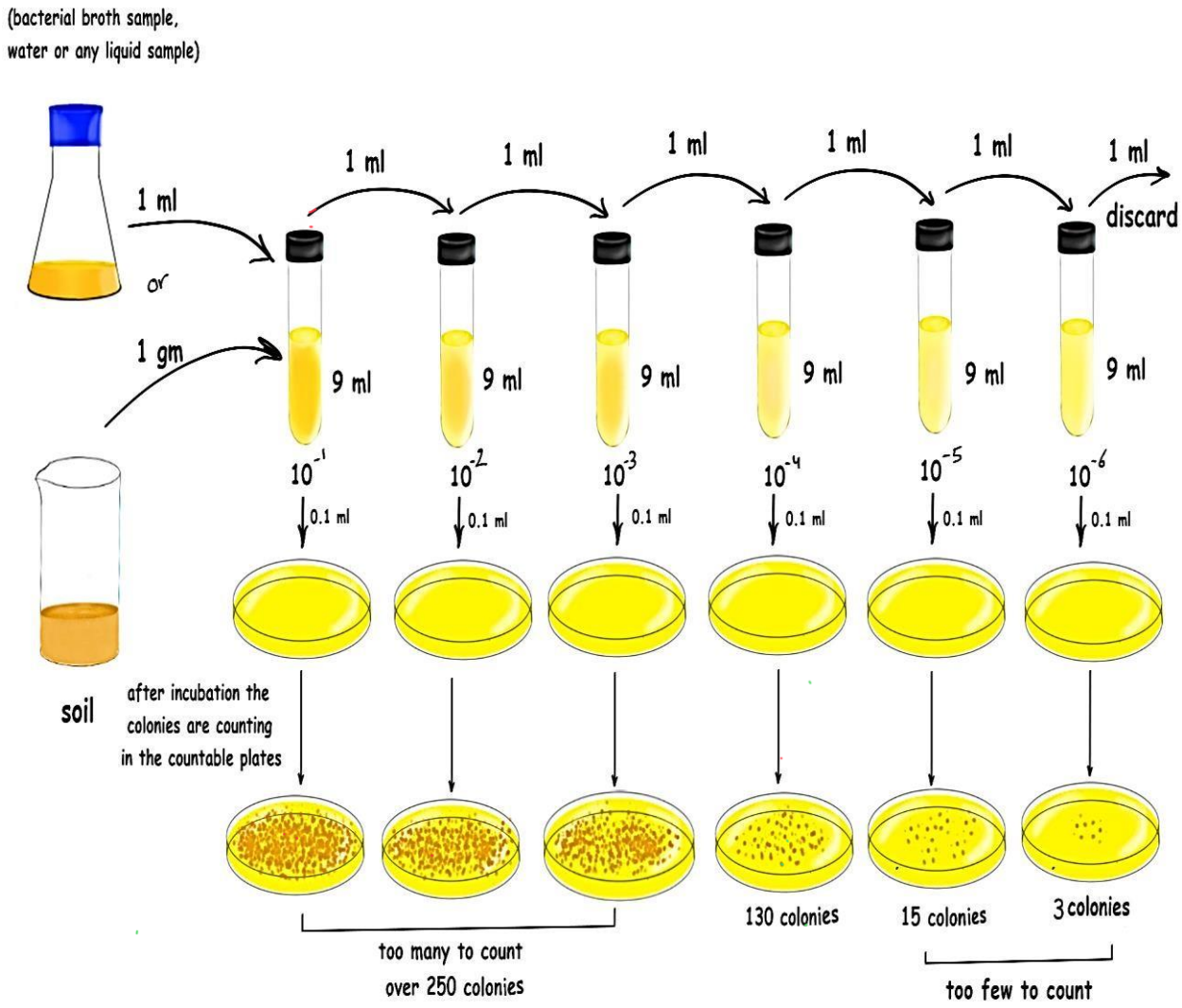


Figure 1.1: Viable plate count technique.

Growth Curve

When fresh liquid medium is inoculated with a given number of bacteria and incubated for sufficient period of time, it gives a characteristic growth pattern of bacteria. Microorganisms grown in closed culture (also known as a batch culture), in which no nutrients are added and most waste is not removed, follow a reproducible growth pattern referred to as the growth curve.

If the bacterial population is measured periodically and log of number of viable bacteria is plotted in a graph against time, it gives a characteristic growth curve which is known as **growth curve.**

Measuring the growth rate of bacteria is a fundamental microbiological technique, and has widespread use in basic research as well as in agricultural and industrial applications.

Principles of bacterial growth curve:

When bacteria are inoculated into a liquid medium and the cell population is counted at intervals, it is possible to plot a typical bacterial growth curve that shows the growth of cells over time. It shows four distinct phases of growth.

- **Lag phase:** Slow growth or lack of growth due to physiological adaptation of cells to culture conditions or dilution of exoenzymes due to initial low cell densities.
- **Log or exponential phase:** Optimal growth rates, during which cell numbers double at discrete time intervals known as the mean **generation time**.
- **Stationary phase:** Growth (cell division) and death of cells counterbalance each other resulting in no net increase in cell numbers. The reduced growth rate is usually due to a lack of nutrients and/or a buildup of toxic waste constituents.
- **Decline or death phase:** Death rate exceeds growth rate resulting in a net loss of viable cells.

Turbidimetric determination is useful for plotting growth curves of bacteria in broth or liquid media. It is one of the simplest methods used to analyze trends in growth because it uses a spectrophotometer to track changes in the optical density (OD) over time. In other words, as the number of cells in a sample increase, the transmission of light through the sample will decrease.

Material required for bacterial growth curve

Bacterial broth culture (E. coli).
Glass wares: Conical flasks, Measuring cylinder, Sterile test tubes, Sterile Petriplates
Reagents: Distilled water
Incubator, Shaker, Spectrophotometer, Micropipettes, Tips, Sterile Loops

- **Generation times for bacteria vary from about 12 minutes to 24 hours or more.** For example, ***Clostridium perfringens,*** one of the fastest-growing bacteria, has an optimum generation time of about 10 minutes; ***Escherichia coli*** can double every 20 minutes; and the slow-growing ***Mycobacterium tuberculosis*** has a generation time in the range of 12 to 16 hours.
- In the intestinal tract, the coliform's **generation time** is estimated to be 12-24 hours.

Procedure:

Day 1:

1. Using sterile loop, streak a loopful of bacterial culture onto the agar plate.
2. Incubate at 37°C for 18-24 hours.

Day 2:

1. Pick up a single colony of each strain from the agar plate and inoculate it into a test tube containing 10 ml of autoclaved broth.
2. Incubate the test tube overnight at 37°C.

Day 3:

1. Take 250 ml of autoclaved broth in a sterile 500 ml conical flask.
2. Inoculate 5 ml of the overnight grown culture in above flask.
3. Take OD at zero hour. Incubate the flask at 37°C.
4. Aliquot 1 ml of the culture suspension at an interval of every 30 minutes and take the optical density (OD) at a wavelength of 600 nm using spectrophotometer, till the reading becomes static.

Alternatively, 50-100 µl of formaldehyde can be added to all the 1 ml aliquots of culture suspension taken after every 30 minutes. Optical density of all the aliquots can be taken at the end of the experiment.

5. At the end of experiment, plot a graph of time in minutes on X axis versus optical density at 600nm on Y axis to obtain a growth curve of bacteria.

Result:

A logarithmic growth curve is obtained showing the changes in size of a bacterial population over time in the culture. The growth curve is hyperbolic due to exponential bacterial growth pattern.