M.Sc. in MEDICAL LABORATORY TECHNOLOGY LAB MANUAL

2nd Semester

Prepared By

Paramedical & Allied Health Science Dept.

MMLT

MIDNAPORE CITY COLLEGE

Code: MMLT 296

FULL MARKS-50

Experiment 1. Demonstration of Autoclave.

Autoclave

The autoclave is a steam-pressure sterilizer. Steam is the vapour given off by water when it boils at 100°C. If steam is trapped and compressed, its temperature rises as the pressure on it increases. As pressure is exerted on a vapour or gas to keep it enclosed within a certain area, the energy of the gaseous molecules is concentrated and exerts equal pressure against the opposing force. The energy of pressurized gas generates heat as well as force. Thus, the temperature of steam produced at 100°C rises sharply above this level if the steam is trapped within a chamber that permits it to accumulate but not to escape. A kitchen pressure cooker illustrates this principle because it is, indeed, an "autoclave." When a pressure cooker containing a little water is placed over a hot burner, the water soon comes to a boil. If the lid of the cooker is then clamped down tightly while heating continues, steam continues to be generated but, having nowhere to go, creates pressure as its temperature climbs steeply. This device may be used in the kitchen to speed cooking of food, because pressurized steam and its high temperature (120 to 125°C) penetrates raw meats and vegetables much more quickly than does boiling water or its dissipating steam. In the process, any microorganisms that may also be present are similarly penetrated by the hot pressurized steam and destroyed.

Essentially, an autoclave is a large, heavy-walled chamber with a steam inlet and an air outlet. It can be sealed to force steam accumulation. Steam (being lighter but hotter than air) is admitted through an inlet pipe in the upper part of the rear wall. As it rushes in, it pushes the cool air in the chamber forward and down through an air discharge line in the floor of the chamber at its front. When all the cool air has been pushed down the line, it is followed by hot steam, the temperature of which triggers a thermostatic valve placed in the discharge pipe. The valve closes off the line and then, as steam continues to enter the sealed chamber, pressure and temperature begin to build up quickly. The barometric pressure of normal atmosphere is about 15 lb to the square inch. Within an autoclave, steam pressure can build to 15 to 30 lb per square inch above atmospheric pressure, bringing the temperature up with it to 121 to 123°C. Steam is wet and penetrative to begin with, even at 100°C (the boiling point of water). When raised to a high temperature and driven by pressure, it penetrates thick substances that would be only superficially bathed by steam at atmospheric pressure. Under autoclave conditions, pressurized steam kills bacterial endospores, vegetative bacilli, and other microbial forms quickly and effectively at temperatures much lower and less destructive to materials than are required in a dry-heat oven (160 to 170°C).

Temperature and time are the two essential factors in heat sterilization. In the autoclave (steam-pressure sterilizer), it is the intensity of steam temperature that sterilizes (pressure only provides the means of creating this intensity), when it is given time measured according to the nature of the load in the chamber. In the dry-heat oven, the temperature of the hot air (which is not very penetrative) also sterilizes, but only after enough time has been allowed to heat the oven load and oxidize vital components of microorganisms without damaging materials.

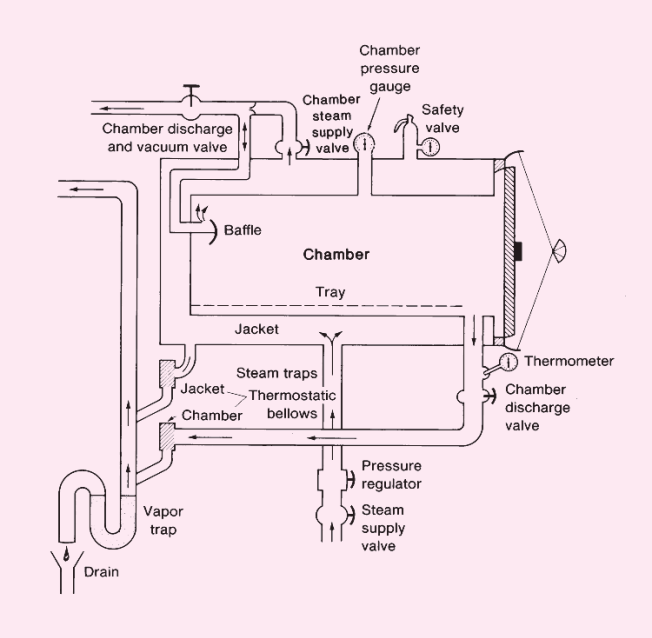


Fig. Autoclave

Table. Pressure-Temperature-Time Relationships in Steam-Pressure Sterilization.

Steam Pressure, Pounds per Square Inch (Above Atmospheric Pressure)	*Temperature*		*Time (Minutes Required to Kill Exposed Heat-Resistant Endospores)*
	Centigrade	*Fahrenheit*	
0	100°	212°	—
10	115.5°	240°	15–60
15	121.5°	250°	12–15
20	126.5°	260°	5–12
30	134°	270°	3–5

Experiment 2. Cleaning and decontamination of glassware and laboratory waste materials.

Glassware for use in microbiological laboratory work should be not merely clean, but *chemically* clean. Test tubes, Petri dishes, flasks, etc., are the receptacles used in the microbiological laboratory for containing the different nutrient substances upon which microorganisms are to subsist. Very frequently free alkali may be present on new glassware in sufficient quantity to prevent microbial growths in the nutrients contained therein. Prescott and Winslow in testing out different glassware say that, "The more soluble glassware yielded sufficient alkali to the medium to inhibit four-fifths of the bacteria present in certain cases."

Glassware which *looks* clean may have been used previously and should be given a thorough cleaning to rid it of possible traces of mercuric chloride, or other chemical having germicidal properties.

Follow directions carefully and clean all new and apparently clean glassware in the order given.

Cleaning New or Apparently Clean Glassware. All new glassware should first be treated with chromic acid cleaning solution (*see appendix for all formulæ*) before proceeding with the directions for cleaning glassware.

Return used cleaning solution to the glass receptacle provided for the purpose. Do not throw it away. This solution may be used until oxidized, i.e., until dark green in color.

Heat will facilitate the action of the cleaning solution.

Small amounts of organic matter adhering to glassware are oxidized by this solution, but will not disappear until removed by a suitable brush and cleaning powder.*

New Petri dishes and test tubes may conveniently be

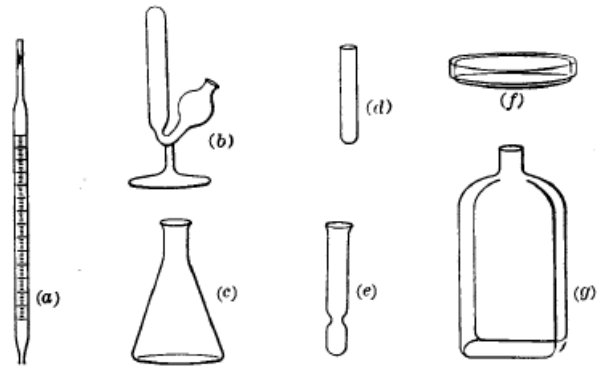


FIG. 1.—(*a*) Pipette, (*b*) Smith's Fermentation Tube, (*c*) Erlenmeyer Flask, (*d*) Test Tube, (*e*) Roux Tube, (*f*) Petri Dish, (*g*) Roux Flask.

placed in a large glass jar, covered with cleaning solution and allowed to stand over night. Heavy glass jars will not stand heating in steam. New flasks may be partially filled with cleaning solution and placed in steam for fifteen minutes.

Test Tubes. New test tubes should be filled with cleaning solution, placed in a wire basket and heated for at least fifteen minutes in the steam. After removing test tubes from the cleaning solution:

1. Wash them in water with a test-tube brush, using cleaning powder if necessary.
2. Rinse with tap water till clean and free from cleaning powder.
3. Rinse with distilled water.
4. Drain.
5. Test tubes and other glassware, flasks, pipettes, etc., may be rinsed with alcohol to facilitate drying, then drained.

Flasks. After treating flasks with cleaning solution:

1. Wash them as clean as possible with tap water and a flask brush; use cleaning powder if necessary. (When using cleaning powder, empty all water out of the flask, wet the flask brush with tap water, dip it in the cleaning powder and then rub the soiled portions vigorously.)
2. Rinse with tap water till clear and free from cleaning powder.
3. Rinse with distilled water.
4. Drain.

Petri Dishes. After removing Petri dishes from the cleaning solution:

1. Wash them in water, using cleaning powder if necessary.
2. Rinse with tap water. (It is not necessary to use alcohol or distilled water.)
3. Wipe immediately with a clean physician's cloth.

Pipettes. 1. Place pipettes delivery end down, in a glass cylinder (graduate) in cleaning solution and allow them to stand over night. (Steam may break the glass cylinder).

2. Pipettes which have been used should be washed immediately. Grease which cannot be removed with water should be treated with 10% NaOH and then with cleaning solution.
3. Rinse with tap water, followed by distilled water.
4. Rinse with alcohol. (Alcohol may be used repeatedly.)
5. Drain.

Fermentation Tubes. 1. Rinse with tap water.

2. Fill with cleaning solution and heat fifteen minutes in steam or allow to stand over night if more convenient.
3. Wash thoroughly in tap water, using a test-tube brush if necessary.
4. Rinse in distilled water and drain.

Cover-glasses and Slides. 1. Immerse the cover-glasses or slides, *one by one* in a 10% solution of sodium hydrate (NaOH) for thirty minutes only. This strength of NaOH will etch the glassware if left longer.

2. Wash *separately* in tap water, *handling with ordinary forceps.**
3. Put, *one at a time*, in cleaning solution, and leave over night as convenient.
4. Wash *separately* in water.
5. Immerse in *clean* alcohol (95%).
6. Wipe with a *clean* physician's cloth.
7. Store in clean Esmarch and deep culture dishes respectively, to keep free from dust.

* Any inexpensive fine-grained cleaning powder as powdered pumice stone, Bon Ami, etc., may be used.

Experiment 3. Sterilization techniques

Sterilisation refers to the anti-microbial process during which all microorganisms are killed or eliminated in or on a substance by applying different processes. Microbes react in their own way to the antimicrobial effects of various physical treatments or chemical compounds, and the effectiveness of treatments depends on many other factors as well (e.g. population density, condition of microorganisms, concentration of the active agent, environmental factors). Sterilisation procedures involve the use of heat, radiation or chemicals, or "physical removal" of microbes. The type of sterilisation should always be chosen as required, by taking into consideration the quality of materials and tools used and the possible adverse effects of sterilisation on them.

Sterilisation by heat

The use of dry heat is based on the removal of the water content of microbes and subsequent oxidation. Open flame can be used for sterilisation if the object is not directly exposed to flame damage. Different laboratory devices (e.g. scalpel, knife, inoculating loop or needle) can be sterilised quickly and safely by crossing over open flame or by ignition.

Dry heat sterilisation is performed in a hot air steriliser. It is an electric box with adjustable temperature like an incubator. In order to achieve uniform chamber temperature, hot air is circulated. Sterilisation with dry heat is limited to devices made of metal, glass or porcelain, and other thermo-stabile-materials, like glycerol, soft paraffin, oils and fats. In the dry heat sterilisation system they have to withstand the temperature needed to kill the spore forming bacteria (at 160°C for 45 minutes; at 180°C for 25 minutes; at 200°C for 10 minutes). The heat conductivity of water is several times higher than that of the air, therefore heat sterilises more quickly and effectively in the presence of hot water or steam than dry heat. Boiling is the simplest and oldest way of using moist heat. The temperature of boiling water does not exceed 100°C at normal atmospheric pressure. Heat resistant, endospore-forming bacteria can survive the 10-30-minute heat treatment of boiling, so no sterilizing effect can be expected from boiling.

Pasteurisation is a widespread method – named after Louis Pasteur – to reduce the number of microorganisms found in different heat sensitive liquids. Milk can be pasteurised by heating to 65°C for 30 minutes or to 85°C for 5 minutes. During ultra-pasteurisation milk is heat-treated at 135-150°C for 2 minutes in a heat exchanger. The temperature and time used for pasteurisation are suitable to control the presence of some pathogenic bacteria, however endospores and cells of heat resistant bacteria e.g. Mycobacterium species, can survive.

Tyndallisation (intermittent sterilisation) is an old and lengthy method of heat sterilisation named after John Tyndall. During this method, a medium or solution is heated to a temperature over 90°C for 30 minutes for four successive days, and the substances are placed in an incubator at 37°C or stored at room temperature in the intermittent periods. Vegetative forms are destroyed during the heat treatments. Endospores which can germinate during the incubation period are destroyed during the consecutive heat treatments. This way, after the fourth day of heat treatment, no living cells remain in the substance.

EXERCISE 1: OPERATION OF THE AUTOCLAVE

The use of saturated steam under high pressure is the most effective method to kill microorganisms. In the laboratories, a sealed heating device called autoclave is used for this purpose. From the inside of the carefully temperature-controlled autoclave, the air is expelled by the less dense steam and

sterilisation takes place in a closed chamber at 121°C and overpressure. The household pressure cooker works on a similar principle but with lower temperature. Autoclaves are widely used in microbiological practise mainly for sterilisation of culture media, glassware and heat-resistant plastic products before their use, and also for contaminated materials prior to disposal as municipal solid waste. To achieve sterilisation, generally 15 minutes of heat treatment at 121°C under 1.1 kg/cm^2 pressures has to be applied. Most microbes are unable to tolerate this environment for more than 10 minutes. However, the time used for sterilisation depends on the size and content of the load.

Object of study, test organisms: culture medium in a flask

Materials and equipment: Distilled water, Heat-proof gloves, Autoclave

Procedure:

1. Open the lid of the autoclave and check that there is sufficient amount of distilled or deionised water in it. If necessary, refill.
2. Place the correctly packaged materials (e.g. laboratory equipment, culture medium in a flask) into the chamber of the autoclave. Stick a piece of autoclave indicator tape onto the surface of materials!
3. Close the lid of the autoclave.
4. Make sure that the bleeder valve is open.
5. Turn on the heating of the autoclave (the indicator lamp is lit).
6. If an intense (a thick, milky white) steam outflow can be detected through the outlet tube of the bleeder valve (100°C on the built-in thermometer), wait for 4-5 minutes and close the bleeder valve (venting).
7. With the help of a built-in thermometer and manometer, check the temperature and pressure increase inside the chamber of the autoclave.
8. The sterilisation time (15 minutes or more) begins only when the temperature equalization (to 121°C) in the chamber has occurred. It is important that the operator stays with the device and controls the process of sterilisation from the time it is turned on until the end of the sterilisation period.
9. Turn off the power switch of the autoclave when the sterilisation cycle/period has ended.
10. Allow the device to cool down to at least 60-70°C.
11. For decompression, slowly open the bleeder valve. Thereafter, carefully open the lid of the autoclave and remove the sterilised materials, using heat-proof gloves. Check the colour of sterilisation indicator controls.

Sterilisation by radiation

Other forms of energy [e.g. ultraviolet (UV) and ionizing radiation] are also used for sterilisation especially for heat-sensitive materials. The full spectrum of UV radiation can damage microbes but only a small part is responsible for the so-called germicidal effect. Very strong "germicidal" effect can be achieved around 265 nm, because maximum UV absorption of DNA occurs at this wavelength. The main cause of cell death is the formation of pyrimidine dimers in nucleic acids. Bacteria are able to repair their nucleic acid after damage using different mechanisms; however, beyond a certain level of damage, the capacity of the enzyme system is not enough and the accumulation of mutations causes death. UV (germicidal) lamps are widely used in hospitals and laboratories (e.g. in biological safety cabinets) for decontamination of air and any exposed surfaces. The disadvantage of the use of UV radiation is that it does not penetrate through glass, dirt films, water, and other substances.

Among the high-energy ionizing radiation, γ-rays from radioactive nuclides ^{60}Co are generally used for sterilisation of disposable needles, syringes, bandages, medicines and certain food (e.g. spices). The advantage of gamma radiation is its deep penetration through the packaging. Its disadvantage is the scattering in all directions, which requires special circumstances for application.

Filter sterilisation

The most commonly used mechanical method of sterilisation is filtration. During filtration, liquids or gases are pressed through a filter, which (depending on its pore size) retains or adsorbs (e.g. asbestos filter pads) microbes, thereby the filtrate becomes sterile. The pore diameter of filters should be chosen carefully so that bacteria and other cellular components cannot penetrate.

Earlier Seitz-type asbestos or different glass filters were commonly used for the filtration of microorganisms. The modern membrane filters are usually composed of high tensile-strength polymers(cellulose acetate, cellulose nitrate or polysulfone, etc.). Their operation is based partly on the adsorption of microbes, partly on a mechanical sieve effect. The pure sieve-based filters can be beneficial because they do not change the composition of the filtered solution. To remove bacteria, membrane filters with pore size of 0.22 μm are the best choice.

Membrane filters are biologically neutral; do not hamper life activities of microorganisms remaining on the filter and do not inhibit their enzyme functions. Furthermore, nutrients can diffuse through the membranes, so bacteria can be cultured on a variety of media also by placing the filters onto their surface.

Sterilisation by chemicals

A wide range of chemicals is suitable to inhibit or kill microbes. Some of the antimicrobial agents only inhibit the growth of microorganisms (e.g. bacteriostatic, fungistatic, and virostatic compounds) while others kill them (e.g. bacteriocidal, fungicidal, and virocidal agents). The -static or -cidal effect of a substance depends on the applied concentration and exposure time in addition to its quality. Only –cidal effect substances are used for chemical sterilisation. These substances have the following requirements: they should have a broad-spectrum effect, they should not be toxic to higher organisms, they should not enter detrimental reactions to the materials being treated with, they should not be biodegradable, they should be environmentally friendly, easy to apply and economical.

The materials used in chemical sterilisation are liquids or gases. Liquid agents are used especially for surface sterilisation. Among sterilising gases, those working at low temperature function by exposing the materials to be sterilised to high concentrations of very reactive gases (e.g. ethylene oxide, beta-propiolactone or formaldehyde). Due to their alkylating effect, these compounds cause the death of microbes by damaging their proteins and nucleic acids. The chemical agents used for sterilisation must be chemically compatible with the substances to be sterilised, therefore they have a great importance in sterilisation of pharmaceutical and thermoplastic materials. The chemicals used by the gas sterilisers are harmful to humans as well. Therefore, the application of gas sterilisers requires compliance with the precautions by the users.

Procedures of disinfection

Any process aimed at destroying or removing the infectious capability of pathogenic microbes that generally occur on inanimate objects, is called disinfection. The chemicals used for disinfection can be classified according to their chemical structure and their mode of action.

Among the alcohols, ethanol and isopropanol are widely used as disinfectants. 50-70% aqueous solution has excellent antiseptic properties. The action mechanism of alcohols depends on the applied concentration. Due to the solubility of lipids in 50-95% ethanol solutions, biological membranes are disintegrated. Alcohols pass through the cell membrane with altered permeability, denature the proteins inside the cell and have a dehydration effect as well. Absolute alcohol (100% ethanol) provides the best dehydration effect but does not coagulate the intracellular proteins. 70% dilution of alcohols is the most effective way to kill the vegetative forms of bacteria and fungi, but less effective against spores and lipid-enveloped viruses.

Phenol called carbolic acid was first used as a disinfectant by Lister. Phenol denatures proteins, and irreversibly inactivates the membrane-bound oxidases and dehydrogenases. Due to the unfavourable physical, chemical and toxicological properties, phenol is no longer used. However, substituted (alkylated, halogenated) derivatives are often used in combination with surfactants or alcohols (e.g. cresol, hexachlorophene, chlorhexidine).

The halogens (F, Cl, I, Br) and their derivatives are very effective disinfectants and antiseptic agents; mainly their non-ionic forms have antimicrobial activity. Chlorine gas is used almost exclusively for the disinfection of drinking water or other waters. In addition, different compounds (e.g. chloride of lime, chloramine-B, sodium dichloroisocyanurate) are among the most widely used disinfectant agents. Sodium hypochlorite (“household bleach” is a mixture of 8% NaClO and 1% NaOH) is one of the oldest high-bleaching and deodorizing disinfectant. The basis of the effect of chlorine and its derivatives is that during decomposition in aqueous solution, a strong oxidant, nascent (atomic state) oxygen ('O'), is released. Nascent oxygen is very reactive and suitable to destroy bacteria, fungi and their spores as well as viruses.

Iodine is also a widely used disinfectant and antiseptic agent. There are two known preparations: tincture of iodine (alcoholic potassium iodide solution containing 5% iodine) and iodophors (aqueous solutions of iodine complexes with different natural detergents). It is applied in alcoholic solution to disinfect skin or in aquatic solution for washing prior surgery.

Aldehydes, such as formaldehyde and glutaraldehyde, are broad-spectrum disinfectants. They are used for decontamination of equipment and devices. Formalin is the 34-38% aqueous solution of formaldehyde gas. Its effect is based on the alkylation of proteins.

Heavy metals such as mercury, arsenic, silver, gold, copper, zinc and lead, and a variety of their compounds are highly efficient disinfectants but they are too damaging to living tissues to apply. They can be used as disinfectants at very low concentrations. Inside the cell, they bind to the sulfhydryl groups of proteins. Primarily, organic and inorganic salts of silver and mercury-containing products are commercially available, which have bactericidal, fungicidal and virocidal effect.

Detergents or surfactants are amphiphilic organic molecules which have a hydrophilic "head" and a long hydrophobic “tail”. Detergents can be non-ionic, anionic or cationic according to the charge of the carbon chain. Nonionic surfactants have no significant biocidal effect and anionic detergents are only of limited use because of their poor efficiency. The latter group includes soaps, which are long-chain carboxylic acids (fatty acids) of sodium or potassium salts. They are not disinfectants on their own, but are efficient cleaning agents due to their lipid-solubilising effect. Cationic detergents, such as quaternary ammonium salts, are the best disinfectants.

Experiment 5. Cultivation of bacteria in laboratory

Principle:

Nutrient agar is used as a general purpose medium for the growth of a wide variety of non-fastidious microorganisms. It consists of peptone, beef extract and agar. This relatively simple formulation provides the nutrients necessary for the replication of a large number of non-fastidious microorganisms. The characteristics of the components used in nutrient agar:

Beef extract is an aqueous extract of lean beef tissues. It contains water-soluble substances of animal tissue, which include carbohydrates, organic nitrogen compounds, water soluble vitamins, and salts.

Peptone is made by digesting proteinaceous materials e.g., meat, casein, gelatin, using acids or enzymes. Peptone is the principal source of organic nitrogen and may contain carbohydrates or vitamins. Depending up on the nature of protein and method of digestion, peptones differ in their constituents, differing in their ability to support the growth of bacteria.

Agar is a complex carbohydrate obtained from certain marine algae. It is used as a solidifying agent for media and does not have any nutritive value. Agar gels when the temperature of media reaches 45°C and melts when the temperature reaches 95 °C.

Materials:

Glass petri plates, conical flasks (250 ml), pipettes (1 ml and 10 ml), spirit lamp, 70% alcohol, fuel alcohol, non-absorbent cotton, tissue paper, distilled water
Media Composition: Nutrient broth 13.0 gm
Agar 20.0 gm
Distilled Water 1000 ml
Final pH 6.8 ± 0.2.

Procedure:

Media preparation:

Required amount (1.3 gm) of nutrient broth was dissolved into 100 ml of distilled water kept in a conical flask.
pH was adjusted to 6.8 ± 0.2.
Then 2.0 gm of agar was added to 100 ml of nutrient broth.
The media was sterilized at 15 lb pressure and 121 ^{o}C for 15 min by using autoclave.
After autoclaving, the media was cooled down to 50 ^{o}C and poured in to the autoclaved petri plates.

Dilution preparation:

Nine milliliter of autoclaved distilled water was added to 6 test tubes.
One milliliter of water sample (Tap water) was mixed with 9 ml of distilled water and thus 10^{-1} dilution was prepared.
Then 1 ml of water from10^{-1} dilution was poured in another test tube containing 9 ml of autoclaved distilled water and thus 10^{-2} dilution was prepared.
Like this, upto 10^{-6} dilution was prepared.

Plating:

One hundred microliter of 10^{-4}, 10^{-5}, 10^{-6} dilutions were spread on the solidified nutrient agar plates.
The plates were incubated at 37 ^{o}C for 24 h.

Result:

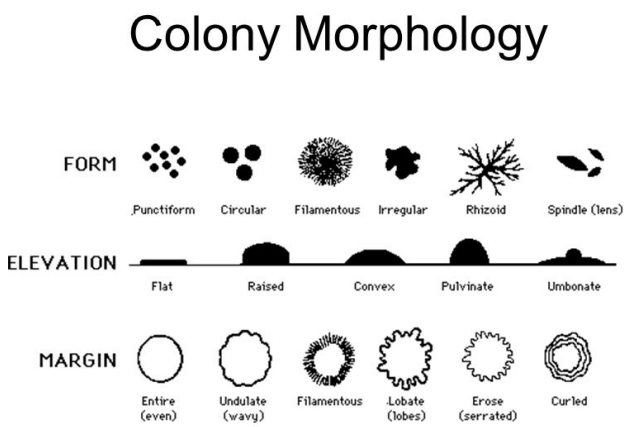
After 24 h, the colony appeared on the petri plates were enumerated and the colony characteristics were observed.

Enumeration of bacteria from water sample:

Sample number	Dilution no.	Sample added (ml)	CFU numbers/ 0.1 ml	CFU numbers/ 1 ml	CFU No./ml in original sample	Average number of CFU No./ml in original sample

Colony characteristics:

Colony number	Colour	Form	Elevation	Margin	Figure



Comment: Hence, the supplied sample contained X CFU/ g of cultivable bacteria.

Experiment 6. Staining of bacteria: Simple staining, Gram staining, and Negative staining.

Simple staining

Principle: Simple staining involved applying a single basic dye to impart colour to the bacterial cell. Basic dyes are positively charged and work well with bacteria because the bacterial cells bear a slight negative charge. Further basic dyes are attached to the acidic part of the cell such as techoic acid. In general there are two main stain types. Positively charged stains have a positive chromophore. The second type, negatively charged stains, has a chromophore that carries a negative charge. Positively charged stains are excellent in binding negatively charged structures such as bacterial cell walls and, if they can enter the cell, many macromolecular structures such as DNA and proteins.

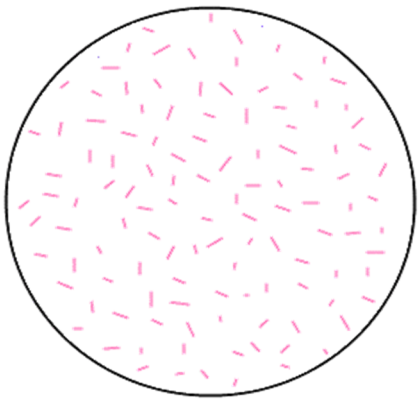
Example of the basic dye includes methylene blue, crystal violet and safranin.

Materials: Glass slide, bacterial culture, cotton, crystal violet, microscope, tray of disinfectant, inoculating loop bunsen- burner

Procedure:

A clean glass slide was obtained.
The smear was prepared by placing a drop of culture by using sterile inoculating loop.
The smear was allowed to air dry and then heat fixed by using Bunsen-burner.
The smear was covered with several drops of crystal violet and incubated for 30 sec-1 min.
The slide was gently washed with drops of tap water.
The slide was air dried and observed under oil immerson microscope (100x).

Observation and Result:

Draw a Representative field:	
Cell Morphology: **Shape:** **Arrangement:** **Cell Colour:**	**Rod Shape** **Single** **Violet**

Comment: According to the above result, the supplied bacterial sample was rod shaped and arranged in single. (Please change accordingly with your result).

Gram Staining

Principle: Differential staining requires the use of at least three chemical reagents that are applied sequentially to a heat fixed smear. The first reagent is called the primary stain. Its function into impart its colour to all cells. In order to establish a colour contrast the second reagent is the discolouring agent. Based on the chemical composition of cellular components the decolourising agent may or may not remove the primary stain from the entire cells or only from certain cell structures. The final reagent, the counter stain has a contrasting than that of the primary stain.

Following decolourisation, if the primary stain is not washed out, the counter stain can't be observed and the cells or their components will retain the colour of the primary stain. If the primary stain is removed, it accepts the contrasting colour of counter-stain. In this way cell type or their structures can be distinguished from each other on the basis of the stain that cells retained.

Purposes: To become familiar with-

i) The chemical and theoritical basis for differential staining procedures.
ii) The chemical basis of gram-stain.
iii) Performance of the procedure for differentiating between the two principle group of bacteria-
   a. Gram positive bacteria.
   b. Gram negative bacteria.

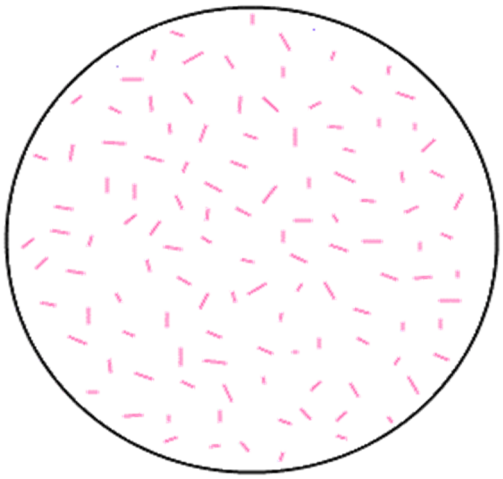
Materials:

- **Culture:** Twenty four hours old culture
- **Reagents:** Crystal violet- Primary stain
  Gram Iodine- Mordant
  Decolourising agent- 70% ethyl alcohol
  Counter Stain- Safranine
- **Equipment:** Bunsen burner, inoculating loop, staining tray, glass slide, lens paper and microscope.

Procedure:

- A clean glass slide was obtained.
- The smear was prepared by placing a drop of culture by using sterile inoculating loop.
- The smear was allowed to air dry and then heat fixed by using Bunsen-burner.
- The smear was covered with several drops of crystal violet and incubated for 30 seconds to 1 minute.
- The slide was gently washed with drops of tap water.
- The smear was then flooded with the Gram's iodine and incubated for one minute.
- The slide was gently washed with drops of tap water.
- The slide was then decolourized with 90% ethyl alcohol.
- The slide was air dried followed by counter staining with safranine for 45 seconds.
- The slide was gently washed with drops of tap water.
- The slide was air dried and observed under oil immerson microscope (100x).

Observation and Result:

Draw a Representative field: **Cell Morphology:** **Shape:** **Arrangement:** **Cell Colour:** **Gram characteristic:**	 **Rod Shape** **Single** **Violet** **Gram Positive**

Comment: Hence the supplied bacterial sample was Gram Positive rod shaped bacteria.

Negative staining

PRINCIPLE

Negative staining procedure is so called because the background gets stained and the organism remains colourless. It is also known as 'Indirect staining. The procedure requires the use of acidic stains such as India ink or Nigrosin. Negative staining finds its utility for the demonstration of capsule and bacteria difficult to stain such as *Treponema palladium*. Wet film India-ink method is the best method for staining capsules of bacteria from cultures in either liquid or solid media.
The acidic dyes such as India ink, nigrosin or eosin have negatively charged chromogen, and will not readily combine with the negatively charged bacterial cytoplasm. Instead it forms a deposit around the organism, leaving the organism itself colourless. Therefore, the unstained cells are easily discernible against the coloured background.

REQUIREMENTS

Equipment: Compound light microscope.
Reagents and glass wares: These include Bunsen flame, staining tray, glass slides and coverslips nigrosin stains. Nigrosin staining solution is prepared by adding 0.03 gram of nigrosin in 100 ml of distilled water.
Specimen: 24 hour broth culture of *Klebsiella pneumoniae* (A capsulated bacterium).

PROCEDURE

1) Take a clean grease free glass slide.
2) Put a small drop of nigrosin close to one end of a clean slide.
3) Using a sterile loop, a loopful of broth culture of the capsulated organism is mixed with the nigrosin drop.
4) With the edge of a second slide, held at 30° angle and held in front of the bacterial suspension mixture, spread the drop along the edge of the applied slide. The slide is then pushed away from the previously spread drop of suspended organism, forming a thin smear.
5) Air dry the preparation without any heat fixation.
6) Observe the stained smear under oil immersion (100x) objective.
7) Record the observations in the note book.

OBSERVATION

The bacterial organism is seen as a clear halo against a black or dark background in the wet film or dry film preparation.

RESULTS AND INTERPRETATION

Hence the supplied bacterial sample contained rod / coccus shaped bacteria.

Experiment 7. IMViC test

Indole Test

OBJECTIVES:

1) Determine the ability of bacteria to degrade the amino acid tryptophan.
2) Distinguish the bacteria based on the indole activity.

PRINCIPLE:

Tryptophan is an essential amino acid that can undergo oxidation by enzymatic activities of some bacteria. Conversion of tryptophan into metabolic products is mediated by the enzyme tryptophanase. The metabolic end products are indole, skatole and indole acetic acid. The ability to hydrolyse tryptophan with the production of indole is not a characteristic of all bacteria. Only some bacteria produce indole.

REQUIREMENTS:

Equipments: Incubator.

IReagents and lab wares: Peptone water / tryptone broth, Kovac's reagent, or Ehrlich's reagent, glass tubes and inoculating wire.

Kovac's reagent consists of para-dimethyl amino benzaldehyde, 5.0 gm; isoamyl alcohol, 75.0 ml; and concentrated hydrochloric acid, 25.0 ml. Ehrlich's reagent consists of p-dimethyl amino benzaldehyde, 4.0 gm; absolute ethyl alcohol, 380.0 ml; and concentrated hydrochloric acid, 80.0 ml.

Specimen: 24 hours to 48 hours peptone water culture of *Escherichia coli* incubated at 37°C.

PROCEDURE

1) Take 0.5 ml of 24 hours to 48 hours peptone water cultures of *E. coli* in a small test tube.
2) Add 0.2 ml of Kovac's reagent to the peptone water and shake.
3) Allow it to stand for few minutes and read the result.

OBSERVATION

In a positive test, a red-violet ring develops within minutes on addition of Kovac's reagent. In a negative test a yellow ring appears.

RESULTS AND INTERPRETATION

Positive indole test is indicated by the appearance of red-violet ring on adding the reagent. Negative reaction is indicated by developing a yellow ring. *E. coli* colonies tested are an indole producing bacteria. *K. pneumoniae* does not produce the indole.

List of Indole positive and negative bacteria

Indole positive bacteria	Indole negative bacteria
1. *Escherichia coli*	*1. Escherichia vulnaris*
2. *Klebsiella oxytoca*	2. *Klebsiella pneumoniae*
3. *Proteus vulgaris*	3. *Proteus mirabilis*
4. *Morganella morganii*	4. Salmonella Typhi
5. *Providencia rettgeri*	5. *Shigella sonnei*
6. *Aeromonas hydrophila*	
7. *Pasteurella multocida*	
8. *Vibrio cholerae*	
9. *Falvobacterium*	
10. *Plesiomonas shigelloides*	

Methyl Red Test

OBJECTIVES

1) Determine the ability of bacteria to oxidise glucose with the production of high concentrations of acidic end products by methyl red test.
2) Differentiate between all glucose oxidizing enteric bacteria particularly *Escherichia coli* and *Enterobacter aerogenes*.

PRINCIPLE

Methyl red is a pH indicator with a range between 6.0 (yellow) and 4.4 (red). The pH at which methyl red detects acid is considerably lower than the pH of other indicators used in bacteriologic culture media. Thus to produce a colour change, the test organism must produce large quantities of acid from the carbohydrate substrate being used.

The methyl red test is a quantitative test for acid production, requiring positive organisms to produce strong acids (lactic acid, acetic acid, formic acid) from glucose through the mixed acid fermentation pathway. Because many species of the Enterobacteriaceae may produce sufficient quantities of strong acids that can be detected by methyl red indicator during the initial phase of incubation, only organisms that can maintain this low pH after prolonged incubation (48–72 hours) overcoming the pH buffering system of the medium can be called methyl red positive.

REQUIREMENTS

Equipments: Incubator.

Reagents and lab wares: Inoculating wire. Methyl red test broth. It consists of poly peptone, 7 gm; glucose, 5 gm; dipotassium phosphate, 5 gm; and distilled water, 1l at a pH of 6.9. Methyl red indicator. It consists of methyl red, 0.1 g in 300 ml of 95% ethyl alcohol.

Specimen: Culture of *E. coli*, *E. aerogenes* and *Klebsiella pneumoniae* in glucose phosphate medium incubated at 30°C for five days.

PROCEDURE

1 Take 0.5 ml of broth cultures of *E. coli* in a small test tube.

2 Add five drops of 0.04% solution of methyl red directly to the broth culture and mix well.

3 Note any change in the colour of medium at once.

OBSERVATION

Look for the development of stable red colour on adding methyl red indicator.

RESULTS AND INTERPRETATION

The development of a stable red colour in the surface of the medium indicates sufficient acid production to lower the pH to 4.4 and constitutes a positive test. Because other organisms may produce smaller quantities of acid from the test substrate, an intermediate orange colour between yellow and red may develop. This does not indicate a positive test. Yellow colour indicates a negative test.

List of MR positive and negative bacteria

MR positive bacteria	MR negative bacteria
1. *E. coli*	1. *K. pneumoniae*
2. *K. ozaenae*	2. *Enterobacter* spp
3. *K. rhinoscleromatis*	
4. *K. ornitholytica*	
5. Edwardsielleae	
6. Salmonellae	
7. Citrobacter	
8. Proteae	
9. Yersinia	

Voges-Proskauer Test

PRINCIPLE

The Voges-Proskauer test determines the capability of some bacteria to produce non-acidic or neutral end products such as acetyl methyl carbinol from the organic acids produced as a result of glucose metabolism. Pyruvic acid, the pivotal compound formed in the fermentative degradation of glucose is further metabolised through various metabolic pathways, depending on the enzyme systems possessed by different bacteria.

One such pathway results in the production of acetoin (acetyl methyl carbinol), a neutral-reacting end product. Enteric bacteria such as members of the Klebsiella-EnterobacterHafnia-Serratia group produce acetoin as the chief end products of glucose metabolism and form smaller quantities of mixed acids.

The test depends on the production of acetyl methyl carbinol from pyruvic acid, as an intermediate product in its conversion to 2: 3 butylene glycol. In the presence of atmospheric oxygen and alkali (40% potassium hydroxide), the small amount of acetyl methyl carbinol present in the medium is converted to diacetyl, which reacts with the peptone of the broth to produce a red colour.

REQUIREMENTS

Equipments: Incubator.

Reagents and lab wares: Inoculating loop. VP broth. It consists of polypeptone, 7 gm; glucose,5 gm; dipotassium phosphate, 5 gm and distilled water, 1 litre at a pH of 6.9. 5% a naphthol. It consists of a naphthol, 5 gm; and absolute ethyl alcohol, 100 ml. It serves as the colour intensifier. 40% potassium hydroxide. It consists of 40 gm potassium hydroxide in 100 ml distilled water. It serves as the oxidising agent.

Specimen: Culture of *Escherichia coli*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* in glucose phosphate medium incubated at 30°C for five days or 37°C for 48 hours.

PROCEDURE

1) Take 1 ml of broth cultures of *E. coli* in a small test tube.
2) First add 40% KOH and then add 0.6 ml of a 5% solution of α-naphthol in ethanol to the broth culture and shake gently. It is essential that the reagents are added in this order.
3) Note any change in the colour of medium within 2-5 minutes.

OBSERVATIONS

Look for the development of pink colour 15 minutes or more after addition of the reagents.

RESULTS AND INTERPRETATION

A positive test is represented by the development of a pink colour 15 minutes or more after addition of the reagents, deepening to magenta or crimson in half an hour. This indicates the presence of diacetyl, the oxidation product of acetoin. A negative test is indicated by colour less reaction for half

an hour. The test should not be read after standing for over 1 hour because negative VP test may produce a copper-like colour, leading to a false positive interpretation.

VP positive and negative bacteria

VP positive bacteria	VP negative bacteria
1. *Klebsiella pneumoniae*	1. *Escherichia coli*
2. *Enterobacter cloacae*	2. *Edwardsiella tarda*
3. *Cedicia netri*	3. Salmonellae
4. *Ewingella americana*	4. Proteae
5. *Serratia marcescens*	5. Yersinieae
6. *Aeromonas sobria*	
7. *Vibrio cholerae*	
8. *Chryseomonas luteola*	
9. *Flavimonas oryzihabitans*	
10. *Sphingomonas paucinobilix*	

Citrate Utilisation Test

OBJECTIVES

Differentiate certain enteric organisms on the basis of their ability to utilize citrate as a sole source of carbon.

PRINCIPLE

In the absence of fermentable glucose or lactose, some bacteria are capable of using citrate as a sole source of carbon for their energy. This ability depends on the presence of the enzyme, a citrate permease that facilitates the transport of citrate in the cell.

Citrate is the first major intermediate in the Krebs cycle and is produced by the condensation of active acetyl with oxaloacetic acid. Citrate is acted on by the enzyme citrase which produces oxaloacetic acid and acetate. These products are then enzymatically converted to pyruvic acid and carbon dioxide (CO_2). During this reaction the medium becomes alkaline because the CO_2 that is generated combines with sodium and water to form sodium carbonate, an alkaline product. The presence of sodium carbonate changes the indicator, bromo thymol blue present in the medium from green at pH 6.9 to deep Prussian blue at pH 7.6. Simmon's citrate and Koser's citrate are two examples of different types of citrate media used in the test.

REQUIREMENTS

Equipments: Incubator.

Reagents and lab wares: Inoculating loop, Simmon's citrate medium (It consists of ammonium dihydrogen phosphate, 1 gm; dipotassium phosphate, 1 gm; sodium chloride, 5 gm; sodium citrate, 2 gm; magnesium sulfate, 0.20 gm; agar, 15 g; bromo thymol blue, 0.08 gm and distilled water 1 litre) pH adjusted to 6.9. The medium is poured into a tube on a slant.

Specimen: Culture of *Escherichia coli*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* in glucose phosphate medium incubated at 37°C for 48 hours.

PROCEDURE

1) Using sterile technique, inoculate each bacteria into its appropriately labeled tube by means of a stab and streak inoculation.
2) Incubate all cultures for 24 hours to 48 hours at 37°C.

OBSERVATIONS

Look for the development of deep blue colour within 24-48 hours of incubation of the inoculated tube.

RESULTS AND INTERPRETATION

A positive test is represented by the development of a deep blue colour within 24 hours to 48 hours, indicating that the test organism has been able to utilize the citrate contained in the medium, with the production of alkaline products. A negative test is indicated by no change of colour of the citrate medium.

List of citrate positive and negative bacteria

Citrate positive bacteria	Citrate negative bacteria
1. *Klebsiella pneumoniae*	*1. Escherichia coli*
2. *Citrobacter diversus*	2. Salmonella Typhi
3. *Enterobacter cloacae*	3. Salmonella Paratyphi A
4. *Serratia marcescens*	4. *Shigella* species
5. *Providencia alcalifaecians*	5. *Yersinia enterocolitica*
6. *Euringella americana*	6. *Edwardsiella tarda*
7. *Acroncobacter oxylosoxidans*	7. *Vibrio holisae*
8. *Vibrio vulnificus*	

Experiment 8. Antibiotic sensitivity test of bacteria

OBJECTIVES

Determine antibacterial sensitivity of bacterial isolates by Kirby –Bauer disc diffusion method.

PRINCIPLE

Due to emergence of many antibiotic resistant strains of bacteria, antimicrobial susceptibility testing is done in order to determine which antimicrobial agent to use against a specific strain of bacteria. The available chemotherapeutic agents vary in their scope of antimicrobial activity. Some have a limited spectrum while others have a wide spectrum of activities against bacteria. The bacterial strains isolated from clinical samples should be tested for antimicrobial sensitivity because it gives the clinician an idea as to what antimicrobial therapy should be started to the patients.

Kirby – Bauer method is a method of determination of antibiotic sensitivity of the bacteria by disc diffusion method. In this method, standard suspension of bacteria to be tested are inoculated on the surface of Mueller Hinton agar plates. Filter paper discs containing specific concentration of antimicrobial agents are pressed on to the surface and incubated at 35°C overnight (18-24 hr.). After incubation, the zone of inhibition of growth of bacteria around each disc is measured and the susceptibility is determined.

REQUIREMENTS

Equipment: Incubator.

Reagents and lab wares: 0.5 McFarland standard, Mueller Hinton agar plates (pH 7.2-7.4), peptone water, filter paper discs impregnated with appropriate concentration of antibiotics, sterile cotton swabs, millimeter ruler, forceps and inoculating wire.

Preparation of 0.5 McFarland standard: Solution A is prepared by adding barium chloride ($BaCl_2$, 2H2O) to 100 ml distilled water. Solution B is prepared by adding 1 ml of sulphuric acid (H_2SO_4 (0.36N) to 100 ml of distilled water. Then 0.5 ml of solution A is added to 99.5 ml of solution B, mixed well and distributed in test tubes with a screw cap. The cap is closed tightly to avoid evaporation. The mixture is stored in the dark. The solution is agitated vigorously before using it.

Specimens: *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, and Pseudomonas aeruginosa*.

Preparation of suspension of bacteria: Approximately, 4-5 well isolated colonies of the bacterial strain to be tested are inoculated into 5 ml of peptone water, and is incubated at 37 °C for 3-4 hours. The turbidity of the suspension is adjusted to match 0.5 McFarland standards. If the density is more it is diluted with sterile saline. The comparison is made against a white back ground with a contrasting black line.

PROCEDURE

1) After standardisation of bacterial suspension, immerse a sterile cotton swab in it and rotate the swab several times with firm pressure on the inside wall of the tube to remove excess fluid.
2) Prepare a Mueller Hinton agar (MHA) plate (pH 7.2-7.4) with a depth of 4 mm.
3) Inoculate the dried surface of the MHA agar plate by streaking the swab three times over the entire agar surface. It is streaked in three directions by rotating the plate 60° after each streak.

4) Place the appropriate antimicrobial impregnated discs on the surface of the agar using sterile forceps.
5) Gently press each disc onto the agar to provide uniform contact. Do not move the disc once it has contacted the agar because some of the antibiotics diffuse almost immediately Discs must be placed in such a way that they are at least 20 mm from one another.
   Note: 6 antibiotic discs may be put in an 85 mm plate.
6) Invert the plates and incubate at 35 °C -37°C for 16-18 hr.

OBSERVATIONS

1) Examine the plates for the presence and size of inhibitory zones.
2) The diameter of the inhibitory zone including the diameter of the disc is measured by using a millimeter scale upto the nearest millimeter.
3) All measurements are made with unaided eye while viewing the back of the petri dish with reflected light against a black non-reflecting background.
4) Measure the inhibitory zones for each antimicrobial agent, compare with the standard Kirby-Bauer's chart and interpret the zone of inhibition as sensitive, intermediate or resistant.

RESULTS AND INTERPRETATION

Each antibiotics produces a specific zone size for each bacteria tested. Depending on the zone size, the bacteria are classified as follows:

Sensitive (S): Infection treatable with normal dosage of the antibiotic.

Intermediate (I): Infection may respond to therapy with higher dosage.

Resistant (R): Unlikely to respond to the antibiotic at the usual dosage.

Experiment 9. Isolation of DNA from bacteria

Purpose: Isolation of Plasmid DNA from a microbial source

Principle: Plasmids are extra chromosomal DNA that replicate independently of the bacterial chromosome. They are normally covalently closed, circular, super-coiled molecules. They carry genes encoding functions (such as antibiotic resistance) which may be useful to the cell but are not essential for normal cellular activities. In the recombinant DNA technology plasmid DNA are used as vectors for carrying any foreign DNA. They can replicate with in host cell and possess phenotypic traits by which they can be detected. Genetic engineering makes use of recombinant DNA technology to fuse genes with plasmid vectors and clone them in the host cells. This way large number of isolated genes and their products can be synthesized and used for industrial, therapeutic and agricultural purposes.

Requirements:

Alkaline lysis solution I: 50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0), deion water

Alkaline lysis solution II: 0.2 N NaOH, 1% (w/v) SDS, de-ion water

Alkaline lysis solution III: 5 M potassium acetate, glacial acetic acid, de-ion water Ethanol 70% (v/v) Isopropanol TE-RNAase pH 8.0

Overnight grown *E. coli* cells

Procedure:

1) Pour overnight grown culture to 1.5 mL labelled falcon tube.
2) Centrifugate at 14.000 rpm for 1 min.
3) Remove the supernatant from the tube.
4) Repeat step 1-3, until leaves bacterial pellet as dry as possible.
5) Add 150 µL resuspension buffer, resuspend the bacterial pellet properly by vortexing.
6) Add 200 µL lysis solution to bacterial suspension (freshly made), close the tube tightly and mix contents thoroughly by inverting the tube 4-6 times until the solution becomes viscous.
7) Add 300 µL neutralization solution and mix contents thoroughly by inverting the tube 4-6 times.
8) Centrifuge at 14.000 rpm for 5 min.
9) Take the supernatant and transfer to a new 1.5 mL falcon max 300 µL.
10) Add equal volume of isopropanol in the supernatant (300 µL) and mix it by inverting the tube couple of times.
11) Incubate in -20°C for 30 min.
12) Centrifuge at 14.000 for 5 min.
13) Remove the supernatant and add 600 µL EtOH 70%.
14) Centrifuge at 14.000 for 5 min.
15) Remove the supernatant and dry the pellet for 10-30 min.
16) Dissolve the pellet in 20-50 µL TE-RNAase pH 8.0. Confirm the plasmid with 5 µL DNA solvent by Agarose Electrophoresis.

Experiment 10. Agarose gel electrophoresis of DNA

Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. Most every molecular biology research laboratory routinely uses agarose gel electrophoresis for the preparation and analysis of DNA. We will be using agarose gel electrophoresis to determine the presence and size of PCR products.

Background:

Electrophoresis is a method of separating substances based on the rate of movement while under the influence of an electric field. Agarose is a polysaccharide purified from seaweed. An agarose gel is created by suspending dry agarose in a buffer solution, boiling until the solution becomes clear, and then pouring it into a casting tray and allowing it to cool. The result is a flexible gelatin-like slab. During electrophoresis, the gel is submersed in a chamber containing a buffer solution and a positive and negative electrode. The DNA to be analyzed is forced through the pores of the gel by the electrical current. Under an electrical field, DNA will move to the positive electrode (red) and away from the negative electrode (black). Several factors influence how fast the DNA moves, including; the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules. DNA itself is not visible within an agarose gel. The DNA will be visualized by the use of a dye that binds to DNA.

Purpose: To determine the presence or absence of PCR products and quantify the size (length of the DNA molecule) of the product.

Materials needed:
- Agarose
- TAE Buffer
- 6X Sample Loading Buffer
- DNA ladder standard
- Electrophoresis chamber
- Power supply
- Gel casting tray and combs
- DNA stain
- Staining tray
- Gloves
- Pipette and tips

Recipes:

TAE Buffer
- 4.84 g Tris Base
- 1.14 ml Glacial Acetic Acid
- 2 ml 0.5M EDTA (pH 8.0)
- bring the total volume up to 1L with water

Add Tris base to ~900 ml H_2O. Add acetic acid and EDTA to solution and mix. Pour mixture into 1 L graduated cylinder and add H_2O to a total volume of 1 L.
Note – for convenience a concentrated stock of TAE buffer (either 10X or 50X) is often made ahead of time and diluted with water to 1X concentration prior to use.

6X Sample Loading Buffer
1 ml sterile H_2O
1 ml Glycerol
enough bromophenol blue to make the buffer deep blue (~ 0.05 mg)

-for long term storage, keep sample loading buffer frozen.

Agarose Gel Electrophoresis Protocol

Preparing the agarose gel

- Measure 1.25 g Agarose powder and add it to a 500 ml flask
- Add 125 ml TAE Buffer to the flask. (the total gel volume well vary depending on the size of the casting tray)
- Melt the agarose in a microwave or hot water bath until the solution becomes clear. (if using a microwave, heat the solution for several short intervals - do not let the solution boil for long periods as it may boil out of the flask).
- Let the solution cool to about 50-55°C, swirling the flask occasionally to cool evenly.
- Seal the ends of the casting tray with two layers of tape.
- Place the combs in the gel casting tray.
- Pour the melted agarose solution into the casting tray and let cool until it is solid (it should appear milky white).
- Carefully pull out the combs and remove the tape.
- Place the gel in the electrophoresis chamber.
- Add enough TAE Buffer so that there is about 2-3 mm of buffer over the gel.

Note – gels can be made several days prior to use and sealed in plastic wrap (without combs). If the gel becomes excessively dry, allow it to rehydrate in the buffer within the gel box for a few minutes prior to loading samples.

Loading the gel

- Add 6 µl of 6X Sample Loading Buffer to each 25 µl PCR reaction
- Record the order each sample will be loaded on the gel, including who prepared the sample, the DNA template - what organism the DNA came from, controls and ladder.
- Carefully pipette 20 µl of each sample/Sample Loading Buffer mixture into separate wells in the gel.
- Pipette 10 µl of the DNA ladder standard into at least one well of each row on the gel.

Note – if you are running multiple gels, avoid later confusion by loading the DNA ladder in different lanes on each gel.

Running the gel

- Place the lid on the gel box, connecting the electrodes.
- Connect the electrode wires to the power supply, making sure the positive (red) and negative (black) are correctly connected. (Remember – "Run to Red")
- Turn on the power supply to about 100 volts. Maximum allowed voltage will vary depending on the size of the electrophoresis chamber – **it should not exceed 5 volts/ cm between electrodes!** .
- Check to make sure the current is running through the buffer by looking for bubbles forming on each electrode.

• Check to make sure that the current is running in the correct direction by observing the movement of the blue loading dye – this will take a couple of minutes (it will run in the same direction as the DNA).
• Let the power run until the blue dye approaches the end of the gel.
• Turn off the power.
• Disconnect the wires from the power supply.
• Remove the lid of the electrophoresis chamber.
• Using gloves, carefully remove the tray and gel.

Gel Staining

• Using gloves, remove the gel from the casting tray and place into the staining dish.
• Add warmed (50-55°) staining mix.
• Allow gel to stain for at least 25-30 minutes (the entire gel will become dark blue).
• Pour off the stain (the stain can be saved for future use).
• Rinse the gel and staining tray with water to remove residual stain.
• Fill the tray with warm tap water (50-55 °C). Change the water several times as it turns blue. Gradually the gel will become lighter, leaving only dark blue DNA bands. Destain completely overnight for best results.
• View the gel against a white light box or bright surface.
• Record the data while the gel is fresh, very light bands may be difficult to see with time.

Note – Gels stained with blue stains are stable for long periods. When destaining is complete, remove gel from water and allow the gel to dehydrate. Dark bands can be seen for in a dried gel for weeks or months.

OBSERVATION

To visualize the DNA after staining, place the gel in white light transilluminator.

RESULT
Put the image of the gel.

Experiment 12. Polymerase Chain Reaction

INTRODUCTION

PCR is a very powerful amplification tool so very little DNA is actually required (usually in the pg range). To copy DNA, all polymerases require a short sequence of nucleotides to provide a free 3'OH group. Within cells of most organisms enzymes unwind the duplex and then RNA polymerase adds priming nucleotides. This priming allows the attachment of DNA polymerase from where extension can occur. In PCR however, there is a different mechanism of replication. PCR requires that the flanking sequences of the target DNA be known, so that single stranded synthetic oligonucleotide primers can be generated. Once these have annealed to the target DNA the polymerase can synthesize the rest of the chain. Primers can be specific to a particular sequence, or they can be universal to sequences that are very common within a DNA molecule allowing for a wide variety of DNA templates.

Deoxyadenylate (A), deoxythymidylate (T), deoxyguanylate (G) and deoxyctidylate (C) are components of the reaction mixture that are the basic building blocks of DNA required for the synthesis of the primers and for their extension. Note that both the primers and the deoxynucleotides are added to the mixture in excess.

The most commonly used polymerase in PCR is referred to as Taq-polymerase. This enzyme was originally found in the 1970's from the thermophilic bacterium Thermus aquaticus. Before its discovery, PCR was a much longer and costlier process since the thermosensitive E.coli DNA polymerase used lost its activity during the heating process and fresh enzyme had to be added at every cycle. Owing that T. aquaticus lives in a hot environment (approx. 75°C), the enzymes that power its internal metabolism have adapted to these conditions resulting in heat-stability. This polymerase will therefore, retains its activity when the mixture is heated and can be used for cycle after cycle.

A disadvantage of Taq-polymerase is that it does this without proofreading the newly synthesized DNA. This means that sometimes the polymerase will include dNTPs that are not complementary to the template DNA which results in errors in coding.

Recently, other thermostable DNA polymerases have been discovered that are less error-prone than Taq. These include Tli and Pfu, which are less efficient than Taq but are used if high fidelity is required. Magnesium ions are cofactors used in PCR specifically by the polymerase. They affect the annealing of the oligonucleotides to the template DNA by stabilizing the interaction, and also stabilize the replication complex of the polymerase with the template primer. Usually in practical situations PCR mixtures contain other components such as reaction buffers, sterile water, EDTA (to chelate the magnesium ions) and other minor reagents. The microfuge tube (containing mixture) is placed into a machine called a thermocycler. The first fully automated PCR machine was developed by Cetus and allowed temperatures to be varied in cycles for different lengths of time within one place. Before the invention of the thermo-cycling machine, the PCR process was slow and cumbersome. Three water baths at different temperatures were required, meaning that constant human intervention was necessary to move the sample between baths.

First Stage of PCR - Denaturation

The two anti-parallel strands of the DNA double helix are held together by hydrogen bonds between the complementary bases. The heating process provides enough energy to disrupt this bonding causing the DNA to "unzip" or denature to single-strands. Note that in reverse (if the DNA is allowed

to cool) the bonds reform and the DNA will renature back to the original double helix conformation. The thermocycler is set to heat at 95°C for 30 to 90 seconds.

Second Stage of PCR - Annealing

This stage involves the annealing of the oligonucleotide primer sequences to the template DNA. The primers cannot bind to the target DNA at the high temperature used in the first stage of PCR, so the microfuge tube is cooled to allow double- strands to form again.

The thermocycler is set to 55-60°C for about 30 to 120 seconds.

The target strands remain denatured since they are at too low a concentration to encounter each other during this stage, but the primers are at a very high concentration and so hybridize with their complementary sequences readily. Reassociation of the parent DNA is therefore successfully avoided.

The annealing temperature is a dynamic variable that affects the yield and specificity of PCR. To increase the yield of PCR the annealing temperature must be decreased (with a reduction in specificity). Conversely, to increase the specificity of PCR the annealing temperature must be increased (with a reduction in yield).

Third Stage of PCR - Extension

The third and final stage of PCR involves the extension of the primed sequences of the target DNA.

The thermocycler is set to raise the temperature of the microfuge tube to 72°C, which is the optimal functioning temperature of the Taq polymerase.

Taq polymerase then binds to the 3’ end of the primers and begins adding dNTPs one at a time as dictated by the template. Extension occurs in the 5' to 3' direction on the growing strand right up to the end (where the polymerase falls off) until there are 2 partial copies of the original DNA fragment.

The incubation period for this stage depends on the size of the target sequence to be polymerized and the polymerase used. For example Taq requires 60 seconds per kb of expected product, therefore this stage would take 3 minutes for a 3kb sequence. This completes one PCR cycle.

Recycling PCR

After the third stage the mixture is heated again to melt the newly formed duplexes and the whole process begins again. After every completed cycle newly synthesized DNA strands can serve as templates for the next cycle. The cycles are generally repeated 30 times. After the last cycle, an incubation period for 10 minutes at 72°C is performed to allow for any uncompleted polymerization and then the product is cooled down for analysis or storage. Since the DNA content is amplified in a logarithmic fashion, theoretically 1 billion copies could be formed in 1 hour. In practice however, (allowing for the time it takes for the thermocycler to change temperatures and for the last completion cycle) 1 million copies are ready in 3 hours.

Lastly, before you can analyze your PCR products they must be separated. This employs a biomolecular separation technique called gel electrophoresis.

Applications: PCR is so efficient at multiplying specific DNA sequences that DNA isolated from a single human cell can be screened for mutations associated with various genetic disorders.

This approach was used to screen fertilized human embryos prepared from sperm and ova from a couple that were carriers of the cystic fibrosis. This genetic disease results from mutation in the CFTR gene located on chromosome seven. The DNA isolated from a single embryonic cell was subjected to amplification and then screened for mutations in one of the two copies of chromosome seven. Embryos that had inherited at least one copy of the wild type gene were selected for implantation into the mother. Therefore, the couple could be assured that they would have a child that would not be at risk from cystic fibrosis.

PCR can also be used to detect the presence of unwanted genetic material, as in the case of a bacterial or viral infection. Conventional procedures that involve culturing microorganisms or antibodies use can take weeks to complete. PCR offers a quick and easy alternative. In the diagnosis of AIDS, PCR can be used to detect the small percentage of cells infected with HIV-1.

PCR Requires:
- DNA Template (up to 3kb)
- Forward and Reverse Oligonucleotide Primers (complementary to 3'-flanking sequences)
- dNTPs (basic DNA building blocks, added in excess to prevent re-association)
- Thermostable Taq-polymerase (to extend primers)
- Magnesium Chloride (cofactor to help annealing and extension)

Stages of PCR:
- Denaturation of Target DNA (heat at 95°C to melt DNA strands)
- Annealing of Primers (cool to 55-60°C to allow hybridization)
- Extension (heat to 72°C, polymerase adds dNTPs to primers)

Procedure:
1) Two PCR tubes were taken and marked as control and test.
2) The test tubes contained 1 µl of each primer (forward and reverse), 2 µl template DNA, 15 µl of 2X PCR mixture, and 12 µl molecular grade water.
3) The control tubes contained 1 µl of each primer (forward and reverse), 15 µl of 2X PCR mixture, and 14 µl molecular grade water.
4) The tubes were placed in the thermal cycler and the following set of conditions will be used: 1 cycle of 95^{o}C for 5 min, then 1 min denaturation (95^{o}C), 1 min annealing (55^{o}C), 1 min extension (72^{o}C). These conditions were repeated for a total of 30 times. Following that, a final 10-minute extension step was used to allow the polymerase to completely finish extending all PCR products. The samples was stored at –20^{O}C or immediately run on an agarose gel.

OBSERVATION

To visualize the DNA after staining, place the gel in white light transilluminator.

RESULT
Put the image of the gel.

Experiment 13. SDS-PAGE analysis

Electrophoresis is the process of migration of charged molecules in response to an electric field. The rate of migration depends on the net charge, size and shape of the molecule, the voltage gradient of the electric field E, and the frictional resistance of the supporting medium f, which impedes their movement. Proteins have a net charge at any pH other than their isoelectric point (pI), thus when placed in an electric field, proteins will migrate towards the electrode of the opposite charge. This principle is used to separate molecules of differing charges.

Electrophoresis in acrylamide gels is referred to as Polyacrylamide gel electrophoresis (PAGE). Polyacrylamide gels which were first used for electrophoresis by Raymond &Weintraub (1959) are chemically inert and particularly stable. By chemical copolymerization of acrylamide monomers with cross linking reagent N-N'-methylene bisacrylamide a clear transparent gel which exhibits little endosmosis is obtained.

The polymerization of acrylamide is an example of free radical catalysis and is initiated by the addition of Ammonium per sulfate and a catalyst N,N,N'N'-Tetramethylenediamine(TEMED).TEMED catalyses the decomposition of the persulfate ion to give a free radical

$$S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^-$$

If this free radical is represented as $R^{\cdot}$ (where the dot represents an unpaired electron) and M as an acrylamide monomer molecule than the polymerization can be represented as follows.

$$R^{\cdot} + M \rightarrow RM^{\cdot}$$
$$RM^{\cdot} + M \rightarrow RMM^{\cdot}$$
$$RMM^{\cdot} + M \rightarrow RMMM^{\cdot}$$

In this way long chains of acrylamide are built up being crosslinked by introduction of bisacrylamide forming a mesh like structure in which the holes of the mesh represent the pores. Overall protein mobility through polyacrylamide gel is proportional to the pore size which is a function of both the acrylamide concentration (%T) and that of bisacrylamide crosslinker (%C.). In general the pore size is inversely proportional to %T.

$$\%T = \frac{\text{Acrylamide (g) + Bisacrylamide (g)}}{100\text{ ml}} \times 100\%$$

$$\%C = \frac{\text{Bisacrylamide(g)}}{\text{Acrylamide (g)+Bisacrylamide (g)}} \times 100\%$$

%T gel	Mr range
5-12	20,000-150,000
10-15	10,000-80,000
>15	<15,000

The proteins may be run in denaturating conditions in presence of SDS or in native condition devoid of denaturants called as native- PAGE of proteins.

In native or non-denaturing gel electrophoresis SDS is not used and the proteins retain their native structure and enzymatic activity. Although the resolution is not as high as that of SDS-PAGE but the technique is useful when the enzymatic activity of a protein need to be assayed following electrophoresis. The migration of proteins in non-denaturating gel is due to both the net charge and the size of the protein.

SDS-PAGE is the most commonly used gel electrophoretic system for analyzing proteins. This method is based on the separation of proteins according to size and can also be used to determine the relative molecular mass of proteins. SDS is an anionic detergent which binds strongly to and denatures proteins to produce linear polypeptide chains. On average one SDS molecule will be present for every two aminoacids. The presence of β-mercaptoethanol assists in protein denaturation by reducing all disulfide bonds. The detergent binds to the hydrophobic region of the denatured protein in a constant ratio of about 1.4g of SDS/gm of protein. The protein-SDS complex carries net negative charges, hence move towards the anode and the separation is based on the size of the proteins. Most SDS-PAGE gels are cast with a molar ratio of Bisacrylamide:Acrylamide of 1:29 which has been shown empirically to be capable of resolving polypeptides that differ in size by little as 3%.

The Polyacrylamide gel is cast as a separating gel topped by a stacking gel. The stacking gel has properties that cause the proteins in the sample to be concentrated into a narrow band at the top of the separating gel. This is achieved by utilizing differences in ionic strength and pH between the resolving buffer and the stacking gel and involves a phenomenon known as isotachophoresis. The stacking gel is of high porosity and buffered with Tris-cl buffer at pH 6.8, whereas separating gel contains high percentage of acrylamide and is cast in Tris-cl buffer at pH 8.8. The upper (and lower) electrophoresis buffers contain Tris at pH 8.3 with glycine as counter ion.

Stacking principle: Glycine at pH 6.8 of the stacking gel remain in neutral zwitterionic form with only a fraction 1% in the negative glycinate form. This prevents glycine to be an effective carrier of current. The Cl^- ions remain effective current carriers at pH 6.8 and migrate rapidly towards the anode. The SDS-coated protein molecules and dye which have charge to mass ratio >glycine but less than that of Cl^- must now migrate to carry the electrophoresis current behind the Cl^- and ahead of the glycine. There is only a small quantity of protein-SDS complexes so they concentrate in a thin band sandwiched between the cl- ions and the glycine molecules at the interface between stacking and separating gels.

The higher pH of the separating gel favours ionization of glycine, carrying a higher charge to mass ratio than that of the proteins. Now the newly formed glycinate ions move faster than the proteins with mobility approaching that of the cl^- ions. The negatively charged protein-SDS move according to

their relative mobilities and are separated by the sieving effect of the separating gel according to size. The high mobility of the tracking dye assures that it will migrate faster than the proteins.

Protein resolved in the gel can be stained with either Coomaassie brilliant blue or with silver stain. Silver staining is the most widely used high sensitivity staining method which is reported to be 100 times more sensitve than Coomassie blue with a detection limit about 0.1-1ng of protein. Coomassie blue are electrostatically attracted to charged groups on the protein, forming strong dye:protein complexes that are further augmented by vanderwaals forces, hydrogen bonding and hydrophobic bonding. On the other hand selective reduction of silver ions to metallic silver at gel sites occupied by proteins is the principle of silver staining. It depends on the differences in the oxidation-reduction potentials in the sites occupied by the proteins in comparison with adjacent sites in the gel that do not contain proteins.

METHODOLOGY:

a) Materials Required:

i) Equipments:

1. Electrophoresis apparatus for vertical slab gels with a size of 0.75mm X 10cm X 12cm.
2. Power supply.
3. Micropipette for loading samples

ii) Chemicals/Reagents/Buffers:

1. Stock acrylamide solution: 30g acrylamide, 0.8g bisacrylamide. Make up to 100ml in distilled water and filter through whatman No1 filter and store in amber bottle at 4□c.

(*CARE: Acrylamide monomer is a neurotoxin. Take care in handling acrylamide (wear gloves)and* avoid breathing).

2. Buffers:

a) Separating gel buffer: 1.875M Tris-cl, pH 8.8

b) Stacking gel buffer : 0.6M Tris-Cl. pH 6.8

3. 10% w/v Ammonium persulfate. Make fresh. Store at 4□c. (*Care: Always use in Fume hood)*

4.10% w/v Sodium dodecyl sulfate (SDS) 5.N,N,N’,N’-tetramethylethylenediamine(TEMED)

6. Sample buffer

0.6M Tris-HCl,pH 6.8	5.0ml
10% SDS	0.5g
Sucrose	5.0g
β-mercaptoethanol	0.25ml Bromophenol blue (0.5% stock) 5.0ml Make up to

50ml with distilled water

7. Electrophoresis buffer: Tris (12g), glycine (57.6g), and SDS (2.0g). Make up to 2l with water. No pH adjustment is necessary

8. Protein Stain: 0.1% Coomassie brilliant blue R250 in 50% methanol, 10%glacial acetic acid. Dissolve the dye in the methanol and water component first, and then add the acetic acid. Filter the solution through whatmann filter paper. (*Note:Coomassie brilliant blue is harmful by inhalation or ingestion. Wear appropriate gloves & safety glasses while handling)*

9. Destaining solution: 10% methanol, 7% glacial acetic acid

10. Protein sample

11. Standard Protein molecular weight markers.

iii) Glasswares and others:
Conical flask Beaker
Graduated cylinder

b) Method:

1. Clean the internal surfaces of the gel plates with methylated spirits, dry, and then join the gel plates together to form the cassette, clamp it in a vertical position.

2. In an Erlenmeyer flask or disposable plastic tube, prepare the separating gel by mixing the following:

(NOTE1)

	For15% gels	For 10%gels
1.875M tris-HCl, pH8.8	8.0ml	8.0ml
Water	11.4ml	18.1ml
Stock acrylamide	20.0ml	13.3ml
10%SDS	0.4ml	0.4ml
Ammonium persulfate (10%)	0.2ml	0.2ml

3. Degas this solution under vacuum for about 30sec. *(NOTE2)*

4. Add 14µl of TEMED and gently swirl the flask to ensure even mixing.

5. Using a Pasteur pipette transfer this separating gel mixture to the gel cassette carefully down one edge. Continue adding this solution until it reaches a position 1cm from the bottom of the comb that will form the loading wells.

6. To ensure that the gel sets with a smooth surface very carefully run distilled water down one edge into the cassette using a Pasteur pipette.

7. While the separating gel is setting prepare the 4% stacking gel solution. Mix the following in a 100ml Erlenmeyer flask or disposable plastic tube.

0.6M Tris-Hcl, pH6.8	1.0ml
Stock acrylamide	1.35ml
Water	7.5ml
10%SDS	0.1ml

Ammonium persulfate (10%) 0.05ml Degas this solution under vacuum for about 30 sec

8. When the separating gel has set, pour off the overlaying water. Add 14 µl of TEMED to the stacking gel. Pour the stacking gel solution directly onto the surface of the polymerized resolving gel. Immediately insert a clean Teflon comb into the stacking gel solution, being careful to avoid trapping of air bubbles. Place the gel in a vertical position at room temperature and allow to set for 20min.

Preparation of samples and running the gel:

9. About 10µl of protein sample and 5µl of sample buffer are mixed by vortexing. The sample is than heated for 5min at 95-100°C to denature the proteins. The sample is than kept in ice *(Note3)*

10. After polymerization is complete, remove the Teflon comb. Rinse out any unpolymerised acrylamide solution from the wells using electrophoresis buffer and assemble the cassette in the electrophoresis tank. Add Tris-glycine electrophoresis buffer to the top and bottom reservoirs. (*Note: Donot prerun the gel before loading the samples, since this procedure will destroy the discontinuity of buffer system.)*

11. Load up to 5-10µl of each of the samples (unknown and standard) in a predetermined order into the wells.

12. Connect the electrophoresis apparatus to the power pack (the positive electrode should be connected to the bottom buffer reservoir), and pass a current of 30mA through the gel (constant current) for large format gels, or 200V (constant voltage) for minigels (Biorad). The gel is run until the bromophenol blue reaches the bottom of the resolving gel. This will take 2.5-3.0h for large format gels (16µm x 16µm) and about 40min for minigels (10µm x7µm) *(Safety care: Always turnoff & disconnect the power supply before removing the lid)*

13. Dismantle the gel apparatus, pry open the gel plates; remove the gel, discard the stacking gel, and place the separating gel in stain solution.

14. Staining should be carried out with shaking, for a minimum of 2h at room temperature. Destain the gel by soaking it in the methanol:acetic acid solution on a slowly rocking platform for 4-8 hrs.

15. After destaining, store the gels in H_2O containing 20% glycerol

16. The gel can now be used for immunoblotting to determine the protein sample

NOTE:

1. Typically15% polyacrylamide gels are used for separating proteins of molecular mass in the range of 100,000-10,000 kd.However,a protein of 150,000for example would be unable to enter a 15% gel.In this case, a large pored gel(eg a 10% or 7.5% gel) would be used.

2. Degassing helps prevent oxygen in the solution “mopping up” free radicals and inhibiting polymerization.

3. □-mercaptoethanol is essential for disrupting disulphide bridges in proteins. However exposure to air decreases the reducing power of □-mercaptoethanol. Thus it should be prepared fresh.

4. Destain solution needs to be replaced at regular intervals since a simple equilibrium is quickly set up between the concentration of stain in the gel and destain solution after which no further destaining

takes place.

5. It is generally accepted that a very faint protein band detected by Coomassie brilliant blue, is equivalent to about 0.1µg(100ng) of protein.

6. Data Analysis Label each lane on the photograph of your gel: The molecular weight of the unknown protein can be determined by running calibration proteins of known molecular weight on the same gel run as the unknown protein.

A standard curve is constructed that plots relative mobility (Rf) versus Log mol weight

R_f =(distance migrated by protein/distance migrated by tracking dye)

The R_f of the standard protein is calculated and plotted on the graph. The R_f value of the unknown protein is calculated by measuring the distance each protein band migrated (Measure from the bottom of the well to the middle of each band) and the distance the tracking dye migrated in each lane. Using the standard curve, the molecular weight of the unknown protein is determined.