

G.T.N. Arts College

(Autonomous)

**Re-accredited by NAAC with 'A++'
Grade in the 2nd Cycle
Dindigul – 624005**

DEPARTMENT OF ZOOLOGY

Under the aegis of DBT Star College Scheme

LAB MANUAL

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Core Practical – II (Principles of Genetics, Molecular Biology and Evolutionary Biology)	20UZOC4P	Mrs. B. Subasri, Dr. S. Dharaneedharan, Dr. N. Renuga Devi
Core Practical –IV (Animal Physiology and Microbiology)	20UZOC6Q	Dr. N. Renuga Devi, Dr. A. Jeevalatha
Core Practical – V (Biotechnology, Immunology and Immunotechnology)	20UZOC6R	Dr. S. Dharaneedharan, Dr. N. Renuga Devi

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Exp. No:1

Study of Mitosis in Onion root tip

Date:

Introduction :

Mitosis is equational cell division. It occurs in all meristematic cells of plants. Root tips are the most actively growing organ and is ideal to study mitosis. Roots consist of different regions. The root cap functions in protection. The apical meristem is the region that contains the highest percentage of cells undergoing mitosis. The region of elongation is the area in which growth occurs. The region of maturation is where root hairs develop and where cells differentiate to become xylem, phloem, and other tissues.

Materials:

Freshly grown onion root tips, Stain Aceto-carmin, 1-M HCl, Fixative - Acetic acid:Alcohol (1:3), Slides and coverslips, Bunsen burner, compound light microscope, Paper towel

Protocol:

Fix the onion root tips in the fixative for 24 hrs.

Remove tips from fixative, wash with distilled water and keep in 1N HCl at 50°C for 10 min. Wash thoroughly with distilled water.

Take the tip on the slide and put 1-2 drops of acetocarmine stain. Warm gently. Repeat the process a few times to allow the stain to permeate the tissues.

Gently place a coverslip on the tip. Tap gently using back, flat side of the pencil between folds of filter paper to allow spread of cells in a single layer.

Observe under 10x and 45X of the microscope to visualize the chromosomes and various stages.

Identify the different stages and record in the journal with diagrams.

Observations:

The non-dividing cell is in a stage called interphase. The nucleus may have one or more dark-stained nucleoli and is filled with a fine network of threads, the chromatin. During interphase, DNA replication occurs.

The first signs of a division is Prophase, in which a thickening of the chromatin threads occurs. Thickening continues until it is evident that the chromatin has condensed into chromosomes. With higher magnification you may be able to see that each chromosome is composed of two chromatids joined at a centromere. As prophase continues, the chromatids continue to shorten and thicken. In late prophase the nuclear envelope and nucleoli are no longer visible, and the chromosomes are free in the cytoplasm. Just before this time the first sign of a spindle appears in the cytoplasm; the spindle apparatus is made up of microtubules, and it is thought that these microtubules may pull the chromosomes toward the poles of the cell where the two

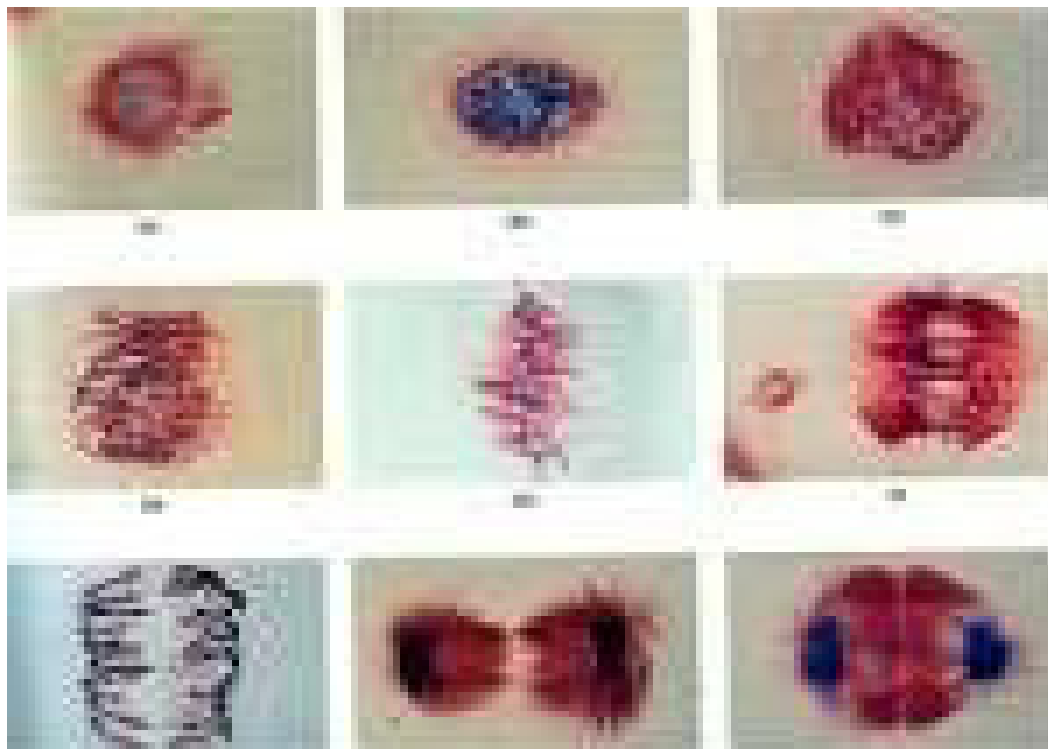
daughter nuclei will eventually form.

At Metaphase, the chromosomes have moved to the center of the spindle. One particular portion of each chromosome, the centromere, attaches to the spindle. The centromeres of all the chromosomes lie at about the same level of the spindle, on an imaginary plane called the metaphase plate. At metaphase you should be able to observe the two chromatids of some chromosomes.

At the beginning of Anaphase, the centromere regions of each pair of chromatids separate and are moved by the spindle fibers toward opposite poles of the spindle, dragging the rest of the chromatid behind them. Once the two chromatids separate, each is called a chromosome. The daughter chromosomes continue poleward movement until they form two compact clumps, one at each spindle pole.

Telophase, the last stage of division, is marked by a pronounced condensation of the chromosomes, followed by the formation of a new nuclear envelope around each group of chromosomes. The chromosomes gradually uncoil to form the fine chromatin network of interphase, and the nucleoli and nuclear envelope appear.

Cytokinesis may occur. This is the division of the cytoplasm into two cells. In plants, a new cell wall is laid down between the daughter cells. In animal cells, the old cell will pinch off in the middle along a cleavage furrow to form two new daughter cells.



Aim:

To visualize the structure of polytene phase chromosome within a nucleus under light microscope.

Materials Required:

Chemicals and glasswares essential to prepare and study the polytene chromosomes :

1. Glacial Acetic Acid
2. Ethyl alcohol (Absolute)
3. Orcein stain
4. Glass slides
5. Cover glasses
6. Fine forceps
7. Fine needle
8. Watch glass
9. Sealing material (Nail polish or paraffin)
10. Dissecting binocular
11. Chironomus larvae

Reagent Preparation

Fixative preparation : Fixative is kind of chemicals which fixes and preserves the cells or animals itself against decomposition and autolysis. In this experiment Aceto- alcohol will act as a fixative. It is prepared by mixing equal volume of glacial acetic acid and absolute ethyl alcohol at the ratio 1:3.

Stain preparation : 02 grams of Orcein powder is dissolved in 100 ml of glacial acetic acid (Remember the orcein powder is a very costly item and do not waste it.)

Protocols

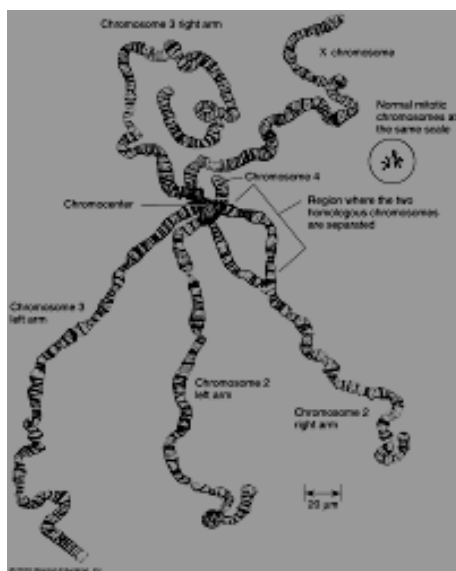
1. The small head is brownish in colour with simple eyes and movable mouthparts. The tail region is characterized by the presence of tufty gills.
2. Hold the bloodworm firmly at a short distance behind the head with a pair of extra fine forceps under a dissecting binocular.
3. Then, with a downward and forward movement of another pair of forceps, pull the head end and separate it from the rest of the body. The salivary gland will generally drawn out completely with the head and can easily be recognized as two flat and whitish structures,

attached to the anterior of the gut, with large cells and prominent nuclei.

4. After isolation of salivary glands add few drops aceto-alcohol(fixative) for 15-20 minutes.
5. Add a drop of aceto-orcein stain on the slide. Leave the gland in the acetoorcein stain for about 30 minutes. If the gland begins to dry up, add another drop of stain.
6. Place a cover slip over the gland in the drop of stain. Lay a paper towel on the desk, turn the slide over, and lay it face down on the towel. Press firmly on the back of the slide with the tip of the finger, so as to blot thoroughly.
7. Place the slide on the desk with cover slip on top, hold the edge of the slide steadily with one hand, and press on the cover slip intermittently with the wooden handle of a dissecting needle.
8. Seal with proper sealing material (Nail-polish).
9. Observation of the giant chromosomes under the microscope.

Salient features of Polytene Chromosome

1. The large size of the chromosome is due to the presence of many longitudinal strands called chromonemata; hence the name polytene (many stranded).
2. They are about 0.5 mm in length and 20 μ m in diameter.
3. The chromosomal strands are formed after repeated division of the chromosome in the absence of cytoplasmic division. This type of division is called endo mitosis.
4. The polytene chromosome contains two types of bands, dark bands and inter bands
5. The bands of polytene chromosomes become enlarge at certain times to form swellings called puffs.
6. The puffs indicate the site of active genes where mRNA synthesis takes place.



Exp. No.:3.

Tissue Preparation for microscopic observation

Date:

Introduction:

Embedding tissue into paraffin blocks supports the tissue structure and enables very thin sections to be cut and mounted onto microscope slides for analysis.

Materials and Reagents:

Water-bath, Container with ice, Glass microscope slides, Microtome and blade, Oven

Procedure:

1. Sectioning: Chill paraffin-embedded tissue blocks on ice before sectioning. Cold wax allows thinner sections to be obtained by providing support for harder elements within the tissue specimen. The small amount of moisture that penetrates the block from the melting ice will also make the tissue easier to cut.
2. Fill a water bath with ultrapure water and heat to 40 - 45°C.
3. Place the blade in the holder, ensure it is secure and set the clearance angle. The clearance angle prevents contact between the knife facet and the face of the block. Follow the microtome manufacturer's instructions for guidance on setting the clearance angle. For Leica blades this is normally between 1° and 5°
4. Insert the paraffin block and orientate so the blade will cut straight across the block.
5. Carefully, approach the block with the blade and cut a few thin sections to ensure the positioning is correct. Adjust if necessary.
6. Trim the block to expose the tissue surface to a level where a representative section can be cut. Trimming is normally done at a thickness of 10-30 µm.
7. Cut sections at a thickness of about 4-5 µm (you will probably need to discard the first few sections as they are likely to contain holes caused by trimming).
8. Using tweezers pick up the ribbons of sections and float them on the surface of the water in the water bath so they flatten out. Use the tweezers to separate the sections.
9. Use microscope slides to pick the sections out of the water bath and store upright in a slide rack.

Place the slide rack into an oven and allow sections to dry overnight at 37°C.

Exp. No.: 4.

Developmental Stages in Chick embryo

Date:

AIM:

Preparation of permanent stained different stages of chick embryo.

REQUIREMENTS:

Egg fertilized having stage, needles, fixatives, grades of alcohol, DPX, glass slide, cover slip, microscope, dye.

PROCEDURE:

1. Take an egg and put in on side undisturbed for 2-3 minutes.
2. Note the blunt end of the egg and make a hole with needle.
3. Again put the egg undisturbed for 15 min so that stage of egg becomes stable on upper side.
4. Make a circle on a egg shell with pencil and then make window by removing the egg shell with forceps.
5. Note the stage of egg and put 2-3 drops of carnoys fixative on the material of egg to fix the stage. Take the fixed stage with the help of forceps and put it in 90% alcohol.
6. Follow the degrading in 90% -70%-50%-30% alcohols for 5 min each.
7. Then put it into dye stain (carmine) for 30 sec and do upgrading 30%-50%-70%-90%.
8. After that mount it in DPX and cover it by cover slip.

Exp. No.: 5.

Study of Mendelian Traits among Class Students.

AIM:

To make a survey for occurrence of certain simple Mendelian traits among class population.

CHARACTERISTICS OF TRAITS:

1. ROLLERS AND NON ROLLERS

The ability to roll the tongue is a dominant trait.

Inability to roll the tongue is a recessive trait.

2. FOLDERS AND NON FOLDERS

The ability to fold the tongue up wards. This is a dominant trait.

Inability to fold the tongue is a recessive trait

3. WINBOW'S PEAK

At the centres of head hair line may dip, down to form a peak which is called winbow's speak.

The trait is inherited as dominant trait is some families.

4. FREE OR ATTACHED FEAR LOBE

Tree or normal ear lobe in man is dominant trait.

Attached or fused ear lobe is recessive trait.

5. CLASPING OF HANDS

Right thumb over left is Dominate trait

Lift thumb over right is recessive trait

6. HITCHHIKER'S THUMB

Ability to bend the thumb backward's is dominant trait.

Inability to bend thumb backwards is recessive trait.

RESULT :

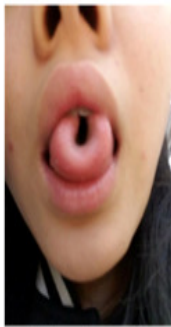
1. TONGUE ROLLING	PRESENCE :	ABSENT :
2. TONGUE FOLDING	PRESENCE :	ABSENT :
3. PRESENCE OR ABSENCE OF WINBOW'S PEAK	PRESENCE :	ABSENT :
4. EAR LOBE	FREE :	ATTACHED :
5. CLASPING OF HANDS	RIGHT OVER LEFT :	ATTACHED :

6. HITCHIKER'S THUMB

PRESENT :

ABSENT

Dominant



Able to roll



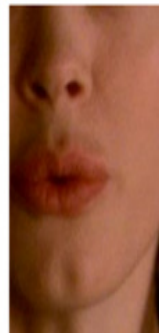
Freckles



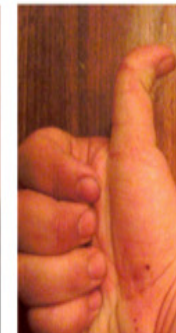
Widow's peak



Free hanging

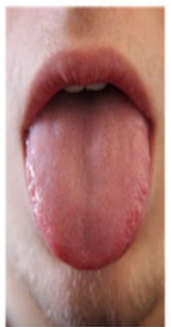


Cleft chin

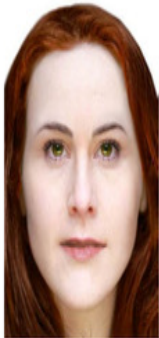


Hitch hikers

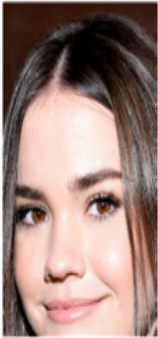
Recessive



Unable to roll



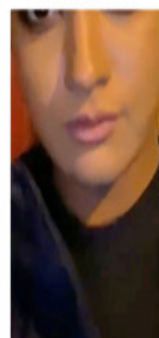
No freckles



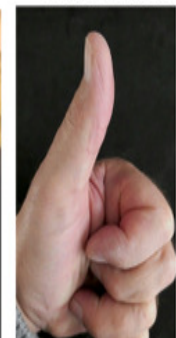
Straight



Attached



Smooth chin



Straight

:

Exp. No.: 6.

Human Karyotype

AIM :

To make an observation of Human karyotype.

PRINCIPLE :

Karyotype is a composite picture of set of chromosome in the mitotic metaphase of an of specially prepared cells and then cutting out of chromosome, matching them and arranging them in descending order of Length.

MATERIALS REQUIRED :

A normal female photomicrograph of chromosome and scissors.

PROCEDURE :

To prepare human karyotype from the culture of blood cells treated with colchine were photomicrographed. The application of colchine was to permanently arrest the cell division at metaphase. A photomicrograph spread out was normal female. The chromosome is nuclear of blood cells were taken to make karyotype. The chromosome were cut out of photomicrograph and paired morphologically arranged in series of decreasing in length.

The chromosomes were arranged according to size and position of centromere. The karyotype was prepared and it is shown in the sketch.

OBSERVATION:

According to the positions of centromere three distinct types of chromosomes are observed. They are given below.

METACENTRIC CHROMOSOME:

The position of the centromere is shown in medium. This gives the chromosome a 'v' shaped configuration.

SUB METACENTRIC CHROMOSOME:

The position of the centromere is terminal and is very close to one end of the chromosome. So that one arm is minute and other is much longer.

A normal human karyotype includes 22 matching pairs of chromosome and is called as autosomes. The autosomes are numbered from 1 to 22 in descending order of length. A part from autosome the female has 2X chromosome of equal size where as male has one 'x' chromosome and unequal sized 'y' chromosome. These sex chromosomes are not numbered. The haploid set found in sperm and egg cell consists of 22 autosomes plus one of the sex chromosome.

When the chromosomes are morphologically paired and are arranged based on their shape and size 7 distinct groups are reorganized these groups have been denoted by letters A, B, C, D, E, F, G.

CHARACTERISTICS OF DIFFERENT GROUPS OF CHROMOSOMES IN HUMAN KARYOTYPES:

GROUP A:

It includes 3 pairs of chromosomes. They are the longest pairs of chromosome. The 1st pair is metacentric having secondary constriction 2nd pair is submetacentric and 3rd pair is metacentric. They are closely distinguished from one another on basis of length and position.

GROUP B:

It includes 4th and 5th pair of chromosomes. They are long but shorter than group A chromosomes. The chromosomes are sub medium. They are distinguished from one another the chromosome is slightly longer than chromosome 5th.

GROUP C:

This group has more number of chromosomes. They include 6 to 12 pairs of chromosomes. The chromosomes of this group are sub metacentric. The 10th chromosome has secondary constriction.

GROUP D:

The chromosome 13 to 15 are included in this group. The chromosomes are acrocentric because position of centromere is terminal. All are denoted as SAT chromosomes. Since satellite is present at shorter of all chromosomes. The 13th chromosome has secondary constriction also.

GROUP E:

This group includes chromosome 16 to 18. 16th is metacentric, 17th and 18th is submetacentric. The 16th pair has secondary constriction. These chromosomes are shorter than group D.

GROUP F:

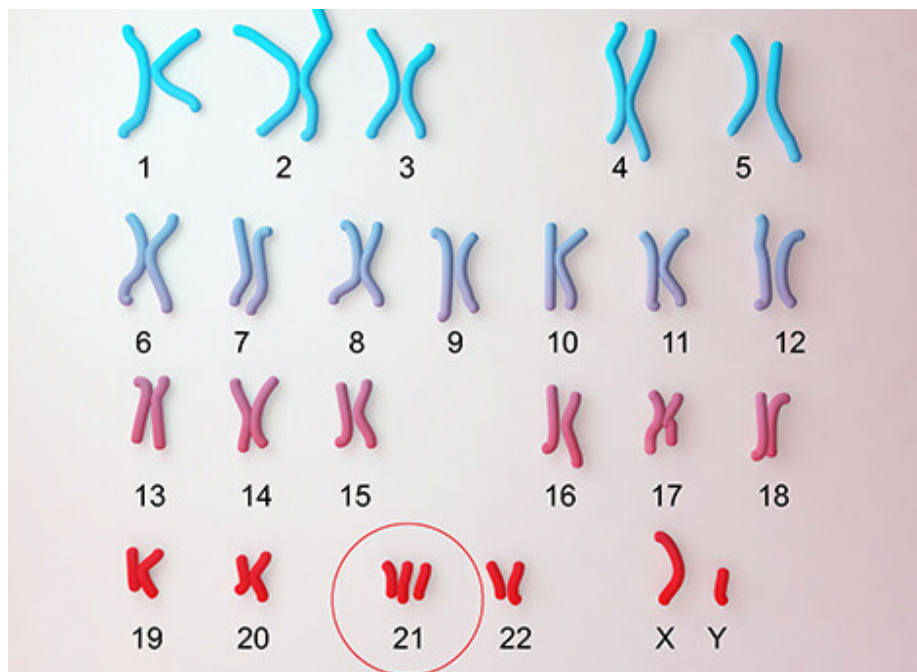
This group includes the chromosome 19 to 20. They are shorter. The centromeres are mainly terminal and chromosomes are metacentric. A satellite is present at short arm of each chromosome. They have nucleolar organizer also.

GROUP G:

'X' and 'Y' chromosomes are included in this group and can be distinguished from 21 and 22 due to greater variability in appearance.

CONCLUSION:

A human karyotype shows metacentric sub metacentric and acrocentric chromosomes. When they are arranged descending order, the normal human karyotype includes 22 matching pairs of autosome plus 2X chromosome is female or One 'X' and 'Y' chromosome in male.



Exp. No.: 7.

Human Chromosomal Abnormalities

AIM:

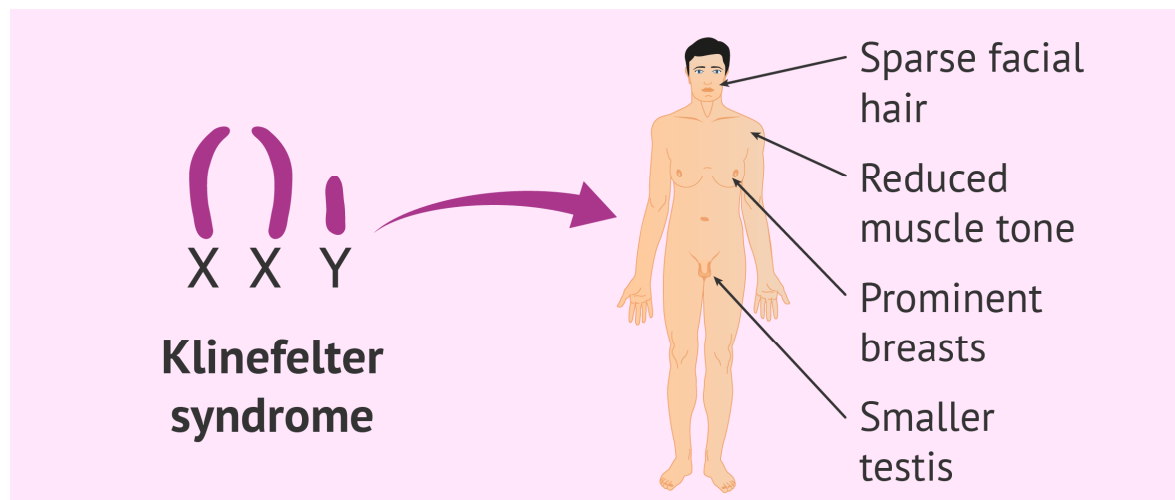
To make an observation of karyotype of human sex chromosomal syndromes.

PRINCIPLE:

Any change on chromosome number will lead to abnormality called syndrome. This is due to addition of sex chromosomes, which is known to be a kind of aneuploidy. There are many sex chromosomal abnormalities and out of them klinefelter's syndrome and turner's syndrome are more common.

KLINFELTER'S SYNDROME:

Klinefelter's syndrome is due to sex chromosomal abnormality in male. It occurs at high as one in 506 births. It is named by Harry F. Klinefelter in 1941. The individual with this syndrome is phenotypically male. But their testes are underdeveloped. The breasts are larger and legs are also longer than average body hair is sparse. They have highly pitched voice. Most patients are sterile mentally defective. The cells of males in Klinefelter's syndrome may have 47 chromosomes instead of 46. Thus this sterile male possesses XXY sex chromosome the possession of Y despite of two X chromosomes the possession of Y enables the patient to have masculine characters. As in mongolism affected one is born more after to older women. This abnormality is due to non-disjunction under abnormal condition older women produce an XX egg on an egg devoid of X chromosome. If an XY egg is fertilized by Y bearing sperm Klinefelter's syndrome results.

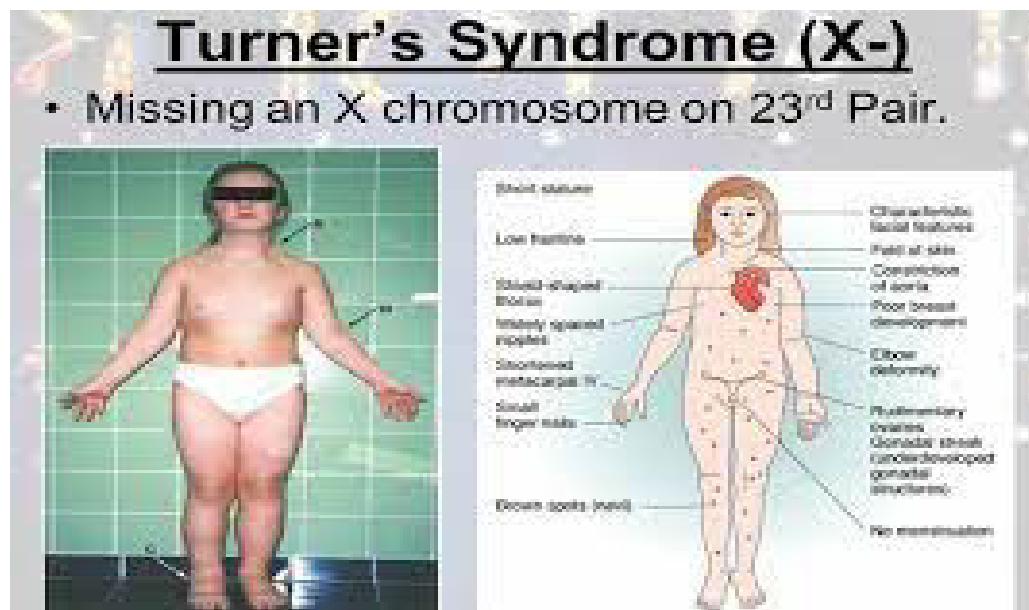


TURNER'S SYNDROME:

The Turner's syndrome is a sex chromosomal abnormality in Female. It was first noticed by Dr.Hendry and H.Turner. Woman with Turner's syndrome has rudimentary ovaries and under development anatomically and physiologically they are female although unable to menstruate and ovulate. Instead of normal ovaries only ridges of whitish tissue occur. i.e. whey many author's use the term "gonadal disgenesis" in place of Turner's syndrome.

The cells of female with Turner's syndrome have 45 chromosomes. Instead of 46. Here there is only one X chromosome instead of two. The absence of one X is due to dis function of sex chromosome during formation of egg by mother.

The mother produces either an XX egg on egg devoid of X chromosome. If the egg which has no chromosome is fertilized by normal X bearing sperm an individual with Turner's syndrome is produced. This single X chromosome woman is sterile and she is under developed female. The incidence of Turner's syndrome is one in 25,000 births.



Exp. No.: 8.

HUMAN BUCCAL SMEAR

AIM:

To observe the human buccal epithelial cells.

MATERIALS REQUIRED:

Glass slide, Cover glass, Physiological saline, Methylene blue stain, Cotton glycerine

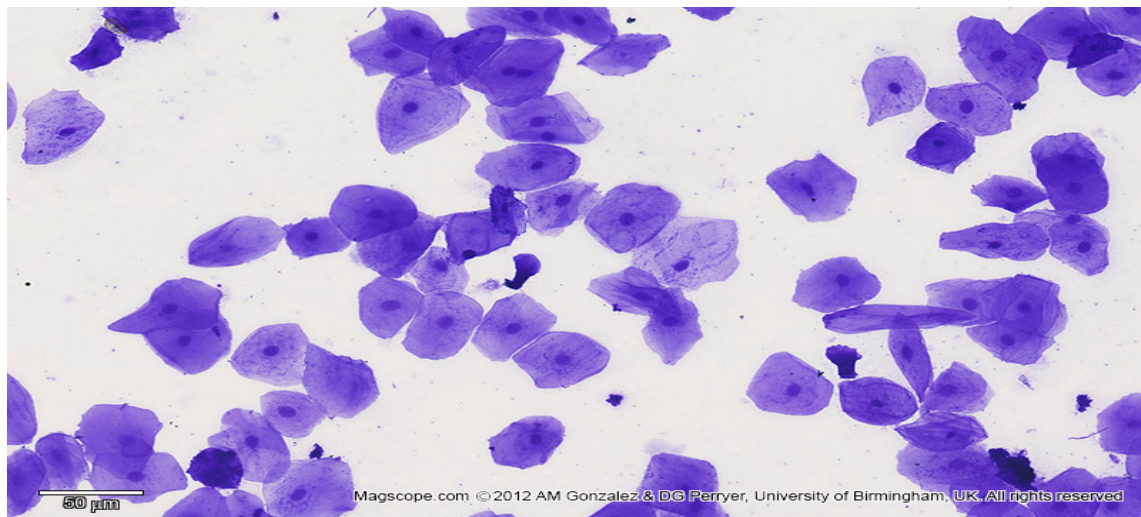
PROCEDURE:

Buccal epithelial cells were collected from inner portion of the cheek by scraping with the help of cotton bud. It was smeared on clean glass slide. A few drop of 0.9% physiological saline was added. After 2 minutes a few drop of methylene blue stain was added. After 5 minutes they are washed in distilled water. The smear was mounted with cover slip using Glycerine. Then the smear was observed and buccal epithelial cells was measured using micrometry procedure under compound microscope.

OBSERVATION:

Rectangular or polygonal shaped nucleated buccal epithelial cells were observed.

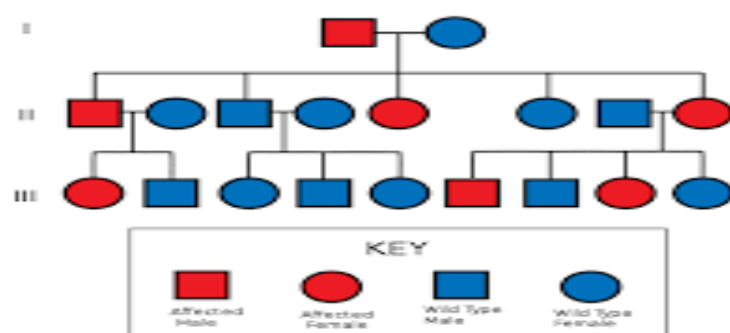
The diameter of one buccal epithelial cell in lower power is 0.4 mm μ



Exp. No.: 9.

PEDIGREE ANALYSIS

1. A pedigree is a systematic listing of the parents and grandparents of a given individual. It is the family tree for a large number of individuals.
2. A pedigree host contains different types of symbols to indicate different types of individuals and they are as follows
3. Normal male members of a family are shown by squares and normal females by circles.
4. Mating is represented by a square and a circle connected by horizontal line.
5. Parents and their children are represented by children connecting with mating line of parents.
6. Identical twins are indicated by the children connected by an inverted 'V' line and horizontal line.
7. Non-identical twins are indicated by children connected by an inverted 'V' line.
8. Affected males are represented by closed squares and females by closed circles.
9. Heterozygous males are represented by half closed squares and females by closed circles.
10. Carrier of sex linked recessive is represented by circles with dot inside.
11. For uncertain phenotype of individual, a question mark is placed in symbol.
12. Death of an individual is represented by crossing square or circle.
13. Different shades of colours added to the symbols can represent various phenotypes.
14. Each generation is listed on spiracle row.
15. From the family tree given below. It is possible to find out the phenotype and genotype of three generations and they are as shown in table in the world.
16. A pedigree chart for inheritance of haemophilia in family is given in figure. This chart gives the phenotypes of individuals in three generations and it clearly indicates that the Haemophilia is a sex linked recessive characters and females are mostly carrier of this disease.



Exp. No.: 10.

ABO BLOOD GROUPING

AIM:

To determine the blood group of ABO and Rh blood group system

MATERIALS REQUIRED:

Glass slide, Spirit, Cotton, Disposable needle, Applicator Sticks, Anti-serum A,B,O, Hand lens and Microscope.

PROCEDURE:

A glass side is cleaned thoroughly. A drop of antiserum 'A' is placed on left top of the slide and another drop of antiserum 'B' is placed on right top of slide. The tip of the index finger is cleaned with a spirit and picked with help of needle. The blood drops are placed near the anti-sera, the blood and the anti-sera. The blood is observed for agglutination.

The blood is observed for agglutination.

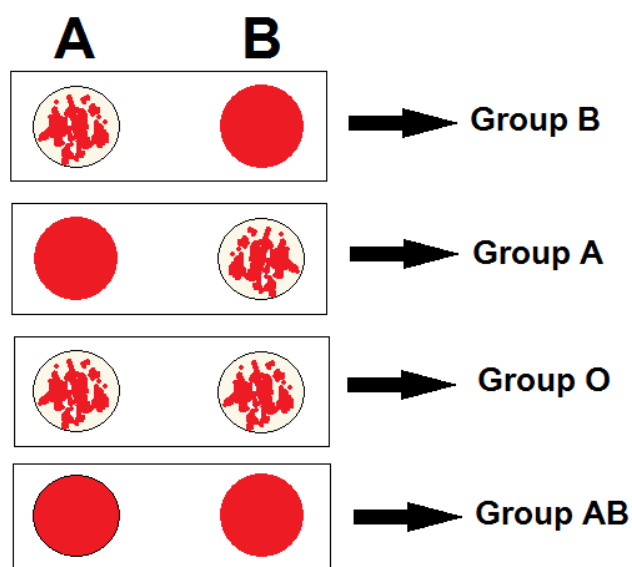
=>When there is agglutination with antiserum 'A' alone the blood tested belongs to groups 'A'

=>When agglutination occurs with antiserum 'B' alone the blood tested belongs to groups 'B'

=> When there is agglutination in both antisera the blood group belongs to group 'AB'

=> When there is no agglutination in both antisera, the blood group belongs to group 'O'

In another cleaned glass slide a drop of antiserum 'D' is placed on centre. The blood drops are placed near the antisera. The blood and antisera are mixed with the help of stick. When there is an agglutination with anti-sera 'D' the blood belongs to (Rh +ve). If there is no agglutination the blood belongs to (Rh -ve)-ve group.



Exp. No.: 11.

Aseptic techniques and Media preparation

Date:

Aim:

To acquire the knowledge on aseptic techniques and develop the skill of media preparation for culturing the microbes

Principle:

Aseptic techniques are a fundamental and important laboratory skill in the field of microbiology. Microbiologists use aseptic techniques for a variety of procedures such as transferring cultures, inoculating media, isolation of pure cultures, and for performing microbiological tests. Proper aseptic technique prevents contamination of cultures from foreign microbes inherent in the environment. For example, airborne microorganisms (including fungal spores), microbes picked up from the researcher's body, the lab bench-top or other surfaces, microbes found in dust, as well as microbes found on unsterilized glassware and equipment, etc. may potentially contaminate cultures, thus interfering with the lab results. Using proper aseptic technique can greatly minimize or even eliminate the risk of microbial contamination. In addition, aseptic technique is of utmost importance to maintain pure stock cultures while transferring cultures to new media. Aseptic technique is also essential for isolation of a single species of microorganism from a mixed culture to obtain a pure culture. Furthermore, proper aseptic technique prevents microbes used in the laboratory from accidentally being released into the environment and/ or infecting people working in the laboratory. This is especially relevant when pathogens are being handled.

As microorganisms are ubiquitous, it is always safe to assume that any environment we are in is filled with them. Before starting today's exercise let us acquaint ourselves with certain important aspects of asepsis.

Work Area Disinfection: Before starting the experiment, the work bench surface must be sprayed with 70% ethanol to disinfect the place. It must be rubbed down into all regions. The mistake of wiping the work bench with towels or tissues to spread the ethanol will only introduce new microbes. Therefore, the best thing to do is to let the volatile ethanol evaporate on its own.

Transfer Instruments - Loops and Needles: Wire loops and needles are made out of inert materials such as platinum. They are extremely durable and can be sterilized by incineration. To do this, place the loop in the blue part of the Bunsen burner flame as it is the hottest and allow the loop to get red hot and then remove. Cool the transfer instrument down before inserting into culture. This procedure has to be carried out prior to any inoculation and after any inoculation.

Culture Tube Flaming: Prior to inserting the loop into the culture tube, remove the lid of the tube and flame the tip of the tube so as to avoid entry of any air contaminants. This has to be carried out before and after insertion of loop into tube culture.

General Sterilization:

Sterilization is the process of destroying all forms of microbial life-vegetative and sporulation. It is important that all equipment used in a microbiological experiment is sterilized in order that a particular organism of interest is grown, without contamination from the surrounding environment.

Several methods are employed in the process of sterilization to sterilize the various equipments used in an experiment. These methods are broadly classified as PHYSICAL and CHEMICAL methods:

I) PHYSICAL AGENTS:

The major physical agents used for the control of microorganisms are TEMPERATURE, RADIATION, and FILTRATION.

TEMPERATURE: Microorganisms can grow over a range of temperatures, from very low

temperatures characteristics of psychrophiles, to very high temperatures characteristics of thermophiles. Temperatures above a maximum generally kill microbes; sterilization can be achieved by high temperatures as DRY HEAT or MOIST HEAT.

DRY HEAT STERILIZATION: Dry heat sterilization is achieved in hot air oven. Laboratory items like petriplates, Erlenmeyer flasks, pipettes, test tubes, dry powders, glycerol and other oils etc. are sterilized in hot air oven. Such material can be sterilized by placing in hot air, at a temperature of 160°C for 120 mins.

Alternatively, equipment like forceps, inoculation needle etc. can be sterilized by direct heating on a flame till red hot. Thus it brings about destruction of unwanted organisms without changing the nature (flavor) of the material. This process involves heating at that temperature for 15 mins and then cooling it quickly to 0-5°C.

PRINCIPLE INVOLVED IN DRY HEAT STERILIZATION: Dry heat oxidizes chemical components of organisms and thus destroying them.

MOIST HEAT STERILIZATION: High temperatures combined with high moisture are one of the most effective ways of sterilization.

PRINCIPLES INVOLVED IN MOIST HEAT STERILIZATION: Moist heat coagulates the microbial proteins, and is hence more rapid in killing microbes.

Moist heat can be applied in the following ways in order to bring about sterility:

STEAM UNDER PRESSURE: Provides temperatures higher than those obtainable by any coagulation of proteins. Autoclave is a device used in the laboratory to sterilize media solution and to kill discarded cultures. It is operated at 151lbs/sq. inch pressure, which yields a temperature of 121°C effective in bringing about sterility in 15 min.

FRACTIONAL STERILIZATION OR TYNDALLISATION: Some microbial solutions cannot be heated over 100°C without being damaged. Such materials are sterilized by tyndallisation, which involves heating at 100°C on three successive days with incubation periods in between. Resistant spores germinate during the period of incubation that is killed on heating the subsequent day.

PASTEURISATION: Milk, cream and other alcohol beverages are subjected to controlled heat treatment which kills microbes of a certain type alone.

RADIATION: When ionizing radiation pass through cells, they create free hydrogen radicals, hydroxyl radicals and peroxides that cause intracellular damage, resulting in destruction of microbes. This method of sterilization is effective for sterilizing heat labile materials.

UV light is the most effective region of the electromagnetic spectrum, and is employed in disinfecting of inoculation chamber and hospital operating rooms. UV light alters nucleic acids and results in a pyrimidine dimer, thus inhibiting DNA replication.

FILTRATION: This technique is used when the material to be used is heat labile and cannot be sterilized by heating for e.g. Solutions of proteins, vitamins etc. Filters of pore size 0.03µ- 0.08µ are used to filter off microbes, thus rendering the filtrate sterile. Pore size, electric charge of filter, charge carried by the organisms and nature of fluid being filtered affect efficiency of filtration. E.g. Acety filters, Berkefeld filter, membrane filter are all microbial filters.

II. CHEMICAL AGENTS:

Ethyl Alcohol (70%) finds indispensable use for disinfecting hands before and after a microbiological experiment, and also to disinfect the inoculation chamber or area where the experiment is conducted.

Several groups of chemicals can be used as antimicrobial agents:

S.No.	Group	Action	Example
1.	Alcohol	Denature proteins and solubilize Lipids	Ethanol, Glutaraldehyde
2.	Aldehyde	Alkylate, reacts with-NH ₂ ,SH-	I ₂
3.	Halogens	COOH	Cl ₂
4	Heavy Metals	I ₂ inactivates proteins oxidize cells.	HgCl ₂
5	Gases	Precipitates and inactivates proteins (used for surface sterilization)	Ethylene Dioxide
6	Detergents	Alkylates organic compounds; Disrupt cell membrane	
7	Phenols	Denature protein & disrupt cell Membrane	

Media Preparation

To prepare the different culture media for growing microorganisms and to learn method for maintaining bacteria in laboratory for long time

GENERAL PURPOSE OF MEDIA PREPARATION

Compare the different types of media.

Describe the different formats of media, plate, tube, etc.

Explain how to sterilize it, and how to distribute it in different formats.

Produce Nutrient agar (NA) plates, NA slants, and Nutrient broths (NB) which will be used in subsequent lab periods.

Know the laboratory equipment and culture media needed to develop and maintain pure cultures.

Carry out aseptic technique for the removal and transfer of microorganisms for culturing.

Correctly sterilize and flame transfer instruments and tubes.

Properly incubate culture media at the appropriate temperature for the organism and the length of incubation.

PROCEDURE

A clean Erlenmeyer flask was taken with 200 ml of distilled water. Appropriate quantities of chemicals are weighed accurately and dissolved one by one taking care to add the next chemical only after the dissolution of the first one, except agar. The total volume of the medium was made up to 1 litre. The medium is then distributed into 250 ml. Erlenmeyer flasks of 100 ml each and were plugged tightly with non-absorbent cotton and wrapped with a paper and tied with thread. Then the flasks were sterilized at 121°C for 15 minutes with 15 lbs/in in an autoclave.

ENRICHED MEDIA

Enriched media are media which allow fastidious organism to grow because of the presence of specific nutrient additives such as blood, serum, hemin, etc. An example of this category is the blood agar and chocolate agar.

Blood agar was prepared by adding 5% of sheep blood or blood from other sources to precooled nutrient agar. It is also a differential medium to differentiate hemolytic and non-hemolytic colonies. It is widely used to isolate *Streptococcus* spp. from clinical specimens.

Chocolate agar is prepared by slightly heating the media in a water bath to about 65- 70°C.

Bright red color of blood agar will change to chocolate color due to the oxidation of haem to hemin. It is generally used to isolate fastidious pathogens like *Neisseria* and *Haemophilus*.

DIFFERENTIAL MEDIA

Differential media are media that aid in the presumptive identification of bacteria based on the organisms appearance in the medium. Common example of differential media is MacConkey agar. MacConkey agar is used to differentiate lactose fermenting gram negative bacteria from lactose non-fermenting bacteria.

MacConkey is a differential and selective medium. The nutritive base included the peptone. The medium is made differential by incorporating lactose and a pH indicator neutral red to detect the acid formation by fermentation of lactose. It is made selective for Gram negative bacteria by incorporating sodium taurocholate. Lactose fermenting colonies appear pink color with a pink halo, against the light pinkish medium. Non- lactose fermenting colonies are colorless or with the color of the medium.

SELECTIVE MEDIA

Selective media are media that contain additives that enhance the presence of the desired bacteria by inhibiting the other bacteria. Example is again MacConkey agar whose preparation is explained in previous section. MacConkey is selective for Gram positive as it contains selective inhibitor of Gram positive bacteria, Sodium taurocholate.

Eosin methylene blue (EMB) agar is another selective medium for isolating *E.coli*. Pancreatic digest is the nutrient component and Eosin and methylene blue are the selective agents inhibiting the Gram positive bacteria. Bacteria that ferment lactose bind to the dye under acidic condition and appear as blue-black colonies with metallic sheen. Under less acidic conditions, other colonies appear pinkish without metallic sheen.

MYCOLOGICAL MEDIA

Sabouraud's Dextrose agar (SDA) is a general purpose media to isolate and cultivate fungi. It has 4% dextrose and a pH of 5.6. This low pH reduces the bacterial contamination. The original composition is now modified by adding antibiotics to prevent bacterial contamination.

PLATES:

The sterilized medium is cooled to 45-50°C and poured into sterile petriplates. About 20 ml of the medium poured into each of the sterile petriplates and allowed to solidify. Incubate in an incubator overnight before use.

SLANTS:

The molten medium after adjusting the pH is poured into test tubes, 5 ml each and the test tubes are placed in a slanted position so that they will solidify with maximum surface area on the slant region and butt region.

DEEPS:

Deeps are prepared by pouring the molten medium in sterile glass test tubes and then placed in a vertical position. The tubes are then cooled in cold water and are used to maintain cultures for long periods of incubation.

Exp. No.: 12

Bacterial Growth Curve

Date:

AIM:

To study the different phases of bacterial growth in the broth culture medium.

MATERIALS REQUIRED:

PRINCIPLE

Bacteria need specific medium for its growth. When bacteria are inoculated into a liquid culture 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 increases, the transmission of light through the sample will decrease.

PROCEDURE:

DAY : 1

Using sterile loop, streak a loopful of bacterial culture onto the agar plate.

Incubate at 37°C for 18-24 hours.

DAY : 2

Pick up a single colony of a specific strain from the agar plate and inoculate it into a test tube containing 10 ml of autoclaved broth.

Incubate the test tube overnight at 37°C.

DAY : 3

Take 250 ml of autoclaved broth in a sterile 500 ml conical flask.

Inoculate 5 ml of the overnight grown culture in above flask.

Take OD at zero hour. Incubate the flask at 37°C.

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 is added to all the 1ml aliquots of culture suspension taken after every 30 minutes. Optical density of all the aliquots is taken at the end of the experiment.

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. Bacterial growth occurs in four distinct phases over time.

Exp. 13. **Genomic DNA extraction and estimation of Nucleic acids**

Date:

Aim:

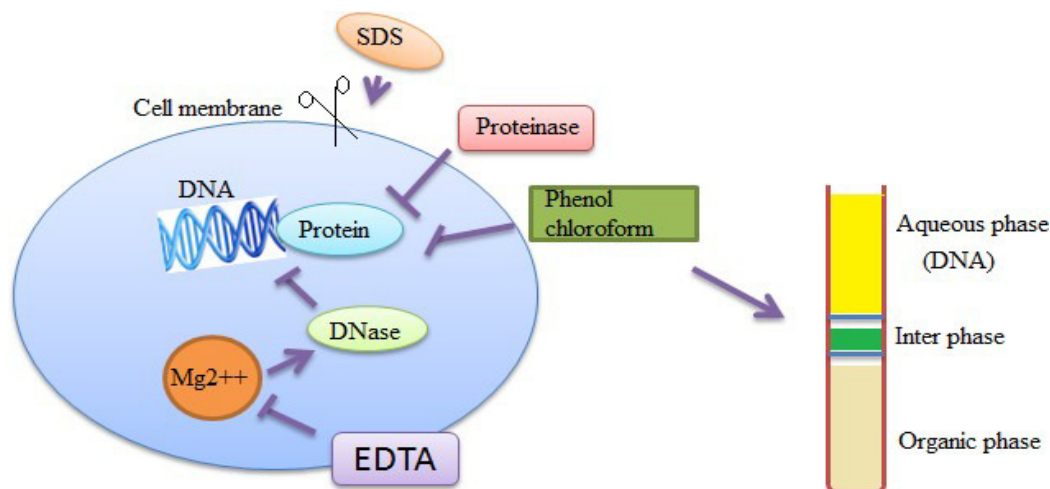
To isolate the genomic DNA from E .coli DH5a cells.

Principle:

The isolation and purification of DNA from cells is one of the most common procedures in contemporary molecular biology and embodies a transition from cell biology to the molecular biology (from in vivo to in vitro). The isolation of DNA from bacteria is a relatively simple process. The organism to be used should be grown in a favorable medium at an optimal temperature, and should be harvested in late log to early stationary phase for maximum yield.

The genomic DNA isolation needs to separate total DNA from RNA, protein, lipid, etc. Initially the cell membranes must be disrupted in order to release the DNA in the extraction buffer. SDS (sodium dodecyl sulphate) is used to disrupt the cell membrane. Once cell is disrupted, the endogenous nucleases tend to cause extensive hydrolysis. Nucleases apparently present on human fingertips are notorious for causing spurious degradation of nucleic acids during purification. DNA can be protected from endogenous nucleases by chelating Mg^{2++} ions using EDTA. Mg^{2++} ion is considered as a necessary cofactor for action of most of the nucleases. Nucleoprotein interactions are disrupted with SDS, phenol or proteinase K. Proteinase enzyme is used to degrade the proteins in the disrupted cell soup. Phenol and chloroform are used to denature and separate proteins from DNA. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. DNA released from disrupted cells is precipitated by cold absolute ethanol or isopropanol.

Schematic diagram showing the principle of isolation of genomic DNA from E. coli



Materials Required:

LB Broth, E. coli DH5α cells

Reagents - TE buffer (pH 8.0), 10% SDS, Proteinase K, Phenol-chloroform mixture, 5M Sodium Acetate (pH 5.2), Isopropanol, 70% ethanol, Sterile Dis.H₂O, Eppendorf tubes, Micropipette and tips

Preparation of Reagents:

TE Buffer (pH 8.0): 10 mM Tris HCl (pH 8.0), 1 mM EDTA (pH 8.0)

10% SDS: Dissolve 10 g of SDS in 100 ml autoclaved distilled water.

Proteinase K: Dissolve 10 mg of Proteinase K in 1 ml autoclaved distilled water.

Phenol – Chloroform mixture: The pH is very important. For RNA purification, the pH is kept around pH 4, which retains RNA in the aqueous phase preferentially. For DNA purification, the pH is usually 7 to 8, at which point all nucleic acids are found in the aqueous phase. Mix equal volume of phenol with chloroform. Keep the mixture on ice and add 20 ml TE buffer, extract by shaking for 15 minutes. Remove the dust on the surface layer using a pipette. Repeat 4-5 times. Add 30-40 ml of TE buffer and store it on ice.

5M Sodium Acetate: Dissolve 41 g of sodium acetate in 100 ml distilled water and adjust pH with dilute acetic acid (pH 5.2).

Isopropanol, 70% Ethanol

Procedure:

1. Take 2 ml overnight bacterial culture and the cells are harvested by centrifugation for 10 minutes.
2. To the cell pellet add 875 µl of TE buffer and the cells are resuspended in the buffer by gentle mixing.
3. Add 100 µl of 10% SDS and 5 µl of Proteinase K to the cells.

4. The above mixture is mixed well and incubated at 37° C for an hour in an incubator.
5. Add 1 ml of phenol-chloroform mixture and mix well by inverting and incubate at room temperature for 5 minutes.
6. The contents are centrifuged at 10,000 rpm for 10 minutes at 4° C.
7. The highly viscous jelly like supernatant is collected using cut tips and is transferred to a fresh tube.
8. The process is repeated once again with phenol-chloroform mixture and the supernatant is collected in a fresh tube.
9. Add 100 µl of 5M sodium acetate and mix gently.
10. To this add 2 ml of isopropanol and mix gently by inversion till white strands of DNA precipitates out.
11. The contents are centrifuged at 5,000 rpm for 10 minutes.
12. The supernatant is removed and 1ml 70% ethanol is added.
13. The above contents are centrifuged at 5,000 rpm for 10 minutes.
14. After air drying for 5 minutes 200 µl of TE buffer or distilled water is added.
15. 10 µl of DNA sample is taken and is diluted to 1 or 2 ml with distilled water.
16. The concentration of DNA is determined using a spectrophotometer at 260/280 nm.
17. The remaining samples are stored for further experiments.

Precautions:

Cut tips should be used so that the DNA is not subjected to mechanical disruption. Depending on the source of DNA the incubation period of Proteinase K should be extended. The phenol chloroform extraction should be repeated depending on the source of DNA to obtain pure DNA. DNase free plastic wares and reagents should be used.

Results and Discussion:

Aim:

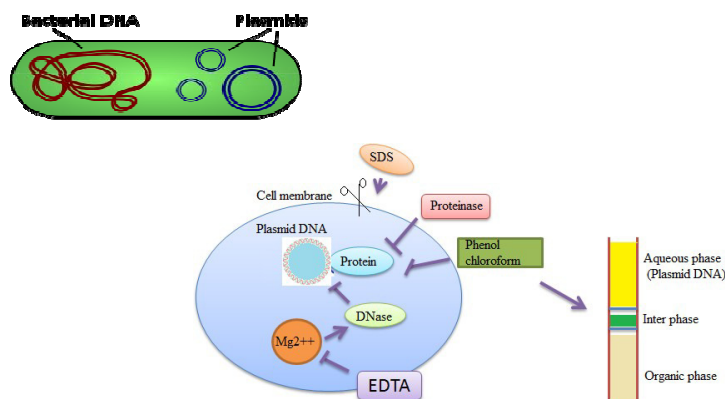
To isolate plasmid DNA from bacterial cells.

Principle:

When bacteria are lysed under alkaline conditions both DNA and proteins are precipitated. After the addition of acetate-containing neutralization buffer the large and less supercoiled chromosomal DNA and proteins precipitate, but the small bacterial DNA plasmids can renature and stay in solution.

In prokaryotes, plasmid is double stranded, circular, and is found in the cytoplasm. The cell membranes must be disrupted in order to release the plasmid in the extraction buffer. Solution 1 contains glucose, Tris, and EDTA. Glucose provides osmotic shock leading to the disruption of cell membrane, Tris is a buffering agent used to maintain a constant pH8. Plasmid can be protected from endogenous nucleases by chelating Mg^{2++} ions using EDTA. Mg^{2++} ion is considered as a necessary cofactor for most nucleases. Solution II contains NaOH and SDS and this alkaline solution is used to disrupt the cell membrane and NaOH also denatures the DNA into single strands. Solution III contains acetic acid to neutralise the pH and potassium acetate to precipitate the chromosomal DNA, proteins, along with the cellular debris. Phenol /chloroform is used to denature and separate proteins from plasmid. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. Once the plasmid DNA is released, it must be precipitated in alcohol. The plasmid DNA in the aqueous phase is precipitated with cold (0oC) ethanol or isopropanol. The precipitate is usually redissolved in buffer and treated with phenol or organic solvent to remove the last traces of protein, followed by reprecipitation with cold ethanol.

Schematic diagram of principle of Plasmid DNA Isolation



Materials Required:

Luria Broth, Bacterial cells containing plasmid

Reagents - TE buffer(pH 8.0), Solution I, Solution II, Solution III

Phenol-chloroform mixture, Isopropanol, 70% ethanol, Autoclaved Distilled Water, Micropipette, Microfuge

Preparation of Reagents:

TE BUFFER (pH 8.0): 10 mm Tris HCl (pH 8.0) 1 mm EDTA (pH 8.0)

Solution I: Lysis solution

Solution II: Denaturing solution

Solution III: Neutralizing solution

Phenol – Chloroform mixture: Mix equal volume of phenol with chloroform. Keep the mixture on ice and add 20 ml TE buffer, extract by shaking for 15 minutes. Remove the dust on the surface layer using a pipette. Repeat 4-5 times. Add 30-40 ml of TE buffer and store it in dark. Isopropanol

Procedure:

1. Take 2 ml overnight culture and harvest cells by centrifugation for 5 minutes. Discard the supernatant carefully.
2. Add 100 µl of solution I to the cell pellet and resuspend the cells by gentle mixing.
3. Incubate the above mixture at room temperature for 5 minutes.
4. Add 200 µl of solution II to the mixture and mix by inverting the tubes for 5 minutes.
5. Incubate for 5-10 minutes at room temperature.
6. Add 500µl of ice cold solution III to the mixture and mix by inverting the tube.
7. Incubate on ice for 10 minutes.
8. Centrifuge at 10,000 rpm for 5 minutes.
9. Transfer the supernatant into fresh tube.
10. Add 400 µl of phenol-chloroform mixture to the contents, mix well by inverting and incubate them at room temperature for 5 minutes.
11. Centrifuge at 10000 rpm for 5 minutes.
12. Collect the supernatant (viscous) using cut tips and transfer to a fresh tube.
13. Add 0.8 ml of isopropanol and mix gently by inversion. Incubate for 30 min at room temperature.
14. Centrifuge the contents at 10,000 rpm for 10 minutes.
15. Discard the supernatant after centrifugation.
16. After air drying for 5 minutes, add 100 µl of TE buffer or autoclaved distilled water to the pellet to resuspend the plasmid DNA. The contaminated salt in the DNA pellet can be removed with 70% ethanol washing.

17. Take 10 μ l of plasmid sample and dilute to 1 ml with distilled water for spectrometric analysis.
18. The concentration of plasmid is determined using a spectrophotometer at 260/280 nm.
19. An aliquot of plasmid DNA is used for agarose electrophoresis for quantitative and qualitative analyses.

Precautions:

Cut tips should be used so that the plasmid is not subjected to mechanical disruption.

The phenol chloroform extraction should be repeated depending on the source of plasmid to obtain pure plasmid.

DNase free plastic wares and reagents should be used.

Results and Discussion:

The most common method of DNA extraction is phenol extraction. The fundamental aim of phenol extraction is deproteinization of an aqueous solution containing the desired nucleic acids. In simple terms the phenol reagent is mixed with the sample under conditions which favor separation of lower organic proteins from nucleic acids. Centrifugation of the mixtures yields two phases; a lower organic protein phase carrying the protein and some lipids, while the aqueous layer containing the nucleic acids remains on the top. The denatured proteins and lipids form the types an insoluble layer at the interphase. The whole theme behind it is that, following extraction with phenol and centrifugation, the protein component of sample is denatured or rendered insoluble, while the nucleic acid remains in solution in the aqueous phase. Used in conjunction with phenol, chloroform improves the efficiency of nucleic acid extraction. This is principally due to its ability to denature protein, thereby assisting the dissociation of nucleic acid from protein. The high density of chloroform also enhances the separation of the phases, facilitating the removal of aqueous phase with little cross contamination with organic materials. Removal of lipid is also another benefit of using chloroform. Usually isoamyl alcohol is added to the chloroform to prevent frothing. The DNA in the aqueous phase can subsequently be recovered by ethanol precipitation.

Materials required:

Tissue sample: Animal and human tissues are a convenient source of DNA. Here, one gram of liver tissue is used for DNA isolation.

Reagents:

1. Homogenizing Buffer or Nuclei acid Extraction Buffer (100 ml, pH 7.4)

- i. 0.35 M Sucrose (MW: 342.30)—11.98gms—Causes rupturing of the cell membrane.
 - ii. 0.05 M TRIS (MW: 121.1)—0.60gms—Maintains buffer capacity of solution & ionic strength.
 - iii. 0.025 M KCl (MW: 74.56)—0.186gms—Maintains ionic strength.
 - iv. 0.05 M Mg acetate (MW: 214.46)—1.07gm—Maintains ionic strength.
- Note: 2ml of 1 M of HCL may be added for the required pH.

2. DNA extraction buffer (100 ml, pH 8.2)

- i. 0.15 M NaCl (MW: 58.44)—0.876 gms. —maintains the ionic strength.
 - ii. 0.1M EDTA (MW: 372.24)—3.724gms—chelating agent, that chelates the divalent cations like Mg^{2+}
 - iii. 0.05 M TRIS (MW: 121.11)—0.606gms— Maintains buffer capacity of solution & ionic strength.
 - iv. Distilled water is added to make up the volume 100 ml.
- Note: 1. NaOH pellets may be added for the required pH.

2. If necessary dissociation of protein can further be improved by incubation of the cell extracts with proteinase-K in the presence of SDS (sodium dodecyl sulphate).

3. 3M Sodium Acetate (MW: 136) 8.16 gms in 20ml of distilled water.

4. 20% SDS (20 gms SDS in 100 ml of distilled water).

5. TRIS saturated phenol (pH 8).

6. Chloroform.

7. Isoamyl alcohol.

8. Chilled ethanol.

9. 70% alcohol.

Procedure:

i. Goat liver is minced and chopped with help of sterile scalpel.

ii. Homogenize the tissue in minimum volume of Homogenizing Buffer or Nuclei Extraction Buffer.

iii. Materials are filtered through cheesecloth and wait for 30 minutes.

iv. Homogenate is spun for 8 minutes at 8000 rpm.

v. Filtrate is discarded and the pellet containing the nuclei is suspended in equal volume of DNA extraction buffer for 15 minutes and vortex it.

vi. To this solution 1/10th volume of 20% SDS is added and mix thoroughly for 10 minutes by rolling at room temperature.

Note: SDS- A detergent that causes rupturing the nuclear membrane and helps in protein denaturation. SDS should be mixed at room temperature and never on ice.

vii. Phenol, chloroform and Isoamyl alcohol (25:24:1) are added and above mixture is mixed gently for 15 minutes.

Note: Phenol: Precipitates the proteins. Phenol is acidic and in acidic pH, RNA will separate in aqueous phase. Insolubilizes the protein in aqueous state. Chloroform: Causes surface denaturation of protein. It also helps to remove lipid and polysaccharides. It stabilizes the two layers the aqueous and the organic phase. It also removes phenol. Isoamyl alcohol: Prevents tanning which is caused by esterification of lipids.

viii. The solution is centrifuged at 8000 rpm for 30 minutes.

ix. The aqueous phase is pipetted out gently and the steps (vii-ix) can be repeated for better extraction.

x. Chloroform isoamyl alcohol (24:1) are then added to the aqueous layer and mixed gently for 10 minutes.

Note: Chloroform brings down the traces of phenol from the DNA and forms the bottom layer.

xi. The mixture is spun at 8000 rpm for 10 minutes.

xii. To this solution 1/10th vol. of 3M sodium acetate is added and mixed thoroughly

and kept at room temperature for 10 minutes.

Note: Sodium acetate removes the bound water from DNA resultant in the precipitation as it reduces dielectric concentration in a suspended form.

xiii. To this chilled ethanol is added in double volume.

xiv. The mixture is kept in freezer for 1 hour and DNA is spooled by a glass rod.

xv. The spooled DNA is washed in 70% ethanol.

xvi. The DNA is spun in 14,000 rpm for 20 minutes.

xvii. The supernatant is discarded

xviii. The DNA is dissolved in least amount of TE buffer possible for further use.

xix. Store in -20°C for further use.

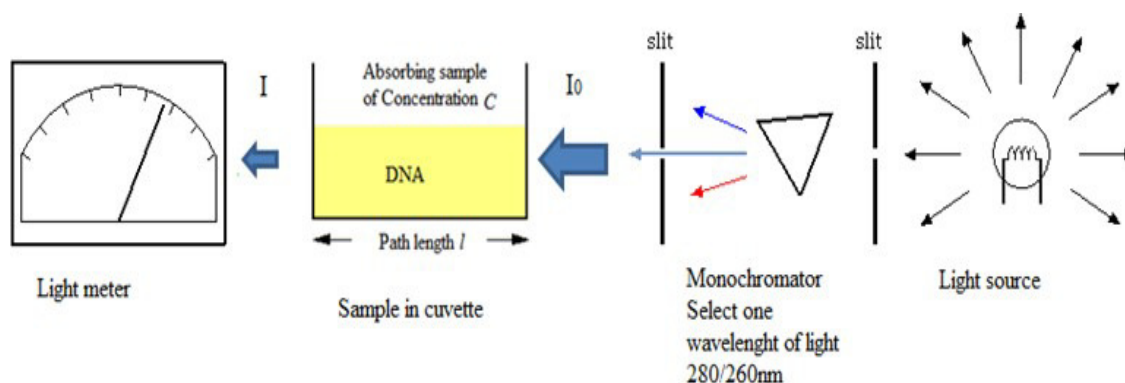
Note: RNase enzyme may also added to remove the RNA from DNA.

Aim:

To determine the amount, concentration and purity of the given DNA sample.

Principle:

This experiment is purely an application of the Beer Lamberts' Law which states that the concentration of the sample is directly proportional to the absorbance of light done by the sample. The device UV spectrophotometer works on this principle and used to find the concentration of the sample. Concentration and quality of a sample of DNA is measured with a UV spectrophotometer. A standard graph can be drawn using different concentrations of DNA and OD (optical density) values.



The diagram above shows that a beam of monochromatic radiation (I_0) is directed to a sample solution. Absorption takes place by the sample and the beam of radiation is leaving out (I).

Materials Required:

DNA sample, TE buffer, UV spectrophotometer

Procedure:

1. Take the DNA sample (10 μ l) in TE buffer.
2. Now dilute the above sample by the factor of 100 i. e, by taking 10 μ l of the sample in 990 μ l of TE buffer.
3. After doing this take the optical density value at A260 & A280 and calculate the amount of DNA recovered.
4. Use the following formula to determine the concentration of DNA:

$$\text{Total DNA (ug)} = (\text{A260}) (50 \text{ ug/ml/A260}) (100) (0.1 \text{ ml})$$

where 100 is the dilution factor and 0.1 ml is the total volume of the DNA;

Quality: DNA quality measurement is based on the fact that OD at 260 nm is twice that at 280 nm if the solution contains pure DNA. If there is a contaminant, there is some additional OD, which decreases the OD ratio between 260 and 280 nm. Clean DNA has a OD₂₆₀/OD₂₈₀ between 1.8 and 2.0

Results and Discussion:

Expr. No.: 17

Separation of DNA By Agarose Gel Electrophoresis Date:

AIM:

To check the presence of the isolated DNA by performing Agarose gel electrophoresis.

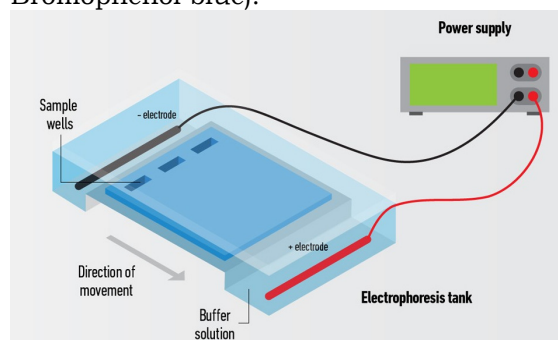
PRINCIPLE:

Electrophoresis refers to the separation of macromolecules of different size by application of a constant electric field on to the DNA fragments placed in a matrix of polymerized agarose. As DNA molecule is negatively charged and travels towards the anode, it is loaded at the cathode end. The speed of migration of the fragments has an inverse relation with the size of DNA. The separated fragments are visualized by staining the gels with an intercalating dye (Ethidium Bromide), which fluoresces under UV light. Acrylamide gel is used for separation of small fragments of DNA (5 to 500bp). Agarose gels can resolve DNA fragments varying in size from 200bp to about 50kb depending upon the concentration of agarose in the gel.

Materials Required:

Glassware and Apparatus - Agarose Gel Electrophoresis Apparatus, Micropipette, Beaker, Conical flask, Test tubes, Eppendorf tubes and Microtips

Chemicals: TAE buffer, Ethidium bromide and Loading Dye (Contains Glycerol and Bromophenol blue).



PROCEDURE:

1. Seal the open ends of a clean gel platform using an adhesive tape so as to form a mould and place it on an even horizontal surface.
2. Place the comb at about one cm from one end of the platform, at a height of about one millimeter from the surface of the plate.
3. Measure out 30 ml of 1X TAE electrophoresis buffer in a conical flask and add 300mg of agarose to give 1% concentration. Dissolve the agarose and melt in the flask by keeping it in a boiling water bath.
4. Cool the melted agarose to about 55 to 60°C and add 0.1µl ethidium bromide.
5. Then pour the agarose solution slowly into the gel platform, without the inclusion of air bubble.
6. Allow the gel to set at room temperature for about 30 minutes and carefully remove the comb and adhesive tap, without disturbing or damaging the slots/wells.
7. Place the gel along with the platform into electrophoresis chamber/tank and pour the TAE buffer into the chamber to a height of about 1mm above the gel.
8. Mix the DNA samples (5-10µl) with a loading dye and slowly load the mixtures into the slots/wells from left to right using a micropipette. Adequate care should be taken to avoid air bubbles and piercing of the gel.
9. Add standard DNA marker (1µg) at the last slot/well.
10. Place the lid and give the power supply. In the beginning, a pulse current of 10 V/cm.

11. Stop the electrophoresis as soon as the bromophenol blue (tracking dye) reaches the appropriate place (ie. 75% of the gel size).

12. Visualize DNA bands under UV transilluminator.

Result and interpretation:

Exp. No.: 18

Restriction Enzyme Digestion

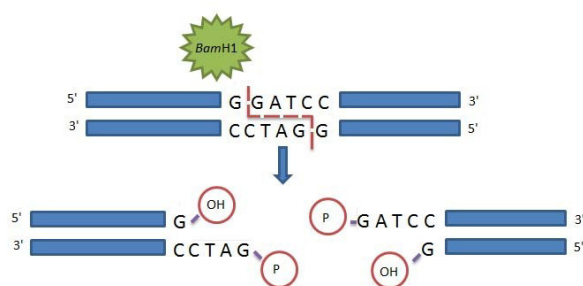
Date:

Aim:

To digest the pUC18 DNA with BamH1 enzyme

Principle:

Restriction endonucleases are the class of enzymes that are used to cleave DNA at specific sites called Restriction sites. Every restriction enzyme has a specific restriction site at which it cuts a DNA molecule. For example restriction sequence for BamHI is GGATCC (type II restriction enzyme). The most abundantly used restriction enzymes are type II restriction enzymes which cleave at specific restriction site only. These endonucleases function adequately at pH 7.4 but different enzymes vary in their requirements for ionic strength usually provided by sodium chloride and magnesium chloride. It is also advisable to add a reducing agent such as dithiothreitol (DTT) which stabilizes the enzymes and prevents their inactivation. Any variation in the concentration of Na or Mg can lead to changes in specificity of enzyme so that it can cleave at additional or non- standard restriction sequences. The phosphodiester bond is cleaved between specific bases, one on each DNA strand, no matter the source of the DNA. The restriction endonucleases produce either sticky or blunt ends upon cleavage. Also based on the number of sequences identified for cleavage they can be tetracutter (4), hexacutter (6) or octacutter (8).



Materials Required:

pUC18 DNA, BamH1 enzyme, 10X buffer, 1Kb Ladder, Sterile water, Agarose 6X loading dye, 1.5 ml Sterile Vials, Ethidium Bromide, 1X TAE buffer

Procedure:

1. Take 1.5 µg of PUC18 DNA (10 µl) in a fresh eppendorf.
2. To this, add 11.5 µl of sterile water followed by 5 µl of 10X buffer.
3. Add 1.5 µl of BamH1 enzyme (1 units) and incubate the mixture at 37°C for 2 hrs.
4. Load the samples including 1 Kb DNA ladder, undigested pUC18 DNA and BamH1 digested PUC18 DNA.
5. Run the gel at 100 V for 1 hr.

6. Visualize the gel under UV illuminator.
7. 10 μ l of the sample and 2 μ l of the dye were mixed
8. Load 10 μ l of this into the gel

Reaction Protocol:

PUC18 DNA : 10 μ l (1.5 μ g)

Sterile water : 11.5 μ l

10X buffer : 2.5 μ l

BamH1 : 1 μ l (1 μ g)

Total : 25 μ l (Incubate at 37° C for 1-2 hrs)

Results and interpretation:

Exp. No.: 19

Determination of Antibiotic Sensitivity of Microbes

Date:

AIM:

To determine the sensitivity of bacterial isolates to antibiotics using Kirby-Bauer method by agar disc diffusion technique.

PRINCIPLE:

An antibiotic is an antimicrobial chemical compound produced by microorganisms against other microorganisms. These antimicrobial compounds can be used for the treatment of microbial infections in human beings. The purpose of the Kirby-Bauer disk diffusion susceptibility test is to determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial compounds. The pathogenic organism is grown on Mueller-Hinton agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit that organism. During incubation, the antibiotic diffuses from the disc into the agar in decreasing amounts the further it is away from the disc. If the organism is killed or inhibited by the concentration of the antibiotic, there will be no growth in the immediate area around the disc which is called as the zone of inhibition. In this test, results are usually reported as sensitive, intermediate, or resistant, based on the size of the zone of inhibition. If the observed zone of inhibition is greater than or equal to the size of the standard zone, the microorganism is considered to be sensitive to the antibiotic. Conversely, if the observed zone of inhibition is smaller than the standard size, the microorganism is considered to be resistant. The antibiotic sensitivity test is also used to identify the organisms based on its sensitivity to various antibiotics.

MATERIALS REQUIRED:

Muller Hinton agar plates, Bacterial strains, Antibiotics Discs, Sterile swabs for lawn cultures

PROCEDURE:

The surface of Muller-Hinton agar plates were inoculated with the well grown culture broth using a sterile cotton swab.

To ensure the uniform and confluent growth, the swab was passed over the entire surface of the plate for at least three times.

Each antibiotic disc was made adhere perfectly to the surface of the agar by gentle pressing. Disc should be at least 2cm apart to prevent the merging of zones of inhibitions.

The plates were incubated at 37°C for 24 hours. The zone of inhibition around individual disc was observed and the diameters were noted down in mm.

RESULTS & INTERPRETATION:

Exp. No.: 20

Extraction of Protein from Bacterial cells

Date:

Aim:

To extract total protein from bacterial cells

Materials required:

Bacterial cells, Tris – EDTA buffer, eppendorf grinder, waterbath, cooling centrifuge.

Protein Extraction (Standard Protocol)

1. Centrifuge a bacterial culture for 15 minutes at 4700xg to spin down the cells. Discard the supernatant.
2. Dilute the cells with TrisHCl-buffer (pH 7.0) . Add to 1 volume cells, 4 volumes buffer.
3. Incubate for 1 hour at 37°C while shaking.
4. Eppendorf grinder can be used to break the cells.
5. Centrifuge the protein suspension for 15 minutes at 4700xg at 4°C. The supernatant is further used for protein extraction.

AIM

To separate protein by SDS PAGE

PRINCIPLE

The separation of macromolecules in an electric field is called electrophoresis. A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. The method is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The most commonly used system is also called the Laemmli method after U.K. Laemmli, who was the first to publish a paper employing SDS-PAGE in a scientific study.

SDS is an anionic detergent, meaning that when dissolved its molecules have a net negative charge within a wide pH range. A polypeptide chain binds amounts of SDS in proportion to its relative molecular mass. The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode (positively-charged electrode) in an electric field.

Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is nearly the same among SDS- denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides. In a gel of uniform density the relative migration distance of a protein (R_f , the f as a subscript) is negatively proportional to the log of its mass. If proteins of known mass are run simultaneously with the unknowns, the relationship between R_f and mass can be plotted, and the masses of unknown proteins estimated.

Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions. The purity of protein samples can be assessed and the progress of a fractionation or purification procedure can be followed. Different staining methods can be used to detect rare proteins and to learn something about their biochemical properties. Specialized techniques such as Western blotting, two-dimensional electrophoresis, and peptide mapping can be used to detect extremely scarce gene products, to find similarities among them, and to detect and separate isoenzymes of proteins.

MATERIALS REQUIRED

Vertical Electrophoresis apparatus Gel plates, Spacers, Comb, Ethanol, Vaseline, Distilled water

BUFFERS AND REAGENTS

Acrylamide (30%):

Dissolve 290g of acrylamide and 10g of N,N'-methylbisacrylamide in 600ml of H₂O. Adjust the volume to 1L with H₂O. Sterilize the solution by filtration (0.45 micron pore size). Check the pH (should be 7.0 or less). Store in dark bottles at room temperature.

Gel casting Buffer:

1.5 M Tris-HCl, pH 8.8 (to prepare resolving gel): Dissolve 18.15 g of Tris base in 80 mL distilled water. Adjust pH to 8.8 using 6N HCl. Make up the final volume to 100 mL with distilled water

0.5 M Tris-HCl, pH 6.8 (to prepare stacking gel): Dissolve 6 g of Tris base in 80 mL distilled water. Adjust pH to 6.8 using 6N HCl. Make up the final volume to 100 mL with distilled water

2X Loading Buffer:

Bromophenol blue 0.004%, 2-mercaptoethanol 10%, Glycerol 20%, SDS 4% and Tris-HCl 0.125 M

10X Running Buffer:

Tris-HCl 25 mM, Glycine 200 mM, SDS 0.1% (w/v), SDS (10%), Dissolve 10 g in 100 ml of distilled water.

APS (10%) - Dissolve 100 mg in 1 ml of distilled water (Need to be prepared fresh for every experiment)

TEMED

Staining solution:

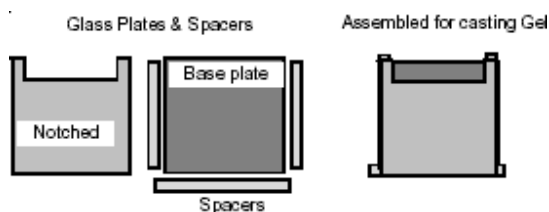
Stain: Dissolve 0.4g of Coomassie blue R350 in 200 mL of 40% (v/v) HPLC grade methanol in water with stirring as needed. Filter the solution to remove any insoluble material. Add 200mL of 20% (v/v) acetic acid in water. The final concentration is 0.1% (w/v) Coomassie blue R350, 20% (v/v) methanol, and 10% (v/v) acetic acid.

Distaining solution:

Add 500mL of HPLC- grade methanol to 300 mL of HPLC grade water. Add 100 mL of reagent grade acetic acid and, after mixing, adjust the total volume to 1000mL with water. The final concentrations are 50% (v/v) methanol in water with 10% (v/v) acetic acid

PROCEDURE

1. Assemble the plates for casting gel as shown below.



2. Clamp the assembly of plates to fix it in a gel casting apparatus.
3. Ensure the assembly is leak proof by filling water between the plates.
4. Silicon grease can be applied to spacer to make a water-tight seal.
5. Prepare the separation gel. Mix in the following order:

Gel %	Water (mL)	30% Acrylamide-Bisacrylamide stock (mL)	Running gel buffer (mL)	10% SDS (μL)	10% APS (μL)	TEMED*(μL)
8%	4.6	2.6	2.6	100	100	10
10%	3.8	3.4	2.6	100	100	10
12%	3.2	4.0	2.6	100	100	10
15%	2.2	5.0	2.6	100	100	10

6. Pour gel, leaving ~2 cm below the bottom of the comb for the stacking gel.
7. Make sure to remove bubbles. Layer the top of the gel with isopropanol. This will help

to remove bubbles at the top of the gel and will also keep the polymerized gel from drying out (In ~30 min, the gel should be completely polymerized).

8. Remove the isopropanol and wash out the remaining traces of isopropanol with distilled water.

9. Prepare the stacking gel (4%). Mix in the following order:

H ₂ O	6.1 mL
Acrylamide/bis (30%)	1.3 mL
Tris-HCl (0.5 M, pH 6.8)	2.5 mL
SDS, 10%	100 µL
TEMED	10 µL
Ammonium persulfate (APS), 10%	100 µL

10. Pour stacking gel on top of the separation gel.

11. Add combs to make wells. In ~30 min, the stacking gel should become completely polymerized.

12. Add 25 µl of sample loading buffer to protein sample.

13. Add 25 µl of sample loading buffer to 25 µl of protein marker.

14. Place it in a boiling water bath for 5 minutes.

15. After the stacking gel has set, carefully remove the comb and the bottom spacer.

16. Wash the wells immediately with distilled water to remove non-polymerized acrylamide.

17. Fill the bottom reservoir with 1X tank buffer and carefully fix the plate to the apparatus without trapping

any air bubbles between the buffer and the bottom of the gel.

18. Fix the plates to PAGE apparatus. Fill the top reservoir with 1X reservoir buffer.

19. Load samples and molecular mass protein markers into wells for separation by electrophoresis.

20. Set voltage at 100 V and switch on the power supply.

21. When the dye front comes to 0.5 cm above the bottom of the gel, turn off the power.

22. This will take approximately 1 to 1 1/2 hours.

23. Remove the gel plates and gently pry the plates apart using a spatula or similar tool, not at the notch. Cut

off the wells and the stacking gel and place the gel in a water containing Tray

24. Wash the gel Gently and discard the water

25. Add 100 ml of staining solution and keep it in the rocker for overnight.

26. Remove the staining solution and add 100 ml of destaining solution and change the destaining solution for

every one hr till the bands are visible.

Results and interpretation:

Exp. No.: 22

White Blood Cell Count

Date:

Principle. A sample of whole blood is mixed with a weak acid solution that lyses nonnucleated red blood cells. Following adequate mixing, the specimen is introduced into a counting chamber where the white blood cells (leukocytes) in a diluted volume are counted.

Reagent. White-count diluting fluid. Either of the following diluting fluids may be used:

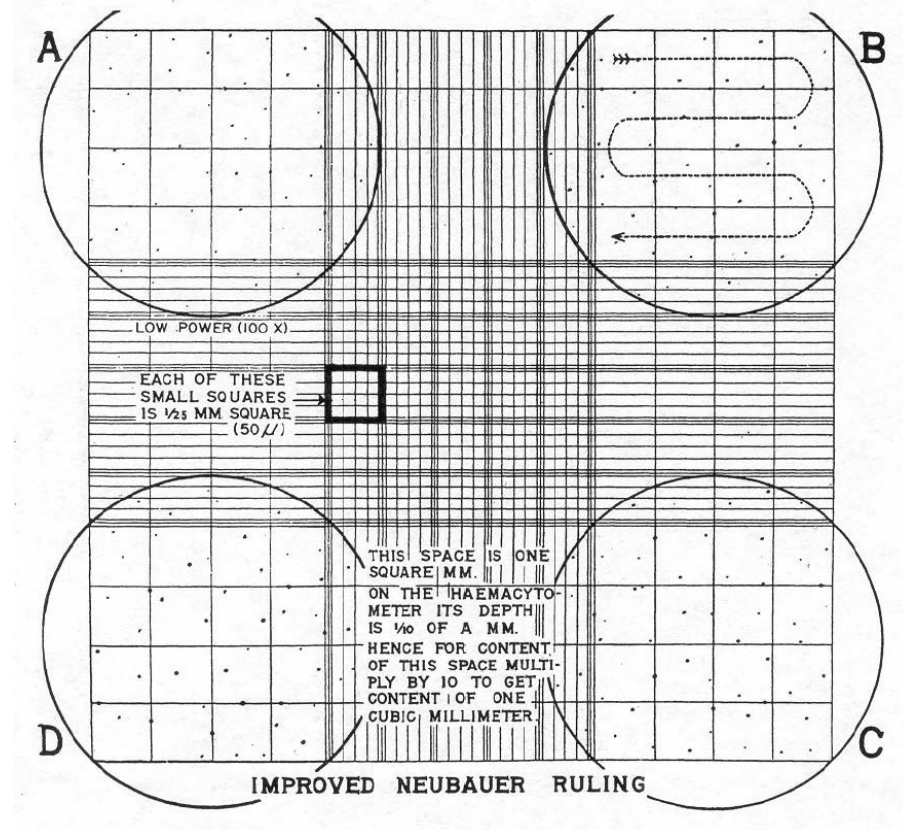
Two percent acetic acid. Add 2 ml glacial acetic acid to a 100 ml volumetric flask. Dilute to the mark with distilled water.

One percent hydrochloric acid. Add 1 ml hydrochloric acid to a 100 ml volumetric flask. Dilute to the mark with distilled water.

Procedure.

1. Draw well-mixed capillary or venous blood exactly to the 0.5 mark in a white blood cell diluting pipet. This blood column must be free of air bubbles.
2. Wipe the excess blood from the outside of the pipet to avoid transfer of cells to the diluting fluid. Take care not to touch the tip of the pipet with the gauze.
3. Immediately draw diluting fluid to the "11" mark while rotating the pipet between the thumb and forefinger to mix the specimen and diluent. Hold the pipet upright to prevent air bubbles in the bulb.
4. Mix the contents of the pipet for 3-5 minutes to ensure even distribution of cells. Expel unmixed and relatively cell-free fluid from the capillary portion of the pipet (usually 4 drops).
5. Place the forefinger over the top (short end) of the pipet, hold the pipet at a 45° angle, and touch the pipet tip to the junction of the cover glass and the counting chamber.
6. Allow the mixture to flow under the cover glass until the chamber is completely charged. Similarly, fill the opposite chamber of the hemacytometer.
Note: If the mixture overflows into the moat or air bubbles occur, clean and dry the chambers, remix the contents of the pipet, and refill both chambers.
7. Allow the cells to settle for about 3 minutes. Under low-power magnification and reduced light, focus on the ruled area and observe for even distribution of cells.
8. Count the white cells in the four 1 sq mm corner areas corresponding to those marked A, B, C, and D of Figure 5-1 in each of two chambers.
9. Count all the white cells lying within the square and those touching the upper and right-hand center lines. The white cells that touch the left-hand and bottom lines are not to be counted. In each of the four areas, conduct the count as indicated by the "snake-like" line in figure 5-1. A variation of more than 10 cells between any of the four areas counted or a variation of more than 20 cells between sides of the hemacytometer indicate uneven distribution and require that the procedure be repeated.

Figure 5-1. Hemacytometer counting chamber (WBCs). Areas marked A, B, C, and D are used to count white blood cells.



Calculation.

Routinely, blood is drawn to the 0.5 mark and diluted to the 11 mark with WBC diluting fluid. All the blood is washed into the bulb of the pipet (which has a volume of 10). Therefore, 0.5 volumes of blood are contained in 10 volumes of diluting fluid. The resulting dilution is 1:20. (These figures are arbitrary and refer strictly to dilution and not to specific volumetric measurements)

The depth of the counting chamber is 0.1 mm and the area counted is 4 sq mm (4 squares are counted, each with an area of 1.0 sq mm therefore, 4×1.0 sq mm = a total of 4 sq mm). The volume counted is: area x depth = volume. Four sq mm x 0.1 mm = 0.4 cu mm.

The formula is as follows:

$$\text{WBCs per cu mm} = \frac{\text{Average number of chambers (2) WBCs counted} \times \text{dilution (20)}}{\text{Volume (0.4)}}$$

For example:

First Chamber Cells counted in each square	Second Chamber Cells counted in each square
35	45
40	37
44	36
39	44
158 WBCs counted	162 WBCs counted

Calculate the average number of WBCs per chamber:

$$\text{Average WBCs} = \frac{158+162}{2} = 160$$

Calculate the number of WBCs per cubic mm:

Discussion.

The available error when four large squares are counted is +20 percent. Counting eight large squares decreases the error to +15 percent.

The importance of clean, dry diluting pipets cannot be stressed too much as the greatest source of error in the counting of WBC is the use of wet and/or dirty pipets.

The counting chamber must be scrupulously clean and free of debris that might be mistaken for cells.

The minimum blood sample recommended for performing routine white blood cell counts is that obtained using one pipet and counting two chambers as previously outlined.

In cases where the WBC count is exceptionally high, as in leukemia, the dilution should be made in the red blood cell diluting pipette. The blood is drawn to the "1.0" mark and the diluting fluid is drawn to the "101" mark. The resulting dilution is 1:100.

Exp. No.: 23

Differential WBC Count

Date:

Aim:

To estimate each type of WBC in whole blood.

Definition:-

It also known as differential leucocyte count, white blood cell morphology, it is performed to determine the relative number of each type of WBC in the blood.

Methods:-

Manual Differential WBC Count

Materials and Instruments

Whole blood drawn from finger, Glass slides, Microscope, Alcohol 70%, Lancet and Leishman's stain.

Procedure:

Preparing blood smear.

1. Clean two slides, one to be covered with the blood film and one to be as spreader.
2. Prick the finger with lancet to obtain a drop of blood. Make a fine touch of one end of a slide with drop of blood.
3. Place the edge of other slide on the surface of the first one at angle of 45 degree.
4. Draw spreader back until contact the drop of blood.
5. Push spreader slowly to the other end of the slide.
6. Allow the film to dry at room temperature.
7. Staining the blood smear
8. Blood smear should be stained within one to two hours.
9. Drop Leishman's stain on to blood film.
10. Stay for 1-2 minutes.
11. Add distilled water to the stain.
12. The diluted stain should act for 15-30 minutes.
13. Wash it with Dis. Water.
14. Shake off excess water and allow drying at room temperature.

Examination of the stained blood smears.

Put the slide on the microscope stage. Examine the blood smear using low power objective. Choose area with plenty of WBC. Place a drop of immersion oil on the selected area and use oil immersion objective. Perform the differential cell count, and examine the morphology of WBC's.

Calculations. Count each WBC seen and record on a dif. cell counter until 100 WBC's have been counted.

Results:-

The normal range of the different types of WBC is as follows :-

Neutrophils ____; Eosinophils ____; Basophils ____; Monocytes ____; Lymphocytes ____;

Aim:

To separate and identify the protein molecules.

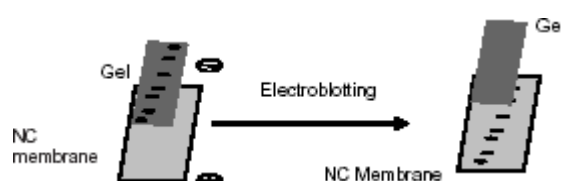
Principle:

Western blotting is an important technique used in cell and molecular biology. By using a western blot, researchers are able to identify specific proteins from a complex mixture of proteins extracted from cells. The technique uses three elements to accomplish this task: (1) Electrophoresis - separation by size, (2) Blotting - transfer to a solid support, and (3) Immunodetection - marking target protein using a proper primary and secondary antibody to visualize.

Electrophoresis

Mixture of proteins is separated based on molecular weight, and separated through polyacrylamide gel electrophoresis (PAGE).

Blotting

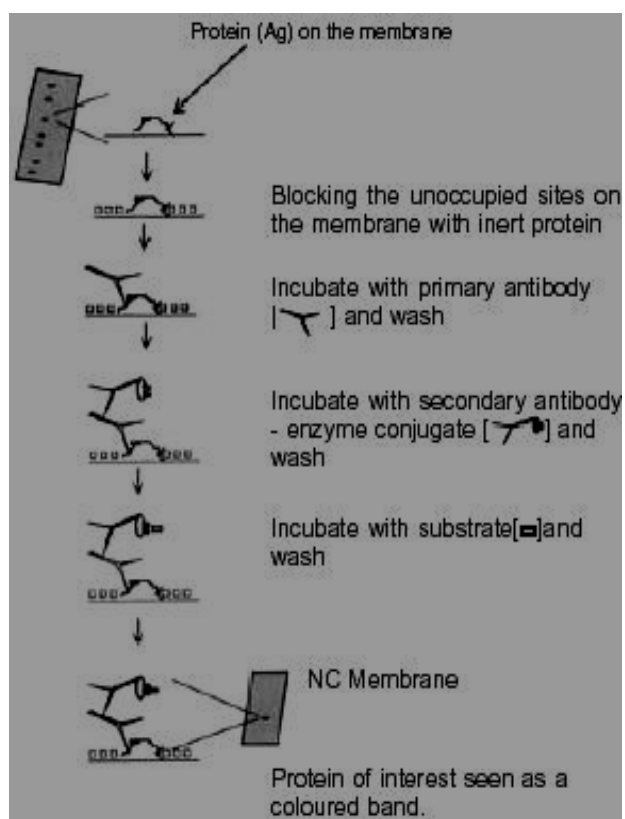


Blotting is transfer of resolved proteins from the gel onto a surface of a suitable membrane, done commonly by electrophoresis and referred to as electroblotting. The gel is placed in contact with nitrocellulose membrane which is then sandwiched between filter paper, two porous pads and two plastic supports. The entire set up is then placed in an electrophoretic tank containing blotting buffer. The proteins get transferred to the corresponding position on the membrane as resolved on the polyacrylamide gel, forming a mirror image of the gel.

Immunodetection

Protein of interest on the membrane is further located by immunodetection. The membrane is then incubated with labels antibodies specific to the protein of interest. The unbound antibody is washed off leaving only the bound antibody to the protein of interest. The bound antibodies are then detected by developing the film. As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of protein present; thus doing a standard can indicate the amount of protein present. The electroblotted sample will then be detected using anti-mouse GAPDH IgG (Cell signaling) as primary antibody and corresponding secondary antibody labeled with Horse Radish Peroxidase (HRP). HRP is then detected using hydrogen peroxide as a substrate and Dimethylamino benzidine (DAB) as a chromogen. HRP acts on hydrogen peroxide to release oxygen, which oxidizes the DAB to DAB oxide. The DAB oxide is deposited wherever enzyme is present and appears as a light brown band on the NC membrane.

Fig 9.1 Principle of Immunodetection



Materials Required:

Gel Rocker, Conical flask, measuring cylinder, Petri dish, Staining tray. Distilled water.

Micropipettes, Tips, Water bath.

Buffers and Reagents:

PBS 10X, 1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, and 18 mM KH₂PO₄.

Transfer buffer (1X)

Tris 3 g, Glycine 14.4 g, Methanol 200 ml.

Dissolve Tris and Glycine in 700 ml of distilled water, adjust the pH to 8.3 and make it up to 800 ml.

Add methanol before the experiment

Blocking solution:

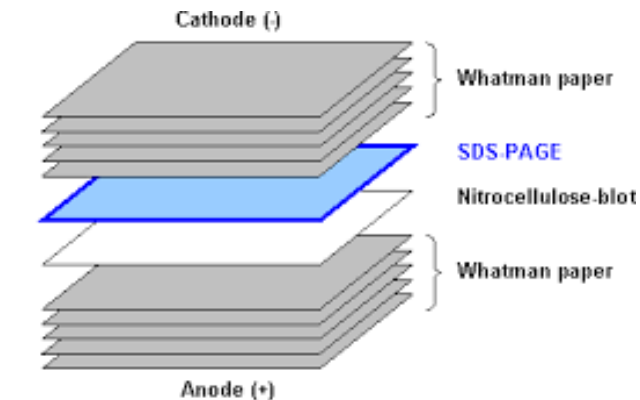
Skimmed milk powder - 10 g, Tween 20 - 200 µl, Glycerol - 10 ml, PBS - 10 ml, Sodium azide - 20 mg.

Dissolve the above contents in 100 ml of millipore water.

Procudeure:

Electroblotting:

1. Assemble the blotting sandwich within the blotting cassette as shown in the figure. Take care to avoid air bubbles between the gel and NC membrane.
2. Insert the cassette into the apparatus filled with blotting buffer and connect blotting unit to power supply as per the convention, red: anode, black: cathode
3. Electrophoreses the sample at 50 V for 2 hours for blotting to occur.
4. Remove the NC membrane gently from the cassette and place the membrane in 10 ml of freshly prepared blocking buffer taken in a petri dish. Leave it overnight at 4°C.



Assembly of a horizontal electroblotting apparatus

Day 2 : Immunodetection

1. Discard blocking buffer.
2. Immerse blot in 10 ml of primary antibody solution and mix gently and incubated for 4 to 5 hours in a rocker (gentle). The primary antibody solution can be used atleast 5 to 6 times.
3. Wash the blot by immersing in 10 ml of 1X PBS for 3-5 minutes. Repeat the wash three times. Discard the buffer each time.
4. Immerse the blot in 10 ml of 1X HRP labeled secondary antibody.
5. Mix gently and incubate for 45 minutes. Discard the HRP labeled antibody.
6. Wash the blot by immersing in 10 ml of T-TBS and TBS for 3-5 minutes.
7. Immerse the washed blot in 10 ml of substrate solution, mix gently for 5-10 minutes, within this time coloured band will appear.
8. Remove the blot, wash with distilled water, discard and dry.

Note: Although the colored band fades with time, the rate of color loss can be retarded if the blots are kept in dark.

Compare the SDS-Polyacrylamide gel with the developed NC membrane.

