AVIT
AARUPADAI VEEDU INSTITUTE OF TECHNOLOGY

VINAYAKA MISSION'S RESEARCH FOUNDATION
(Deemed to be University under section 3 of the UGC Act 1956)

Accredited by NAAC

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DEPARTMENT OF BIOTECHNOLOGY

NAME OF THE LAB

481172L1- GENETIC ENGINEERING LAB

HOD

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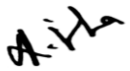
481172 L1-GENETIC ENGINEERING LAB

LIST OF EXPERIMENTS

1. Leishman staining
2. Giemsa staining
3. Osmosis and tonicity
4. Tryphan blue assay
5. Staining for different stages of mitosis in Allium cepa (Onion)
6. Staining for different stages of meiosis using (Grasshopper)
7. Blue and White selection for recombinants
8. Isolation of Genomic DNA from Plant / Animal / Bacterial Cells
9. Isolation of Total RNA
10. Isolation of Plasmid DNA
11. Quantification of DNA and RNA
12. Gel Electrophoresis of DNA – Agarose Gel, Polyacrylamide gel.
13. Southern Blotting.
14. Polymerase Chain Reaction.
15. Elution of Plasmid DNA from Agarose gel.
16. Restriction digestion of Bacterial Genomic and Plasmid DNA.
17. Ligation of DNA.
18. Preparation of Competent Cells.
19. Transformation in E. Coli.
20. Screening and selection of Recombinants and Confirmation of Insert DNA in Plasmid.
21. SDS-PAGE.

22. Western Blotting.



HOD

EXPT. NO. :

DATE :

LEISHMAN'S STAINING

AIM

To demonstrate different types of leucocytes in human blood by Leishman's staining.

PRINCIPLE

Leishman's stain is used in microscopy for staining blood smears. It is used to differentiate blood cells and to stain intracellular parasites in red blood cells and plasma, e.g. *Plasmodium falciparum* (malaria parasite). Leishman stain is named after its inventor, the Scottish pathologist William Boog Leishman. It is similar to and partially replaceable with Giemsa stain, Jenner's stain and identical to Wright's stain. Like them, it is a version of Romanowsky stain.

Romanowsky staining was a prototypical staining technique that was the forerunner of several distinct but similar methods including Giemsa, Jenner, Wright, and Leishman stains which are used to differentiate cells in pathological specimens. Romanowsky and Malakowsky independently developed a technique using a mixture of Eosin Y and oxidized Methylene blue.

The original Romanowsky stain was made by dissolving in methyl alcohol, the precipitate formed by the interaction of watery solutions of eosin and zinc free methylene blue. The stain imparts a reddish purple colour to the chromatin of malaria and other parasites.

REAGENTS REQUIRED

(i) Blood sample

(ii) Leishman's stain

(iii) Double distilled water

MATERIALS REQUIRED

(i) Microscope

(ii) Microscope slides

(iii) Lancet

(iv) Wash bottle

(v) Other standard lab ware

PROCEDURE

(i) Place a sterile 1by 3 – in. glass microscope slide on a horizontal surface.

(ii) Add a drop (30 to 40 μL) of blood onto one end of the slide about 0.5 inches. from the end.

(iii) Place a second glass slide 45 degree to the first slide and make a blood smear from one end to the other end.

(iv) Allow the film to air dry for at least 2 – 3 minutes.

(v) Pour the undiluted stain on the film and allow it to act for 1 minute.

(vi) Add double the volume of double distilled water with the help of a pipette to the slide and mix.

(vii) Leave the slide undisturbed for 12 minutes.

(viii) Wash off the stain by gently flooded in double distilled water until the film appeared bright pink in colour (usually in 30 seconds).

(ix) Remove the excess water with a blotting paper.

(x) Air dry the slide and observe under a microscope

OBSERVATION

Microscopically the RBC's appear as pinkish grey, platelets appear as deep pink and WBC's show blue nuclei and lighter cytoplasm. The nuclei of neutrophils appear as

dark purple multi-lobed nucleus with pale pink cytoplasm, bilobed blue nuclei for eosinophils, trilobed purple to dark blue nucleus for basophils and a dark purple to deep bluish purple nuclei for the lymphocytes (Fig. 1).

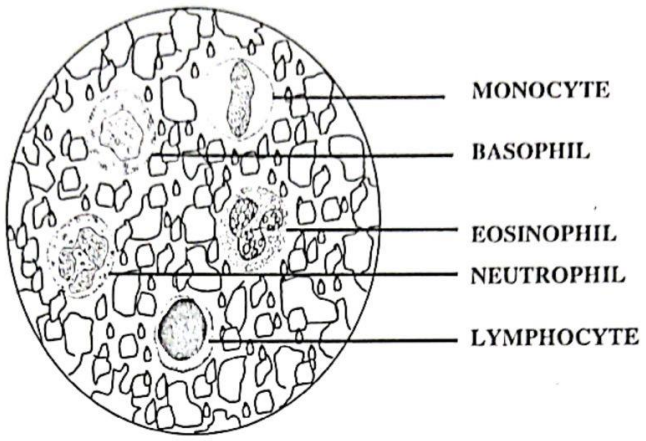


Fig 1. Geimsa stained WBCs

RESULT

In the slide Neutrophils, Eosinophils, Lymphocytes and Monocytes are identified.

EXPT. NO. :

DATE :

GIEMSA STAINING

AIM

To differentiate the different types of WBC's using Giemsa Staining

PRINCIPLE

Giemsa stain named after Gustav Giemsa (1867 – 1948), an early malariologist is used for the histopathological diagnosis of malaria and other parasites. It is a member of the "Romanowski" group of stains and consists of a mixture of a stock glycerol methanol solution of eosinates of Azure B and methylene blue with some excess of the basic dyes. The stain is usually prepared from commercially available Giemsa powder.

Giemsa stain is a differential stain. It is used to differentiate nuclear and / or cytoplasmic morphology of platelets, RBCs, WBCs, and parasites. Erythrocytes stain pink, platelets show a light pale pink, lymphocyte cytoplasm stains sky blue, monocyte cytoplasm stains pale blue, and leukocyte nuclear chromatin stains magenta. It is also used to study the adherence of pathogenic bacteria to human cells. It differentially stains human and bacterial cells with purple and pink respectively. Hence used for histopathological diagnosis of malaria, some other spirochete and protozoan blood parasites.

It is also specific for the phosphate groups of DNA and attaches itself to regions of DNA where there are high amounts of adenine – thymine bonding. Hence used in Giemsa banding commonly called G-banding to stain chromosomes and often used to create a karyotype. It can identify chromosomal aberrations such as translocations and interchanges.

REAGENTS REQUIRED

(i) Blood sample

(ii) Giemsa stain – 1: 20 dilution (2 mL of Cuimsa + 40 mL of buffered water)

(iii) Fixative – (Ethanol : Acetic acid in 3:1 ratio)

(iv) Double distilled water

MATERIALS REQUIRED

(i) Microscope

(ii) Microscope slides

(iii) Cover slips

(iv) Staining trough

(v) Lancet

(vi) Other standard lab ware

PROCEDURE

(i) Place a sterile 1by 3 – in. glass microscope slide on a horizontal surface.

(ii) Add a drop (30 to 40 µL) of blood onto one end of the slide about 0.5 inches. from the end.

(iii) Place a second glass slide 45 degree to the first slide and make a blood smear from one end to the other end.

(iv) Allow the film to air dry for at least 30 minutes to 1 hour.

(v) Fix the air-dried film in the fixative for 2 – 3 minutes.

(vi) After fixing, air dry the slide for 2 – 3 minutes.

(vii) Stain the air dried film with diluted Giemsa stain (1 : 20, v/v) for 30 min.

(viii) Briefly wash the slide by dipping the slide in and out of a Coplin jar of buffered water (one or two dips) for one or two seconds. Note : Excessive washing will decolourize the film.

(ix) Air dry the slide in a vertical position.

(x) Observe the slide under a microscope

OBSERVATION

Microscopically the RBC's appear pinkish grey, platelets appear deep pink and WBC's show blue nuclei and lighter cytoplasm. The nuclei of neutrophils appear as dark purple multi-lobed nucleus with pale pink cytoplasm, bilobed blue nuclei for eosinophils, trilobed purple to dark blue nucleus for basophils and a dark purple to deep bluish purple nuclei for the lymphocytes (Fig 1).

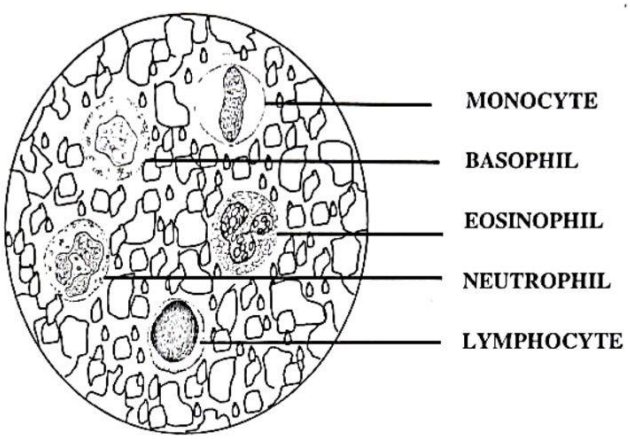


Fig 1. Geimsa stained WBCs

RESULT

In the slide, Neutrophils, Eosinophils, Lymphocytes and Monocytes are identified.

EXPT. NO. :

DATE :

OSMOSIS USING POTATO

AIM

To demonstrate osmosis in living plant membrane using potato.

PRINCIPLE

Osmosis is the net diffusion of water molecules from a dilute solution or pure water itself to a more concentrated solution across a semipermeable membrane that permits the diffusion of water but not of a solute. Thus if an aqueous solution of sucrose is separated from water by a semipermeable membrane, water flows into the sucrose solution across the membrane. Osmosis requires (a) semipermeability of the membrane separating the two solutions, so that the membrane is permeable to water but not to the solute and (b) a difference in concentration of the solute on the two sides of the membrane.

Water continues to flow into the more concentrated solution across the membrane until the hydrostatic pressure rises so high on the concentrated side of the membrane as to cause a transmembrane diffusion of water in the opposite direction at the same rate as the osmotic inflow. This excess of hydrostatic pressure, which exactly balances the osmotic influx of water from pure water to the concentrated solution is called the osmotic pressure of that solution. Osmotic pressure may also be defined as that pressure that has to be exerted on the concentrated solution, separated from pure water by a semipermeable membrane in order to counteract and stop the osmotic inflow into that solution. Osmotic pressure is a colligative property of a solution and not dependent on their nature or shape of the solutes. According to vant Hoff's, the osmotic pressure of a solution is directly proportional to the molar concentration (c) of the solute as long as the temperature is maintained constant. The more concentrated a solution is, the higher is its osmotic pressure.

REAGENTS REQUIRED

(i) 1% Sucrose solution

(ii) Coloured water

MATERIALS REQUIRED

(i) Potato

(ii) Beaker

(iii) Other standard lab ware

PROCEDURE

(i) Take a large size potato tuber.

(ii) Remove its skin and cut the base to make it flat.

(iii) Make a hollow cavity and fill with concentrated sugar solution.

(iv) Place the tuber in a beaker filled with coloured water.

OBSERVATION

After few hours the level of the solution within the potato cavity rises and turns pink colour (Fig. 1).

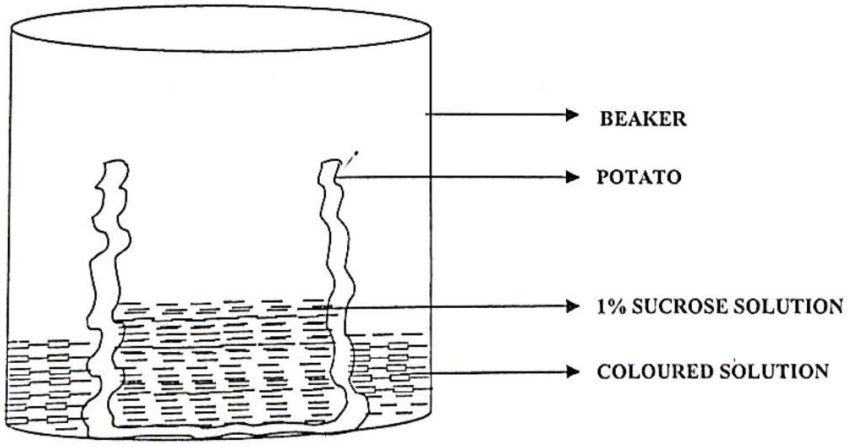


Fig 1 : Demonstration of Osmotic inflow

RESULT

The level of solution within potato cavity is raised. The raise in the level of the solution inside the potato cavity is due to the net movement of water through the living cell membrane of potato tubers.

EXPT. NO. :

DATE :

TRYPAN BLUE ASSAY

AIM

To count the number of viable cells in the given RBC population by Trypan blue assay.

PRINCIPLE

Viability assays measure the percentage of a cell suspension that is viable. The measurement of cell viability and growth is a valuable tool in a wide range of research areas. This is generally accomplished by a dye exclusion stain, where cells with an intact membrane are able to exclude the dye while cells without an intact membrane take up the colouring agent. A dye uptake stain can be used to measure viability as well. In this case the dye is normally taken up by viable cells but not by the non-viable cells.

The dye used for exclusion stain is usually trypan blue but erythrosin and naphthalene black have also been used. Diacetyl fluorescein is an example of a dye used for dye uptake assays. The cells are very selectable when it comes to allowing or baring compounds to pass through the cell membrane. In viable cells, trypan blue stain is not absorbed. However, in nonviable cells the damaged membrane allows trypan blue to become absorbed. Hence, dead cells are shown as a distinctive blue colour under a microscope.

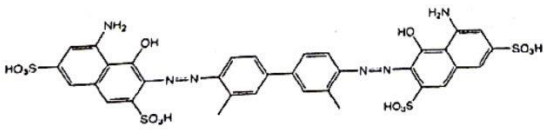


Fig 1. Chemical Structure of Trypan Blue

Trypan blue is derived from Toludine. Trypan blue is so-called because it can kill trypanosomes, the parasites that cause sleeping sickness. The dye is also known as diamine blue or Niagara blue. The reactivity of trypan blue is based on the fact that the chromophore is negatively charged and does not interact with cells unless the cell

membrane is damaged. The blue stain is easily visible and the cells can be counted using a light microscope.

REAGENTS REQUIRED

(i) Blood sample

(ii) RBC diluting fluid

(iii) 0.4% Trypan blue solution

MATERIALS REQUIRED

(i) Microscope

(ii) Haemocytometer

(iii) Test tubes

(iv) Microscope slides

(v) Slide staining stand

(vi) Cover slips

(vii) Other standard lab ware

PROCEDURE

(i) Dilute 0.5mL of the given RBC sample with RBC diluting fluid to a concentration of 2×10^5 to 4×10^5 cells / mL.

(ii) Aseptically transfer the diluted suspension to a fresh tube.

(iii) Add 0.5mL of trypan blue solution (0.1% w/v) to the diluted blood sample and mix well.

(iv) Allow the cells to remain in the dye solution for not less than 3 minutes and no longer than 10 minutes.

(v) Apply a drop of the above sample to the haemocytometer or a slide.

(vi) Count the total number of cells, keeping a separate count of blue cells.

(vii) Determine the frequency of the blue cells (that is cells that are not excluded the dye).

(viii) The viability of the given cell population was determined by the following calculation.

CALCULATION

Total number of cells counted = X

Number of cells failed to exclude and are blue = Y

Number of cells that excluded the dye and are white in colour = Z

Percentage Viability = $\frac{Z}{X}$ x 100

OBSERVATION

Blue coloured non-viable cells and white coloured viable cells are observed (Fig.1).

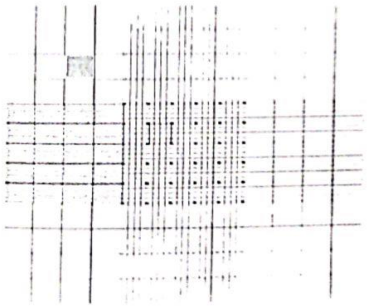


Fig 2. Haemocytometer grid

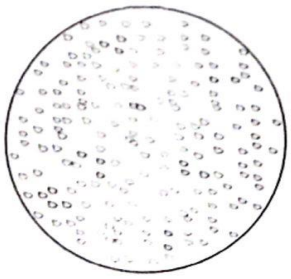


Fig 3. Blue coloured non viable and white coloured viable cells

RESULT

The percentage viability of the given cell population is ----------%

EXPT. NO. :

DATE :

MITOSIS

AIM

To demonstrate the different stages of mitosis in *Allium cepa* (Onion)

PRINCIPLE

During normal cell activity the chromosomes are unwound and too thin to be seen. Chromosomes of most plant and animal cells are visible with light microscopy only in the most condensed state i.e. during the various phases of cell division (mitosis or meiosis). Mitosis is the process by which a cell duplicates the chromosomes in its cell nucleus of eukaryotes, in order to generate two identical daughter nuclei. It is generally followed immediately by cytokinesis, which divides the nuclei, cytoplasm, organelles and cell membrane into two daughter cells containing roughly equal shares of these cellular components. The process of mitosis is complex and highly regulated. The sequence of events is divided into stages, corresponding to the completion of one set of activities and the start of the next. These stages are prophase, metaphase, anaphase and telophase.

In plants, the root tips are meristematic regions; i.e. root tips continue to grow as they search for water and nutrients. The meristematic region is located just behind the root cap, a protective covering on the root. This can be seen as the bright white tip in the preserved onion root tips. These regions of growth are good for studying the cell cycle because at any given time, cells that are undergoing mitosis can be found.

Onions are excellent material for studying mitosis, because lot of roots can be generated in a few days simply by placing an onion in a dish of water and it is fairly easy to see mitosis in onion root tips. The root tips are fixed prior to staining. The fixation brings about the death of the cells in such a way that the structure of the living cells is preserved in the same form as it was before death therefore the cells do not undergo any alterations. The fixative used is an acetic acid: ethanol solution. Acetic acid is a powerful precipitant of nuclear protein and it prevents cell shrinkage. Ethanol acts as a dehydrant (removes water form tissues) which hardens the tissue. 1 N HCl breaks down the calcium

pectate and magnesium pectate (which cement or attach the cells to each other) in the root tip tissues to pectic acid which cannot cement cells together. As a result after gently tapping, the cells separate out and form a mono layer in which cells are easily observable. The stain carmine is a pigment of a bright red colour. It is a basic dye extracted from the *Coccus* cacti and is taken up well by nucleic acids. Ferric chloride added to the stain acts as a mordant.

REAGENTS REQUIRED

(i) Freshly grown onion root tip

(ii) 1N Hydrochloric acid

(iii) Carnoy's fixative solution (Absolute alcohol : Glacial acetic acid – 3 : 1)

(iv) Acetocarmine stain (45 mL of glacial acetic acid and 55 mL of distilled water was taken into a 1 liter flask. Heat to boiling and add 0.5 grams of carmine, shake well, cool and filter).

(v) Ferric chloride

(vi) Double distilled water

MATERIALS REQUIRED

(i) Petri plates

(ii) Plastic pipettes

(iii) Razor blade

(iv) Microscope

(v) Microscope slides

(vi) Coverslips

(vii) Forceps

(viii) Other standard lab ware

PROCEDURE

(i) Using forceps take an onion root and remove the root tip.

(ii) Place the root tip in the 1N HCl kept in the Petri plate for 3 minutes (agitate the plate during fixation).

(iii) Transfer the hydrolyzed root tip to a Petri plate containing a small amount of Carnoy's fixative.

(iv) Fix the root tip for 3 minutes (agitate the plate during fixation).

(v) Place the fixed root tip in a Petri plate containing double distilled water (washing) for 30 seconds.

(vi) Transfer the root tip on a sterile glass slide.

(vii) Add a few drops of acetocarmine dye to the root tip and allow to stand for 5 minutes.

(viii) Place a cover slip over the stained tissue.

(ix) Remove the stain with a blotting paper.

(x) Press the cover slip firmly onto the slide to spread the cells in a very thin layer.

(xi) Observe the slide under a microscope

OBSERVATION

The various stages of mitosis are observed. In the interphase, chromosomes (DNA) exist as chromatin. During prophase, the chromatin is condensed. In metaphase, the duplicated chromosomes each consisting of two sister chromatids are lying midway between the two poles. In the early and late anaphase – the sister chromatids are seen separating and moving towards the poles. In the telophase, the chromosomes are located at opposite poles (Fig 1).

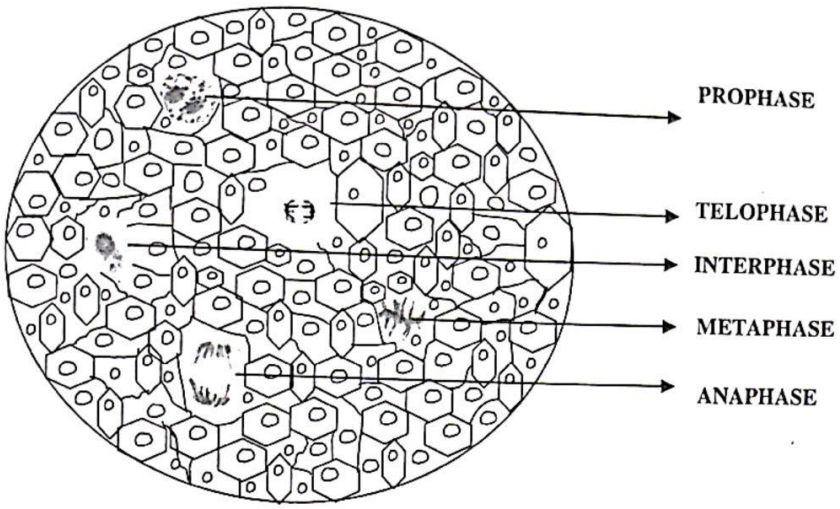


Fig 1. Mitosis – Different steps

RESULT

The interphase, prophase, metaphase, anaphase and telophase stages are identified.

BLUE WHITE SCREENING OF RECOMBINANTS

Blue-white screening is a rapid and efficient technique for the identification of recombinant bacteria. It relies on the activity of β-galactosidase, an enzyme occurring in *E. coli*, which cleaves lactose into glucose and galactose.

PRINCIPLE

For screening the clones containing recombinant DNA, a chromogenic substrate known as X-gal is added to the agar plate. If β-galactosidase is produced, X-gal is hydrolyzed to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerizes to produce an insoluble blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo. The colonies formed by non-recombinant cells, therefore appear blue in color while the recombinant ones appear white. The desired recombinant colonies can be easily picked and cultured.

Isopropyl β-D-1-thiogalactopyranoside (IPTG) is used along with X-gal for blue-white screening. IPTG is a non-metabolizable analog of galactose that induces the expression of lacZ gene. It should be noted that IPTG is not a substrate for β-galactosidase but only an inducer. For visual screening purposes, chromogenic substrate like X-gal is required.

SCREENING

A Wide range of chromogenic substrates that aid screening of recombinant bacteria. Some products may be used to spread on LB agar plates (screening protocol 1), while the others are incorporated into the microbial medium (screening protocol 2). The products are used along with IPTG wherever required. The protocols for both the procedures are given below.

SCREENING PROTOCOL

Spread 40 µL or appropriate amount of stock solution of chromogenic substrate and 10 µL of IPTG solution on LB agar plates using a sterile spreader.

1. The plates should include those with appropriate antibiotic and without antibiotic as controls.
2. Leave the plates to dry in laminar flow chamber with lids slightly open.
3. Spread 10-100 µL of transformed *E. coli* cells onto the LB agar plates using sterile spreader.
4. Incubate the plates at 37°C for 24-48 hours.
5. Blue and white colonies appear on the agar surface. Select the recombinant cells in the white colonies to culture.

Blue-white color selection of recombinant bacteria using X-gal.

EXPT. NO :

DATE :

GENOMIC DNA ISOLATION FROM THE GIVEN *E. coli* CULTURE BY KIT METHOD

AIM

To isolate the genomic DNA from the given *E. coli* Bacterial cells by Kit Method.

PRINCIPLE

The isolation of DNA is one of the most commonly used procedures in many areas of bacterial physiology, genetics, molecular biology and biochemistry. Purified DNA is required for many applications such as studying DNA structure and chemistry, examining DNA-protein interactions, carrying out DNA hybridizations, cloning, sequencing or performing various other genetic studies. The genetic material (genome) in bacteria is not very well organized as compared to eukaryotic genome, which is highly condensed and is present as nucleosomes. So extraction of bacterial genomic DNA is fairly simple and do not involve elaborate protocols. 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 cells can then be lysed and the DNA is isolated by one of the several methods. The method of choice depends in part on the organism of interest and purpose of which the DNA will be used for after purification. Following the lysis, other cellular constituents are selectively removed. Once this is accomplished, DNA can be precipitated from solution with alcohol and dissolved in an appropriated buffer.

The steps involve the separation of the DNA from other macromolecules in the lysate. Both phenol (that has been equilibrated with Tris buffer) and chloroform (with isoamyl alcohol as a defoaming agent) are commonly used to dissociate protein from nucleic acids. These reagents also remove lipids and some polysaccharides. Proteolytic enzymes such as pronase or Proteinase K are often added to further remove protein. Proteinase K is a particularly useful enzyme which is not denatured by SDS and in fact works more effectively in the presence of SDS. The nucleic acids (including RNA) may then be precipitated in ice-cold ethanol if the ionic strength of the solution is high. This is

followed by RNAse treatment to degrade the RNA. The solution may then be reprecipitated with ethanol. In this precipitation, the ribonucleotides from RNase treatment will remain in solution leaving purified DNA in the pellet. The pellet can then be dissolved in an appropriate buffer.

REAGENTS REQUIRED

(i) Overnight *E. coli* Bacterial culture

(ii) Solution A

(iii) Solution B

(iv) Absolute ethanol

(v) 70% Ethanol

(vi) Control DNA

(vii) 5 x TBE Buffer (Stock solution) – 27g Tris, 13.75g Boric acid, 1.8629g EDTA (Tris base, Boric acid and EDTA were dissolved in 400mL of double distilled water, adjusted the pH to 8.3 and made up to the final volume). The solution was stored at room temperature.

(viii) 1x TBE Working solution – 100mL of the 5 x TBE stock was diluted to 500mL using double distilled autoclaved water.

(ix) 0.7% Agarose gel solution – 0.7g of agarose was dissolved in 100mL of 1 x TBE buffer

(x) Loading dye – 50mg Bromophenol Blue, 3mL of glycerol, 6mL of TE (pH 8.0), 0.5mL of Ethidium bromide.

(xi) Ethidium bromide – 10mg / mL (10mg of Ethidium bromide was dissolved in 1mL of double distilled water)

MATERIALS REQUIRED

(i) Microcentrifuge

(ii) Eppendorf tubes

(iii) Micropipettes and tips

(iv) Gel casting apparatus along with the comb

(v) Electrical adhesive tape

(vi) Electrophoretic tank

(vii) Power pack with leads

(viii) Transilluminator

(ix) Other standard labware

PROCEDURE

(i) Thaw the bacterial cells and resuspend in 700µL of solution A.

(ii) Incubate the sample at room temperature for 5 minutes.

(iii) Spin at 10,000 rpm for 10 minutes.

(iv) Collect 500µL of the supernatant in a fresh vial.

(v) To the above, add double the volume of (1mL) absolute alcohol, mix by inverting the tube till the white strands of DNA precipitate out.

(vi) Spin at 10,000 rpm for 10 minutes.

(vii) Discard the supernatant and wash the pellet with 70% ethanol.

(viii) Air dry and dissolve the pellet in 50µL of 1x TE buffer.

(ix) Check the dissolved sample on a 0.7% agarose gel.

(x) Mix 10µL of the sample with 3µL of gel loading dye and load to the wells.

(xi) Run electrophoresis at 50 volts for 1 – 2 ½ hours.

(xii) Visualize and document the gel under an UV transilluminator.

OBSERVATION

A single band of high molecular weight Bacterial genomic DNA is observed in the gel (Fig.1).

Fig. 1. 0.7% Agarose gel electrophoregram of Bacterial Genomic DNA isolated by kit method

Lane : 1-2 : Bacterial Genomic DNA

Run Voltage : 50V

Run Buffer : 1X TBE

Run Time : 1 – 1 ½ hours

RESULT

The Bacterial genomic DNA isolated is found to be of high molecular weight and pure.

EXPT. NO. :

DATE :

ISOLATION OF PLANT CELL GENOMIC DNA BY CTAB KIT METHOD

AIM

To isolate genomic DNA from the Plant tissue by CTAB Kit Method.

PRINCIPLE

The application of molecular biology techniques to the analysis of complex genomes, depends on the ability to prepare pure, high molecular weight DNA. The isolation of plant DNA is the fundamental requirement for most of the genomic characterization and mapping procedure in plant molecular biology studies. The degree of purity and quantity required for the DNA isolation varies from application to application. DNA extraction from plant tissues, unlike DNA isolation from mammalian tissues, remains difficult due to the presence of a rigid cell wall surrounding the plant cells. Currently used methods inevitably require a laborious mechanical grinding step, necessary to disrupt the cell wall for the release of DNA.

In general, a good extraction procedure for the isolation of DNA should fill three major criteria.

i) It should yield DNA of reasonable purity.

ii) DNA should be sufficiently intact.

iii) The yield of DNA should degenerate.

The present protocol describes the isolation of plant genomic DNA by CTAB kit method. CTAB is a cationic detergent that has the property of precipitating nucleic acids and acidic polysaccharides from solutions of low ionic strength. Under these conditions, proteins and acidic polysaccharides remain in solution. In solutions of high ionic strength, the CTAB forms complexes with proteins and all polysaccharides especially the acid polysaccharides; but will not precipitate nucleic acids. CTAB is therefore particularly

useful for purification of DNA from organisms that produce large quantities of polysaccharides eg. Plants.

The nucleic acid – CTAB complex is only soluble in high salt. TE solution detergent is removed by raising the sodium chloride concentration and precipitating the nucleic acid. The residual CTAB is removed by washing the nucleic acid pellet by 70% ethanol.

REAGENTS REQUIRED

(i) Solution A (CTAB extraction solution)

(ii) Solution B (CTAB – 1M NaCl solution)

(iii) Solution C (CTAB precipitation solution)

(iv) Solution D (High salt TE)

(v) 1 x TE (pH 8.0)

(vi) 24 : 1 (Chloroform, Isoamyl alcohol)

(vii) Ethanol

(viii) β – Mercaptoethanol

(ix) RNase A

(x) Polyvinyl pyrolidone (PVP)

(xi) Sodium acetate

(xii) 5 x TBE Buffer (Stock solution) – 27g Tris, 13.75g Boric acid, 1.8629g EDTA (Tris base, Boric acid and EDTA were dissolved in 400 mL of double distilled water, adjusted the pH to 8.3 and made up to the final volume). The solution was stored at room temperature.

(xiii) 1x TBE Working solution – 100mL of the 5 x TBE stock was diluted to 500mL using double distilled autoclaved water.

(xiv) 0.7% Agarose gel solution – 0.7g of agarose was dissolved in 100mL of 1 x TBE buffer

(xv) Loading dye – 50mg Bromophenol Blue, 3mL of glycerol, 6mL of TE (pH 8.0), 0.5mL of Ethidium Bromide.

(xvi) Ethidium Bromide – 10mg / mL (10mg of Ethidium bromide was dissolved in 1mL of double distilled water)

MATERIALS REQUIRED

(i) Mortar and pestle (pulverizer)

(ii) Microcentrifuge

(iii) Dry ice

(iv) 65°C Water bath

(v) Eppendorf tubes

(vi) Micropipettes and tips

(vii) Gel casting apparatus along with the comb

(viii) Electrical adhesive tape

(ix) Electrophoretic tank

(x) Power pack with leads

(xi) Transilluminator

(xii) Other standard labware

PROCEDURE

Prior to the experiment β – Mercaptoethanol was added to the required amount of solution A to give a final concentration of 2% (v/v). The solution A and solution B was warmed to 65 °C.

(i) A sterile pulverizer is chilled with liquid nitrogen or dry ice.

(ii) In the chilled pulverizer reduce the plant tissue to a fine powder

(iii) Transfer the powder to an organic solvent resistant test tube.

(iv) Add 4mL of solution A to the pulverized tissue, mix to wet thoroughly.

(v) Incubate the tube for 10 – 60 minutes at 65°C in a water bath.

(vi) After the incubation, extract the homogenate with an equal volume of 24 : 1 chloroform : isoamyl alcohol.

(vii) Mix well by inversion and centrifuge for 5 minutes at 8,000 rpm at 4°C.

(viii) Recover the aqueous phase and add one-tenth volume of solution B to the aqueous phase, mix well by inversion.

(ix) Extract the above solution with an equal volume of chloroform: isoamyl alcohol.

(x) Mix well the contents by inversion and centrifuge at 8,000 rpm at 4°C for 5 minutes.

(xi) Recover the top aqueous phase and add to that exactly 1 volume of solution C, mix well by inversion (If precipitate is visible proceed to step viii. if not incubate the mix for 30 minutes at 65°c).

(xii) Spin the sample for 5 minutes at 10,000 rpm at 4°C.

(xiii) Discard the supernatant and resuspend the pellet in solution D (high salt TE buffer).

(xiv) Precipitate the nucleic acid by adding 0.6 volume of absolute alcohol, mix well and keep at -20°C for 30min.

(xv) Centrifuge for 15 minutes at 10,000 rpm at 4°c.

(xvi) Discard the supernatant and wash the pellet with 70% ethanol.

(xvii) Air dry and dissolve the pellet in 50µL of 1x TE buffer.

(xviii) Check the dissolved sample on a 0.7% agarose gel.

(xix) Mix 10µL of the sample with 3µL of gel loading dye and load to the wells.

(xx) Run electrophoresis at 50 volts for 1 – 2 ½ hours.

(xxi) Visualize and document the gel under an UV transilluminator.

OBSERVATION

A single band of high molecular weight Plant genomic DNA is observed in the gel (Fig.1).

Fig.1. 0.7% Agarose gel electrophoregram of Plant Genomic DNA isolated by kit method

Lane : 1-3 : Plant Genomic DNA

Run Voltage : 50V

Run Buffer : 1X TBE

Run Time : 1 – 1 ½ hours

RESULT

The Plant genomic DNA isolated is found to be of high molecular weight and pure.

EXPT. NO. :

DATE :

ISOLATION OF HUMAN GENOMIC DNA BY KIT METHOD

AIM

To isolate the Human genomic DNA from the given blood sample by Kit Method.

PRINCIPLE

For cloning, southern blotting and other purposes it is necessary to isolate high molecular weight (HMW >50 kilobases) genomic DNA free of proteins and other compounds that will interfere with subsequent enzymatic reactions.

DNA extraction kit from whole blood is designed for quick, reliable and reproducible higher yields of genomic DNA from fresh blood and frozen blood. The steps include lysis of the red blood cells using solution A, followed by lysis of the white blood cells and nuclei by chaotropic solution B. Finally the genomic DNA is concentrated and desalted by ethanol precipitation. The isolated DNA can be used directly for PCR amplification and restriction digestion.

REAGENTS REQUIRED

(i) Solution A

(ii) Solution B

(iii) Absolute ethanol

(iv) 70% Ethanol

(v) Solution C

(vi) 5 x TBE Buffer (Stock solution) – 27g Tris, 13.75g Boric acid, 1.8629g EDTA (Tris base, Boric acid and EDTA were dissolved in 400mL of double distilled water, adjusted the pH to 8.3 and made up to the final volume). The solution was stored at room temperature.

(vii) 1x TBE Working solution – 100mL of the 5 x TBE stock was diluted to 500mL using double distilled autoclaved water.

(viii) 0.7% Agarose gel solution – 0.7g of agarose was dissolved in 100mL of 1 x TBE buffer

(ix) Loading dye – 50mg Bromophenol blue, 3mL of glycerol, 6mL of TE (pH 8.0), 0.5mL of Ethidium bromide.

(x) Ethidium bromide – 10mg / mL (10mg of Ethidium bromide was dissolved in 1mL of double distilled water)

MATERIALS REQUIRED

(i) Microcentrifuge

(ii) 15mL Centrifuge tubes

(iii) Pasture pipettes

(iv) Eppendorf tubes

(v) Micropipettes and tips

(vi) gel casting apparatus along with the comb

(vii) Electrical adhesive tape

(viii) Electrophoretic tank

(ix) Power pack with leads

(x) Transilluminator

(xi) Other standard labware

PROCEDURE

Prior to use solution A was diluted to 1X.

(i) Pipette out about 300µL of peripheral venous blood into an EDTA coated 1.5mL vial and centrifuge at 5,000rpm for 8 minutes at room temperature.

(ii) Remove the supernatant (serum) by pipetting.

(iii) Resuspend the pellet in 1mL of 1X solution, mix by inversion and keep at RT for 10 minutes.

(iv) Spin the above sample at 8,000rpm for 5 minutes at RT.

(v) Discard the supernatant and resuspend the pellet in 1mL of solution A.

(vi) Centrifuge at 8,000rpm for 5 minutes.

(vii) To the WBC pellet, add 600µL of solution B, resuspend and keep at RT for 5 minutes for complete lysis.

(viii) Centrifuge the sample at 10,000rpm for 10 minutes at RT.

(ix) To the supernatant add 1mL of absolute ethanol, mix by gentle inversion and centrifuge at 10000rpm for 25 minutes.

(x) Discard the supernatant and wash the pellet with 70% ethanol.

(xi) Air dry and dissolve the pellet in 50µL of 1x TE buffer.

(xii) Check the dissolved sample on a 0.7% agarose gel.

(xiii) Mix 10µL of the sample with 3µL of gel loading dye and load to the wells.

(xiv) Run electrophoresis at 50 volts for 1 – 2 ½ hours.

(xv) Visualize and document the gel under an UV transilluminator.

OBSERVATION

A single band of high molecular weight Human Genomic DNA is observed in the gel (Fig.1).

Fig. 1. 0.7% Agarose gel electrophoregram of Human Genomic DNA isolated by kit method

Lane : 1-3 : Human Genomic DNA

Run Voltage : 50V

Run Buffer : 1X TBE

Run Time : 1 – 2 ½ hours

RESULT

The Human Genomic DNA isolated is found to be of high molecular weight and pure.

EXPT. NO. :

DATE :

TOTAL RNA ISOLATION

AIM

To isolate total RNA from the given *E. coli* Bacterial cells by Kit Method.

PRINCIPLE

Ribonucleic acid (RNA) is a nucleic acid polymer consisting of covalently bound ribonucleotides which contains ribose sugar, a phosphate and a nitrogeneous base. It is transcribed from DNA. There are three types of RNA – messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA).

The basic steps involved in the isolation of RNA is – disruption of cells or tissues, denaturation and removal of proteins and DNA, purification of endogenous ribonucleases and finally purification of RNA from contaminating denatured DNA and proteins. Most methods employ the use of strong denaturants (e.g.guanidine thiocynate) or detergents like SDS that inhibit ubiquitous RNase. Since RNase is found almost everywhere, it is essential that any item that could contact the purified RNA is RNase free. All surfaces, glassware and gel equipments are decontaminated by treating with diethyl pyrocarbonate (DEPC) to prevent degradation of RNA.

A pure intact RNA is the starting template for the elucidation of structure, amount, size and synthesis rate of the RNA produced. Isolation of pure, intact RNA is important for cDNA cloning and is essential for analyzing gene expression.

REAGENTS REQUIRED

(i) Overnight *E. coli* Bacterial culture

(ii) Extraction buffer

(iii) 3M Sodium acetate

(iv) DTT

(v) Proteinase K

(vi) Absolute ethanol

(vii) 70% Ethanol

(viii) 5 x TBE Buffer (Stock solution) – 27g Tris, 13.75g Boric acid, 1.8629g EDTA. (Tris base, Boric acid and EDTA were dissolved in 400mL of double distilled water, adjusted the pH to 8.3 and made up to the final volume). The solution was stored at room temperature.

(ix) 1X TE 1x TBE Working solution – 100mL of the 5 x TBE stock was diluted to 500mL using double distilled autoclaved water.

(x) 0.7% Agarose gel solution – 0.7g of agarose was dissolved in 100mL of 1 x TBE buffer

(xi) Loading dye – 50mg Bromophenol blue, 3mL ofglycerol, 6mL of TE (pH 8.0), 0.5mL of Ethidium bromide.

(xii) Ethidium bromide – 10mg / mL (10mg of Ethidium bromide was dissolved in 1mL of double distilled water)

MATERIALS REQUIRED

(i) Microcentrifuge

(ii) Eppendorf tubes

(iii) Micropipettes and tips

(iv) Gel casting apparatus along with the comb

(v) Electrical adhesive tape

(vi) Electrophoretic tank

(vii) Power pack with leads

(viii) Transilluminator

(ix) Other standard labware

PROCEDURE

(i) Harvest the o/n culture by centrifugation at room temperature at 10,000rpm for 2 minutes.

(ii) Resuspend the cell pellet in 330µL of extraction buffer and incubate at 37°C for 20 minutes.

(iii) After the incubation, add 30µL of sodium acetate and an equal volume of chloroform : isoamyl alcohol, mix and centrifuge at 10,000rpm for 10 minutes at 4°C.

(iv) Transfer the aqueous phase into another eppendorf tube.

(v) Add an equal volume of chloroform : isoamyl alcohol, mix and centrifuge at 10,000rpm for 10 minutes at 4°C.

(vi) Collect the aqueous phase in a fresh vial and add $1/10^{th}$ volume of sodium acetate, mix well and incubate on ice for 10 minutes.

(vii) After the incubation, centrifuge the above sample at 10,000rpm for 10 minutes at 4°C.

(viii) Collect the supernatant in a fresh vial and add double the volume of absolute alcohol, mix well and incubate at – 20°C for 2 hours.

(ix) After the incubation, centrifuge the above sample at 10000rpm for 10 minutes at 4°C.

(x) Discard the supernatant and wash the pellet with 70% ethanol.

(xi) Air dry and dissolve the pellet in 50µL of 1x TE buffer.

(xii) Check the dissolved sample on a 1.0% agarose gel.

(xiii) Mix 10µL of the sample with 3µL of gel loading dye and load to the wells.

(xiv) Run electrophoresis at 50 volts for 1 – 2 ½ hours.

(xv) Visualize and document the gel under an UV transilluminator.

OBSERVATION

The total RNA is seen as 2 sharp bands of ribosomal RNA with 18s rRNA migrating ahead of 28s rRNA (Fig.1).

Fig. 1. 1.0 % Agarose gel electrophoregram of total RNA isolated from *E.coli*

Lane : 2	: Low range marker
Lane : 1	: Total RNA isolated from *E.coli*
Run Voltage	: 50V
Run Buffer	: 1X TBE
Run Time	: 1 – 2 ½ hours

RESULT

The total RNA is isolated. The predominant forms 28s rRNA and 18s rRNA resolves into two separate sharp bands according to their size.

EXPT. NO. :

DATE :

ISOLATION OF PLASMID DNA USING SPIN PREP ™ PLASMID KIT METHOD

AIM

To isolate the Plasmid DNA from *E. coli* cells using Bangalore genei Spin prep ™ plasmid kit.

PRINCIPLE

The spin column method used for plasmid preparation is a simple, rapid and cost effective method. The spin column method uses the alkaline lysis method for plasmid isolation and silica based DNA binding membrane technology for plasmid isolation and purification. The DNA of interest can be isolated by virtue of its ability to bind silica in the presence of high concentrations of chaotropic salts. These salts are then removed with an alcohol-based wash and the DNA is eluted in a low-ionic-strength solution such as TE buffer or water. The binding of DNA to silica seems to be driven by dehydration and hydrogen bond formation, which competes against weak electrostatic repulsion. Hence, a high concentration of salt will help to drive DNA adsorption onto silica, and a low concentration will release the DNA.

Bacterial cells are harvested, lysed by alkaline lysis and then are subjected to adsorption onto a silica membrane. In the first few elutions the cell debris and the alcohol are removed and the final elution with the elution buffer elutes the DNA from the column. The recovered plasmid DNA has predominantly supercoiled form. The DNA preparation is free of RNA and genomic DNA contamination. The purified DNA can be used for molecular biology applications like restriction digestion, cloning PCR, radio labelling and automated sequencing.

REAGENTS REQUIRED

(i) Solutiong1 – Bacteril resupension buffer

(ii) Solution g2 – Bacterial Lysis buffer

(iii) Soluction g3 – Neutralization buffer

(iv) Wash Buffer I & II

(v) Elution Buffer 5 x TBE Buffer (Stock solution) – 27g Tris, 13.75g Boric acid, 1.8629g EDTA. (Tris base, Boric acid and EDTA were dissolved in 400mL of double distilled water, adjusted the pH to 8.3 and made up to the final volume). The solution was stored at room temperature.

(vi) 1x TBE Working solution – 100mL of the 5 x TBE stock was diluted to 500mL using double distilled autoclaved water.

(vii) 0.7% Agarose gel solution – 0.7g of agarose was dissolved in 100mL of 1 x TBE buffer.

(viii) Loading dye – 50mg Bromophenol Blue, 3mL of glycerol, 6mL of TE (pH 8.0), 0.5mL of Ethidium Bromide.

(ix) Ethidium Bromide – 10mg / mL (10mg of Ethidium bromide was dissolved in 1mL of double distilled water)

MATERIALS REQUIRED

(i) Cooling centrifuge

(ii) Spin columns

(iii) Eppendorf tubes

(iv) Micropipettes and tips

(v) Gel casting apparatus along with the comb

(vi) Electrical adhesive tape

(vii) Electrophoretic tank

(viii) Power pack with leads

(ix) Transilluminator

(x) Other standard labware

PROCEDURE

(i) Harvest the o/n culture by centrifugation at room temperature at 10,000 rpm for 2 minutes.

(ii) Decant the supernatant and centrifuge for 30 seconds.

(iii) Remove the liquid with a pipette tip.

(iv) Gently resuspend the cell pellet in 250µL of solution g2 (suspension buffer) by vortexing.

(v) Add 250µL of solution g2 (lysis buffer) and invert the tube once to mix (Inverting more than once causes chromosomal DNA contamination).

(vi) Add 350µL of solution G3 (neutralizing solution) and invert once to mix.

(vii) Centrifuge the sample for 10 minutes at 10000 rpm.

(viii) Transfer the clear supernatant to spin column placed over a collection tube (avoid the white precipitate).

(ix) Centrifuge the spin column for 1minute at 10000 rpm.

(x) Lift out the column, discard the liquid from the collection tube and replace the column to the collection tube.

(xi) Add 500µL of Buffer I (wash buffer) to the spin column and centrifuge for 2 minutes at 10,000 rpm.

(xii) Discard the flow through and centrifuge the spin column at 10000 rpm for 1 minute.

(xiii) Repeat the above steps with 0.75mL of wash buffer II.

(xiv) Place the spin column in a new 1.5mL collection tube.

(xv) Add 25µL of elute buffer (Buffer C) directly onto the middle of the white spin filter membrane.

xvi) Keep the tube at room temperature for 2 minutes.

xvii) Centrifuge the spin column for 30 seconds.

xviii) Remove the spin column and close the tube lid.

xix) Check the isolated plasmid DNA on a 1.0% agarose gel.

xx) Mix 10µL of the sample with 3µL of gel loading dye and load to the wells.

xxi) Run electrophoresis at 50 volts for 1 – 2 ½ hours.

xxii) Visualize and document the gel under an UV transilluminator.

OBSERVATION

A single band of covalently closed circular Plasmid DNA is observed in the gel Fig.1).

Fig. 1. 1.0% Agarose gel electrophoregram of Plasmid DNA isolated by manual method

Lane : 1 : Plasmid DNA

Run Voltage : 50V

Run Buffer : 1X TBE

Run Time : 1 – 2 ½ hours

RESULT

The Plasmid DNA isolated is found to be covalently closed circular form.

EXPT. NO. :

DATE :

QUANTIFICATION OF DNA USING SPECTROPHOTOMETRY

AIM

To determine the Concentration and Purity of the given DNA sample by using UV-spectrophotometry

PRINCIPLE

An essential element of cellular and molecular biology is the ability to quantify nucleic acids in large number of samples at a sensitivity that enables the determination of small amount of sample. Earlier method utilized measurement of hydrolyzed nucleic acid by quantification of the various components from hydrolysis such as the phosphorus or the ribose and or deoxyribose sugar of the backbone, to estimate total nucleic acid concentration. These assays are time consuming and or relatively insensitive.

Nucleic acids (i.e., DNA and RNA) are often characterized and quantified using their *absorption spectra*, as measured by spectrophotometer. UV absorbance spectroscopy has been the traditional method of quantifying DNA in molecular biology laboratories. It is also used to estimate the purity of the DNA solution. The absorptivity constant for nucleic acid depends on its base composition and on whether it is single-stranded or double-stranded. Purines absorbance maximum is slightly below 260; pyrimidines maximum is slightly above 260. Purines have a higher molar absorptive than pyrimidines. Therefore, the absorbance maximum and absorptivity of a segment of DNA depends on its base composition.

Nucleic acid has an absorption peak at 260nm and proteins have an absorption peak at 280nm. DNA analysis reveals that DNA absorbs UV light maximally at 260nm, owing to the chemical nature of the nitrogenous bases. Deoxyribose and phosphate groups do not contribute appreciably to absorption. Proteins have little absorbance at this wavelength. The absorptivity constant for a particular protein at 280nm depends on its composition. Proteins have two absorbance peaks in the UV region, one between 215 –

230nm, where peptide bonds absorb, and another at about 280nm due to light absorption by aromatic amino acids (tyrosine, tryptophan and phenylalanine). Proteins that contain a higher percentage of aromatic amino acids have higher absorption at 280nm than those with fewer.

The reading at 260nm allows calculation of the concentration of the nucleic acids in the sample. An OD of 1 corresponds to 50µg for double stranded DNA and 40µg for single stranded DNA and RNA, 33µg for single stranded oligonucleotides. The ratio between the reading at 260nm and 280nm provides an estimate of the purity of the nucleic acids. A pure preparations of DNA and RNA have an OD 260 : 280 values between 1.8 and 2.0. If there is significant protein or phenol the OD 260 : 280 ratio will be less than the values given above.

It is important to quantify DNA in a sample in order to perform cloning, translation studies, southern blotting, restriction analysis and transformation studies. The accuracy and precision of estimates of DNA concentration are critical factors for efficient use of DNA samples in high-throughput genotype and sequence analyses.

REAGENTS REQUIRED

(i) DNA Sample

(ii) 1 x TE buffer

MATERIALS REQUIRED

(i) UV Spectrophotometer

(ii) Quartz cuvette

(iii) Eppendorf tubes

(iv) Micropipettes

(v) Other standard labware

PROCEDURE

(i) Pipette out 20µL of the given DNA sample in a sterile eppendorf tube.

(ii) Mix with 980µL of 1 x TE buffer, vortex briefly and spin at room temperature for 1 minute.

(iii) Using UV Spectrophotometer, 1 x TE buffer as a reference, take the absorbance at 260nm and 280nm.

(iv) Calculate the concentration of the given DNA sample using the formula : If OD at 260nm = 1, the concentration is 50 µg / mL ds DNA.

The concentration of the unknown sample = Measured OD at 260nm x $\frac{50\ \mu g / mL}{1}$ x Dilution factor

(v) Find out the purity of the given sample by the ratio OD at 260nm / OD at 280nm.

(vi) Obtain the total yield of the given sample by :

DNA concentration in µg / mL x total volume in mL.

OBSERVATION

The observed values at 260nm and 280nm are given in table I.

Table 1 : OD Values at 260nm and 280nm

S. No.	OD at 260nm	OD at 280nm	Purity (OD 260 / 280nm)	Concentration (µg / mL)

RESULT

The given DNA samples are of high concentration and with less or nil protein contamination.

EXPT. NO. :

DATE :

DNA EXTRACTION FROM AGAROSE GEL

AIM

To extract the DNA from the agarose gel using Genei Gel Extraction kit.

PRINCIPLE

The Gel Extraction Kit is designed for efficient extraction of DNA from agarose gels and reaction mixtures. It provides a rapid method to separate DNA fragment of interest from RNA, proteins, primers, unincorporated nucleotides, excess linkers, enzymes and salts, residual phenol, chloroform or dyes (ethidium bromide, bromphenol blue, etc.). It is also an alternative method to ethanol precipitation. The Silica Bead DNA Gel Extraction Kit utilizes the modified glass beads protocol of Vogelstein and Gillespie. A chaotropic salt is used to dissolve agarose gel and denature enzymes. In the presence of chaotropic salts, DNA binds to the specially prepared glass particles. The chaotropic salts and impurities are washed from the glass particles containing bound DNA. The washing steps are followed by elution of the DNA in TE buffer or water.

The Silica based DNA Gel Extraction Kit can be used to purify a wide range of DNA fragments with recoveries of >80%. Typically, recoveries are 80-90% for Gel Extraction. The entire procedure can be completed in 20 minutes and the eluted DNA is ready to use in restriction digestion, ligation, PCR, and sequencing reactions.

REAGENTS REQUIRED

(i) Specially Formulated Glass Powder.

(ii) Sodium Iodide

(iii) Wash Buffer

(iv) 5 x TBE Buffer (Stock solution) – 27g Tris, 13.75g Boric acid, 1.8629g EDTA (Tris base, Boric acid and EDTA were dissolved in 400mL of double distilled

water, adjusted the pH to 8.3 and made up to the final volume). The solution was stored at room temperature.

(v) x TBE Working solution – 100mL of the 5 x TBE stock was diluted to 500mL using double distilled autoclaved water.

(vi) 0.7% Agarose gel solution – 0.7g of agarose was dissolved in 100mL of 1 x TBE buffer

(vii) Loading dye – 50mg Bromophenol Blue, 3mL of Glycerol, 6mL of TE (pH 8.0), 0.5mL of Ethidium Bromide.

(viii) Ethidium Bromide – 10mg / mL (10mg of Ethidium bromide was dissolved in 1mL of double distilled water)

MATERIALS REQUIRED

(i) Centrifuge

(ii) 15mL Centrifuge tubes

(iii) Eppendorf tubes

(iv) Micropipettes and tips gel casting apparatus along with the comb

(v) Electrical adhesive tape

(vi) Electrophoretic tank

(vii) Power pack with leads

(viii) Transilluminator

(ix) Other standard lab wares

PROCEDURE

Prior to use solubilise the glass solution by vortexing it, till it forms a homogeneous mixture.

(i) The DNA band of interest was excised from ethidium bromide stained agarose gel with a sharp blade.

(ii) To the above 2.5 volumes of sodium iodide solution was added

(iii) The above sample was incubated at 45° – 55°C for 2 to 3 minutes.

(iv) The contents were mixed thoroughly by inverting 2 – 5times.

(v) The sample was again incubated at 45° – 55°C for 5 minutes.

(vi) 15µL of glass solution was added to the above sample.

(vii) The contents were mixed thoroughly and left at room temperature for 10 – 15 minutes with occasional mixing.

(viii) Spun at 12000rpm for 1 minute.

(ix) The supernatant was discarded and to the pellet (DNA bound to the glass) 200µL of wash buffer was added, vortexed and spun at 12000rpm for 1 minute.

(x) The above step was repeated.

(xi) The supernatant was discarded and to the pellet, 30 – 40µLof 1xTE buffer was added.

(xii) The pellet was resuspended by mild vortexing and incubation at 45° – 55°C for 5 minutes.

(xiii) The above sample was spun at 12000 rpm for 1 minute and the supernatant was collected in a fresh tube.

(xiv) The efficiency of elution was checked on a 1% gel.

(xv) 10µL of the eluted sample was mixed with 3µL of gel loading dye and loaded to the well.

(xvi) Electrophoresis was carried out at 50volts for 1 – 2 ½ hours.

(xvii) The gel was visualized under an UV transilluminator and documented.

(xviii) Eluted DNA is now ready for downstream applications like PCR, sequencing etc.

OBSERVATION

A single band of high molecular weight Genomic DNA was observed in the gel. Absence of smearing indicated the absence of salt contaminations (Fig.1).

Fig. 1. 1.0% Agarose gel electrophoregram of Plasmid DNA isolated by manual method

Lane : 1 : DNA before elution

Lane : 2 DNA after elution

Run Voltage : 50V

Run Buffer : 1X TBE

Run Time : 1 – 2 ½ hours

RESULT

The DNA extracted was found to be free of salt and other contaminations.

EXPT. NO. :

DATE :

SOUTHERN HYBRIDIZATION

AIM

To demonstrate Southern hybridization by a) Electrophoretically separating the DNA molecules by agarose gel electrophoresis, b) Transferring the separated DNA molecules from agarose gel to nylon membrane, c) Immobilizing the DNA to a nylon membrane and d) Hybridizing and non-isotopic detection of DNA of interest by kit Method.

PRINCIPLE

The Southern blot (named for its inventor – Prof. E. M. Southern) usesgel electrophoresis together with hybridization probes to characterize restriction fragments of genomic DNA (or DNA from other sources, such as plasmids). A probe is prepared that will hybridize with a particular sequence, which might be the cDNA coding for one protein or a repetitive sequence that occurs more than once in a genome. The DNA to be analyzed is digested to completion with a restriction endonuclease (and sometimes sequentially with two or more restriction enzymes). It is applied to an appropriate gel and electrophoresis is performed in a manner that will maximally separate restriction fragments in the expected size range. A set of standards of known size is run in one lane of the gel. The fragments are then "blotted" onto a nitrocellulose membrane and hybridized with the probe. After pre-hybridization to reduce non-specific hybridization with the probe, the membrane is hybridized with the desired labelled nucleic acid probe. The probe is labelled before hybridization either radioactively or enzymatically (e.g. Alkaline pospatase or Horseradish peroxidase). The membrane is subsequently washed to remove unbound and weakly bound probe, and is then exposed to X-ray film or incubated with substrate solution to develop colour. .

Southern blots are used to identify specific DNA sequences, in the analysis of genetic diseases, in DNA fingerprinting and analysis of PCR products. It also allows in

determining the molecular weight of a restriction fragment, to measure relative amount in different samples and to locate a particular sequence of DNA within a complex mixture.

REAGENTS REQUIRED

(i) DNA marker

(ii) Bioatinylated probe

(iii) Pre - hybridization buffer : 1g of blocking powder was added to 10mL of pre - hybridization buffer.

(iv) Hybridisation buffer: 1g of blocking powder was added to 10mL of hybridization buffer.

(v) 2 x Wash buffer

(vi) Blocking buffer: 1g of blocking powder was added to 10mL of blocking buffer.

(vii) Streptavidin HRP conjugate : 9mL of conjugate dilution buffer was prepared by adding 9µL of Tween–20 to the conjugate dilution buffer. From this, 9mL of conjugate dilution buffer was mixed with 3µL of streptavidin – HRP conjugate for each experiment.

(viii) Conjugate dilution buffer

(ix) 10 x Substrate buffer : 0.5mL of substrate solution was mixed with 4.5mL of double distilled water togive a final volume of 5mL substrate solution of 1 x concentration.

(x) 10 x Electrotransfer buffer : 25mL of 10 x Electrotransfer buffer was diluted with 225mL of double distilled water to give a final volume of 250mL electrotransfer buffer at 1x concentration.

(xi) Blocking powder

(xii) Tween - 20

(xiii) Agarose

(xiv) Ehidium bromide

(xv) Gel loading dye

(xvi) 10 x TAE buffer

MATERIALS REQUIRED

(i) Electroblot

(ii) Hot air oven

(iii) Shaker incubator (45°C)

(iv) UV-transilluminator

(v) Filter paper

(vi) Nylon membrane

(vii) Petridish

(viii) Eppendorf tubes

(ix) Micro pipettes and tips

(x) Other standard labware

PROCEDURE

Carry out the experiment by wearing gloves. All the buffers are to be used at 1x concentration.

(i) Prepare 1.0% agarose gel containing ethidium bromide.

(ii) Load 20µL of ready to use DNA marker to the well.

(iii) Run electrophoresis at 50 - 100 volts until the dye reaches 4.5cm from the well.

(iv) Cut the DNA marker lane from the agarose gel in such a way that the gel measures about 4 to 4.5cm.

(v) Cut the filter paper (2 No.) and nylon membrane exactly to the size of the cut gel.

(vi) Wet the cut gel, nylon membrane, filter papers and the electrotransfer cassette in 1x electro transfer buffer.

(vii) Assemble the electrophoretic transfer apparatus as given below – First place filter paper on the cathode cassette cover followed by the cut gel and nylon membrane (the soft side of the nylon membrane was placed to the cut gel). Place the wet filter paper on the nylon membrane followed by anode cassette cover. Tightened the cassette using the screws provided (Ensure that no air bubbles are present between any of the layers of filter paper, cut gel and the nylon membrane).

(viii) Insert the cassette into the apparatus filled with 250 mL of 1x electrotransfer buffer.

(ix) Run the electrophoretic transfer at 50 volts for 3 ½ hours.

(x) After the completion of transfer, turn off the power supply and the remove the cassette from the apparatus. Discard the buffer.

(xi) Gently take the nylon membrane from the cassette and place on a thin transparent polythene sheet.

(xii) Place the nylon membrane with the transparent polythene sheet on a UV transilluminator (expose the soft side of the membrane containing transferred DNA to UV light) with UV lamps switched on for 20 minutes.

(xiii) After 20 minutes, switch off the lamps and place the membrane in a petridish.

(xiv) Incubate in a hot air oven at 70°C for 30 minutes.

(xv) After incubation, bring the temperature of the petridish containing the membrane to room temperature.

(xvi) Add 10mL of pre - hybridisation buffer to the petridish and incubate at 45°C in incubator shaker with mild shaking (70 - 90 rpm) for 45 minutes.

(xvii) Discard the pre - hybridisation buffer after the incubation and add 10mL of hybridization buffer to the petridish membrane.

(xviii) Mean while keep one vial of biotinylated probe for 10 minutes in a boiling water bath and immediately chill by placing it on ice for 5-10 minutes.

(xix) Add this probe to the hybridization buffer in the petridish (Rinse the empty vial with 300µL of hybridization buffer and add it to the petridish).

(xx) Incubate the petridish at 45°C incubator shaker with mild shaking at 70 rpm for 16 hours.

(xxi) After incubation, decant off the hybridization buffer and add 10mL of wash buffer A to the

(xxii) petridish with gentle swirling for 5 minutes at room temperature.

(xxiii) Repeat the wash twice. Discard the buffer during each step.

(xxiv) Add 10mL of 1x pre warmed (70°C) wash buffer B to the petridish and gently swirl.

(xxv) Incubate the petridish at 70°C for 5 minutes in a hot air oven and swirle gently.

(xxvi) Repeat the wash twice. Discard the buffer during each step.

(xxvii) Add 10mL of blocking buffer to the petridish and incubate at room temperature for 1 hour with gentle rocking.

(xxviii)Discard the blocking buffer.

(xxix) Add 9mL of diluted HRP-streptavidin conjugate to the petridish and incubate at room temperature for 20 minutes with gentle rocking.

(xxx) Discard the conjugate buffer.

(xxxi) Add 10mL of 1 x wash buffer C to the petridish and incubate the petridish at room temperature for 5 minutes with gentle rocking.

(xxxii) Repeat the wash twice. Discard the buffer during each step.

(xxxiii) Add 10mL of 1x wash buffer D to the petridish and incubate the petridish at room temperature for 5 minutes withg entle rocking.

(xxxiv) Repeat the wash twice. Discard the buffer during each step.

(xxxv) Add 5mL of substrate solution and swirl gently at room temperature for 15 – 20 minutes until a blue colour band develops.

(xxxvi) Arrest the reaction once the blue colour develops by placing the membrane in distilled water.

OBSERVATION

A single blue band of the marker DNA is observed on the nylon membrane (Fig.1).

RESULT

The transferred marker DNA is appeared as a single blue band on the nylon membrane.

EXPT. NO. :

DATE :

POLYMERASE CHAIN REACTION

AIM

To amplify the given DNA sample using Polymerase Chain Reaction by Kit Method.

PRINCIPLE

PCR is a technique used to amplify the number of copies of a specific region of DNA in order to produce enough DNA to be adequately tested. PCR was invented by Karris Mullis. PCR has become one of the most widely used techniques in molecular biology. It is a rapid and simple means of producing relatively large number of copies of DNA molecules from minute quantities of source DNA material.

The reaction requires two oligonucleotide primers, flanking the sequence of interest that is to be amplified and the heat-stable *Taq* DNA polymerase from *Thermophilus aquaticus*. A mixture of DNA containing the desired sequence, a large excess of primers, *Taq* polymerase, and deoxyribonucleoside triphosphates is subjected to the following sequence of steps :

(i) **Denaturation of DNA at 90 – 95^{0}C :** During this step the DNA is melted by disrupting the hydrogen bonds between complementary bases of the DNA strands.

(ii) **Annealing of Primers :** In this step the reaction temperature is lowered so that the primers can anneal to the single-stranded DNA template. Brownian motion causes the primers to move around, and DNA-DNA hydrogen bonds are constantly formed and broken between primer and template. Stable bonds are only formed when the primer sequence exactly matches the template sequence, and to this short section of double-stranded DNA, the DNA polymerase binds and begins DNA synthesis. The temperature at this step depends on the melting temperature of the primers, and is usually between 50 – 64°C for 20 – 40 seconds.

(iii) **DNA Extension** : The DNA polymerase synthesizes new DNA strands complementary to the DNA template strands. The temperature at this step depends on the DNA polymerase used. *Taq* polymerase has a temperature optimum of 70 – 75°C, thus in most cases a temperature of 72°C is used. The hydrogen bonds between the extended primer and the DNA template are now strong enough to withstand forces breaking these attractions at the higher temperature. Primers that have annealed to DNA regions with mismatching bases dissociate from the template and are not extended. The DNA polymerase condenses the 5'-phosphategroup of the dNTPs with the 3'-hydroxylgroup at the end of the nascent (extending) DNA strand, i.e., the polymerase adds dNTP's that are complementary to the template in 5' to 3' direction, thus reading the template in 3' to 5' direction. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases in one minute.

The cycle is then repeated by simply changing the temperature of the reaction mixture between these values. Since the newly-synthesized DNA serves as additional template for DNA synthesis, each subsequent round of synthesis will have twice as much DNA template for primer annealing and extension, resulting in the geometrical amplification of the original DNA sequence. 25 to 35 cycles can amplify a DNA sequence more than a million times. Other sequences, contained in the original DNA sample but, not bound by the primers, are not amplified. Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene. Suppose there is only one copy of the wanted gene before the cycling starts, after one cycle, there will be 2 copies, after two cycles, there will be 4 copies, three cycles will result in 8 copies and so on. After 20 cycles, roughly 1 million copies exist, or enough material to detect the desired DNA by conventional means such as colour reaction. A final elongation is given to ensure that any remaining single-stranded DNA is fully extended.

PCR technology is used in bio-analytical applications that include amplification of human – specific DNA sequences, direct sequencing of PCR products, detecting mutations, disease diagnostics, drug development, monitoring cancer therapy, detecting bacterial and viral infections, sex determination of prenatal cells and in forensic fingerprinting from collected evidence and also in identifying individuals with cancer susceptible genes.

REAGENTS REQUIRED

(i) PCR Kit components –

(a) Double distilled Water.

(b) 10 x Assay Buffer

(c) dNTPs Mix

(d) Forward Primer

(e) Reverse Primer

(f) Template DNA

(g) *Taq* DNA Polymerase

(ii) 5 x TBE Buffer (Stock solution) – 27g Tris, 13.75g Boric acid, 1.8629g EDTA. (Tris base, Boric acid and EDTA were dissolved in 400mL of double distilled water, adjusted the pH to 8.3 and made up to the final volume). The solution was stored at room temperature.

(iii) 1x TBE Working solution – 100mL of the 5 x TBE stock was diluted to 500mL using double distilled autoclaved water.

(iv) 0.7% Agarose gel solution – 0.7g of agarose was dissolved in 100mL of 1 x TBE buffer

(v) Loading dye – 50mg Bromophenol blue, 3mL of glycerol, 6mL of TE (pH 8.0), 0.5mL of Ethidium bromide.

(vi) Ethidium bromide – 10mg / mL (10mg of Ethidium bromide was dissolved in 1mL of double distilled water).

MATERIALS REQUIRED

(i) Thermocycler

(ii) Centrifuge

(iii) PCR tubes

(iv) Micropipettes and Tips

(v) Gel casting apparatus along with the comb

(vi) Electrical adhesive tape

(vii) Electrophoretic tank

(viii) Power pack with leads

(ix) Transilluminator

(x) Other standard labware

PROCEDURE

(i) Thaw and spin all the buffers prior to use.

(ii) Take a sterile 0.5mL PCR tube and add the following components in a step wise manner as given in Table I.

Table I : PCR Reaction Mixture

S. No.	PCR Kit components added	Volume added (µL)
(i)	Double distilled water	38.0
(ii)	10 x *Taq* Buffer	5.0
(iii)	dNTP Mix	3.0
(iv)	Primer F	1.0
(v)	Primer R	1.0
(vi)	Template DNA	1.0
(vii)	Taq DNA polymerase	1.0
	Total reaction volume	50.0

(iii) Set the thermal cycler with the cycle conditions as given in Table II.

Table II : PCR Reaction Condition

S. No.	Steps	Temperature	Reaction time	No of cycles
(i)	Initial denaturation	94°C	2 minutes	N = 1
(ii)	Denaturation	94 °C	30 seconds	N = 30
(iii)	Annealing	48°C	30 seconds	
(iv)	Extension	72 °C	60 seconds	
(v)	Final extension	72 °C	2 minutes	N = 1
	Hold at 4°C until use			

(iv) After the completion of amplification, remove the PCR tubes from the thermocycler

(v) Check the amplified products along with 100bp ladder on a 1 % Agarose gel.

(vi) Mix 10µL of the PCR products with 3µL of gel loading dye and load to the wells.

(vii) Load 10µL of 100bp DNA Ladder to one of the wells.

(viii) Run electrophoresis at 50 volts for 1 – 2 ½ hours.

(ix) Visualize and document the gel under an UV transilluminator.

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	Hold at 4°C until use			

(iv) After the completion of amplification, remove the PCR tubes from the thermocycler

(v) Check the amplified products along with 100bp ladder on a 1 % Agarose gel.

(vi) Mix 10µL of the PCR products with 3µL of gel loading dye and load to the wells.

(vii) Load 10µL of 100bp DNA Ladder to one of the wells.

(viii) Run electrophoresis at 50 volts for 1 – 2 ½ hours.

(ix) Visualize and document the gel under an UV transilluminator.

OBSERVATION

The PCR amplified product is observed to migrate along with 0.8kb (800bp) fragment of 100bp ladder (Fig.1).

Fig. 1. 1.0 % Agarose gel electrophoregram of PCR product

Lane : 1	: 100bp DNA Ladder
Lane : 2	: PCR Product (800bp)
Run Voltage	: 50V
Run Buffer	: 1X TBE
Run Time	: 1 ½ – 2 ½ hours

RESULT

The amplified product size is found to be 800bp

EXPT. NO. :

DATE :

AGAROSE GEL ELECTROPHORESIS

AIM

To perform Agarose gel Electrophoresis using the given DNA sample

PRINCIPLE

Electrophoresis is an electrochemical separation process in which molecules, such as proteins, enzymes, RNA or DNA fragments are made to move through some medium, under the influence of an electric current. In agarose gel electrophoresis the molecules are separated on the basis of their molecular size charge and / or pH by migration through an agarose gel.

Agarose is an uncharged high molecular weight polysaccharide obtained and purified from red sea weeds (*Rhodophyta*). It is a polysaccharide of alternate 1,4 – linked α–D–galactopyranose and 1,4 – linked 3,6 anhydro α–L–galactopyranose residue and arranged into double helix. It forms a clear gel when dissolved in hot water and allowed to cool.

Agarose gels are hydrocolloids, held together by hydrogen and hydrophobic bonds. They are also chemically inert and easily stained. Agarose gel have a large pore size and can be used to separate macro molecules such as nucleic acids, large proteins and protein complexes. The pore size and sieving characteristics of agarose gel to a certain extent is determined by its concentration. Higher the concentration, smaller the pore size. At agarose concentrations of 0.3 to 2.0% the pores are of the proper size to retard the movement of DNA molecules during electrophoresis. A DNA fragment of a given size migrates at different rates through gels containing different concentrations of agarose. Thus, by using gels of different concentration, it is possible to resolve a wide size-range of DNA fragments.

The separation of DNA on the gels is carried out under an electric field applied to the gel matrix. DNA molecules migrate towards the anode due to the negatively charged

phosphates along the backbone of DNA. Fragments of linear DNA migrate through agarose gel with a mobility that is inversely proportional to the log 10 of their molecular weight. Thus the larger DNA molecules travel at a slower rate than the smaller molecules.

Circular forms of DNA migrate in agarose distinctly from linear DNAs of the same mass. Typically uncut plasmids will appear to migrate more rapidly than the same plasmid when linearized. Additionally most preparations of uncut plasmid contain at least topologically different forms of DNA, corresponding to super coil forms and nicked circles.

After the DNA has been separated across the agarose gel, it is necessary to locate the bands of DNA. Ethidium bromide is commonly used staining agent because it intercalates with the DNA and will fluoresce when exposed to UV light.

Agarose gel electrophoresis is employed to determine the yield and purity of DNA isolated or to check the PCR products, restricted products and to size fractionate the DNA molecules which then could be eluted from the gel.

REAGENTS REQUIRED

(i) 5 x TBE Buffer (Stock solution) – 27g Tris, 13.75g Boric acid, 1.8629g EDTA(Tris base, Boric acid and EDTA were dissolved in 400 mL of double distilled water, adjusted the pH to 8.3 and made up to the final volume). The solution was stored at room temperature.

(ii) 1 x TBE Working solution – 100mL of the 5 x TBE stock was diluted to 500mL using double distilled autoclaved water.

(iii) 0.7% Agarose gel solution – 0.7g of agarose was dissolved in 100mL of 1 x TBE buffer

(iv) Loading dye – 50mg Bromophenol Blue, 3mL of glycerol, 6mL of TE (pH 8.0), 0.5mL of Ethidium Bromide.

(v) Ethidium Bromide – 10mg / mL (10mg of Ethidium bromide was dissolved in 1mL of double distilled water)

MATERIALS REQUIRED

(i) Gel casting apparatus along with the comb

(ii) Electrical adhesive tape

(iii) Electrophoretic tank

(iv) Power pack with leads

(v) Transilluminator

(vi) Micropipettes and tips

(vii) Other standard labware

PROCEDURE

(i) Weigh 700mg of agarose and add to 100mL of 1X TBE buffer (0.7% Agarose)

(ii) Boil the mixture to get a uniform solution (transparent).

(iii) Cool the solution to 60°C.

(iv) Meanwhile prepare the cast by sealing the open ends of the gel platform by cello tape.

(v) Fix the comb in the cast.

(vi) On cooling, add ethidium bromide to the gel solution, swirl gently so that no air bubbles are formed and pour into the cast.

(vii) Leave the gel undisturbed to set.

(viii) After solidification, remove the comb and the cello tape.

(ix) Transfer the gel to the Electrophoretic tank and add buffer to the tank in such a way that the gel is immersed in the buffer to a depth of around 1 mm.

(x) Mix 5µL of the sample and 3µL of the loading dye and load into the wells separately.

(xi) Run Electrophoresis at 50 volts for 1 – 2 ½ hours.

(xii) Visualize and document the gel under an UV transilluminator.

OBSERVATION

A single band of high molecular weight genomic DNA is observed in the gel (Fig.1).

Fig 1. 0.7% Agarose gel electrophoretogram of the given Genomic DNA.

Lane 1 : High molecular weight DNA

Run Voltage : 50 V

Run Buffer : 1 X TBE

Run Time : 1 ½ hour.

RESULT

The given genomic DNA sample is found to be pure and of high molecular weight.

RESTRICTION DIGESTION OF LAMDA PHAGE GENOMIC DNA USING *Eco RI* AND *Hind* III

AIM

To demonstrate the digestion of λ phage DNA using Restriction endonuclease type II enzyme *Eco*RI and *Hind*III.

PRINCIPLE

Restriction enzymes are DNA cutting enzymes found in bacteria. Because they cut within the molecule, they are often called restriction endonucleases. Restriction endonucleases provide an anti-viral protection for bacteria by cleaving the DNA of invading bacteriophages. A restriction enzyme recognizes and cuts DNA only at a particular sequence of nucleotides.

Five major types of restriction enzymes are widely used in recombinant technology. The type II enzymes are homodimers and recognize the specific complementary sequence known as palindrome sequence or palindromes, which have same sequence when read from both the 5′-3′ ends. According to the number of nucleotides recognized in a site subjected to cut, the enzymes are classified as tetra cutter (4bp), hexa cutter (6bp) and octa cutter (8bp). Different enzymes cleave at different positions within the recognition sequence, and may generate 2 types of cut ends namely blunt fragments, or fragments with 5' or 3' overhanging ends. When the restriction enzymes make a cut in the middle of the recognition sequence of the double stranded DNA, blunt ends are generated. The enzymes, which generate blunt ends, are called blunt end cutter. For example, the bacterium *Hemophilus aegypticus* produces an enzyme named *Hae*III that cuts DNA wherever it encounters the sequence :

5' –G G C C – 3'

3' – C C G G – 5'

Some restriction enzymes make staggered cut in both the strands of the DNA resulting in sticky or cohesive ends. The staggered cut results in hanging ends. *Eco*RI isolated from *Escherichia coli* was the first restriction endonuclease to be described and structure to be determined. It recognizes the sequence :

5' – G A A T T C – 3'

3' – C T T A Ag – 5'

Cleaving both strands on the 3' side of the, leaving a 5' overhanging end. These are called "sticky ends" because they are able to form base pairs with any DNA molecule that contains the complementary sticky end. Any other source of DNA treated with the same enzyme will produce such molecules. Mixed together, these molecules can join with each other by the base pairing between their sticky ends. The union can be made permanent by another enzyme, DNA ligase that forms covalent bonds along the backbone of each strand. The result is a molecule of recombinant DNA (rDNA). The ability to produce recombinant DNA molecules has not only revolutionized the study ofgenetics, but has laid the foundation for much of the biotechnology industry. The availability of human insulin, human factor VIII and other proteins used in human therapy, were all made possible by recombinant DNA.

One enzyme unit is defined as the amount of enzyme required for producing a complete digest of 1µg of λDNA in reaction volume of 50 µL in 60 minutes under optimal conditions of salt, pH and temperature.

REAGENTS REQUIRED

(i) Substrate DNA (λ DNA)

(ii) Nuclease free double distilled water

(iii) Restriction enzymes a) *Eco*RI b) *Hind*III.

(iv) 2X Assay buffer

(v) Stock TBE buffer (5X)

(vi) 1X TE buffer

(vii) 5 x TBE Buffer (Stock solution) – 27g Tris, 13.75g Boric acid, 1.8629g EDTA. (Tris base, Boric acid and EDTA were dissolved in 400mL of double distilled water, adjusted the pH to 8.3 and made up to the final volume). The solution was stored at room temperature.

(viii) 1x TBE Working solution – 100mL of the 5 x TBE stock was diluted to 500mL using double distilled autoclaved water.

(ix) 0.7% Agarose gel solution – 0.7g of agarose was dissolved in 100mL of 1 x TBE buffer

(x) Loading dye – 50mg Bromophenol Blue, 3mL of glycerol, 6mL of TE (pH 8.0), 0.5mL of Ethidium Bromide.

(xi) Ethidium Bromide – 10mg / mL (10mg of Ethidium bromide was dissolved in 1mL of double distilled water)

MATERIAL REQUIRED

(i) Water bath

(ii) Electrophoresis unit and power supply

(iii) UV Transilluminator

(iv) Eppendorf tubes

(v) Micropipettes and tips

(vi) Gel casting apparatus along with the comb

(vii) Electrical adhesive tape

(viii) Electrophoretic tank

(ix) Power pack with leads

(x) Transilluminator

(xi) Other standard labware

PROCEDURE

(i) Prepare the restriction digestion mixture to a total volume of 50µl by adding the following components into a 1.5mL autoclaved Eppendrof tube in the order of addition mentioned in Table I (a) & (b).

Table I (a) : Restriction Digestion Reaction Mixture

S. No.	Components added	Volume added (µL)
(i)	Double distilled water	2.0
(ii)	2X Assay buffer	25.0
(iii)	λ DNA	20.0
(iv)	*Eco*RI	3.0
Total reaction volume		50.0

Table I(b) : Restriction Digestion Reaction Mixture

S. No.	Components added	Volume added (µL)
(i)	Double distilled water	2.0
(ii)	2X Assay Buffer	25.0
(iii)	λ DNA	20.0
(iv)	*Hind*III	3.0
Total reaction volume		50.0

(ii) Spin the above reaction mixture.

(iii) Incubate at 37°C for 1 hour.

(iv) Arrest the reaction by incubating at 65°C for 5 minutes.

(v) Mix 10µL of the restricted sample with 3µL of gel loading dye and load to the wells.

(vi) Mix 10µL of control DNA and 10µL of λ*mLu* digest with 3µL of gel loading dye and load.

(vii) Run electrophoresis at 50 volts for 1 – 2 ½ hours.

(viii) Visualize and document the gel under an UV transilluminator.

OBSERVATION

The substrate λ DNA is restricted into bands of different mobility rate. On digestion with *Eco*RI and *Hind*III, six and eight bands are observed respectively. The control DNA appears as a single uncut DNA band (Fig.1).

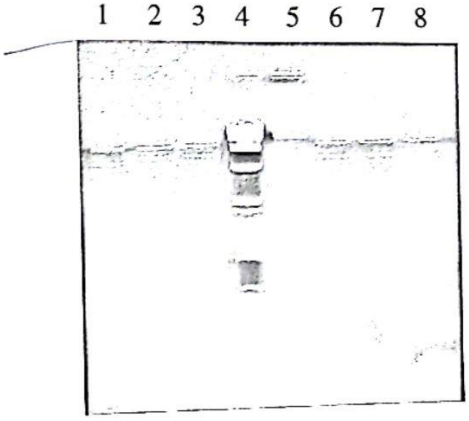


Fig. 1. λ DNA digested with ***EcoR*** I and *Hind* III Restriction enzymes, checked on a 1.0 % Agarose gel

Lane : 1 : λ / *EcoR* I digest
Lane : 2 & 3 : λ / *Hind* III digest
Lane : 4 : λ / *mlu* I size marker
Lane : 5 : Control DNA
Lane : 6 & 7 : λ / *EcoR* I digest
Lane : 8 : λ / *Hind* III digest

RESULT

The λ DNA is restricted by *Eco*RI and *Hind*III into six and eight fragments respectively.

EXPT. NO. :

DATE :

LIGATION

AIM

To perform ligation of λ / *EcoR*I digest using T_4 DNA Ligase.

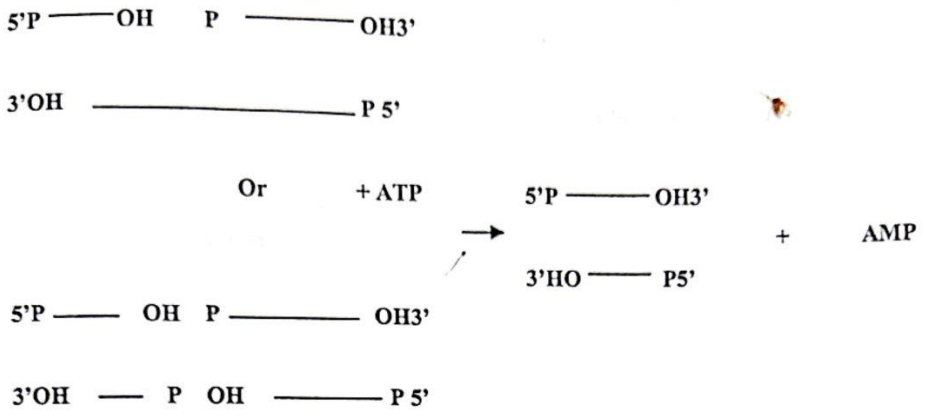
PRINCIPLE

Construction of recombinant DNA molecule is dependent on the ability to covalently seal single stranded nicks in DNA. This process is accomplished both *in vivo* and *in vitro* by the enzyme DNA ligase. *In vivo,* DNA ligases play a pivotal role in the replication, repair, and recombination of DNA. It catalyzes the formation of phosphodiester bonds between juxtaposed 5' phosphate and 3' hydroxyl terminus of double stranded DNA. It can repair single stranded DNA and can join double stranded DNA restriction fragments having either blunt ends or homologous cohesive ends.

E.Coli ligase and T_4 DNA ligase are the two DNA ligases used in nucleic acid research. They can be classified according to their adenylation cofactor requirement as either ATP-dependent or NAD^+-dependent ligases and in their ability to ligate blunt ends. T_4 DNA ligase, the prototype of ATP-dependent DNA ligases, is one of the workhorses of molecular biology. T4 DNA ligase is approximately, 6, 00,000-Dalton (60KD) protein produced by Bacteriophage T_4. T_4 DNA ligase has the unique ability to join sticky and blunt end fragments. One factor that contributed to the widespread use of T_4 DNA ligase is the fact that it catalyzes efficiently the joining of blunt-ended dsDNA in contrast with all other DNA ligases studied so far. It has been shown that T_4 DNA ligase seals dsDNA substrates containing an abasic site or a gap at the ligation junction, joins branched DNA strands, and forms a stem-loop product with partially double stranded DNA.

Cohesive end ligation is carried out at 12°C to 16°C to maintain a good balance between annealing of ends and activity of the enzyme. If reaction is set at higher temperature annealing of the ends become difficult, while at lower temperatures diminishes the ligase activity. Lack of cohesive termini makes blunt end ligation more

complex and significantly slower. Since annealing of ends is not a factor, the reaction is carried out at 24°c. However, 10 – 100 times more enzyme is required to achieve similar ligation efficiency as that of cohesive end ligation.



Schematic representation of ligation reaction

DNA ligases have become indispensable tools for *in vitro* DNA manipulation in a wide range of applications in molecular biology in the detection of specific nucleic acid sequences (DNA or RNA) or protein analytes in DNA nanotechnology and in DNA computation.

REAGENTS REQUIRED

(i) *EcoR*I digest

(ii) 2 x Ligase assay buffer

(iii) T_4 DNA Ligase

(iv) 5 x TBE Buffer (Stock solution) – 27g Tris, 13.75g Boric acid, 1.8629g EDTA. (Tris base, Boric acid and EDTA were dissolved in 400mL of double distilled water, adjusted the pH to 8.3 and made up to the final volume). The solution was stored at room temperature.

(v) 1x TBE Working solution – 100mL of the 5 x TBE stock was diluted to 500mL using double distilled autoclaved water.

(vi) 0.7% Agarose gel solution – 0.7g of agarose was dissolved in 100mL of 1 x TBE buffer

(vii) Loading dye – 50mg Bromophenol blue, 3mL of glycerol, 6mL of TE (pH 8.0), 0.5mL of Ethidium bromide.

(viii) Ethidium bromide – 10mg / mL (10mg of Ethidium bromide was dissolved in 1mL of double distilled water)

MATERIALS REQUIRED

(i) Centrifuge

(ii) Thermometer

(iii) Water bath

(iv) Eppendorf tubes

(v) Micropipettes and tips

(vi) Gel casting apparatus along with the comb

(vii) Electrical adhesive tape

(viii) Electrophoretic tank

(ix) Power pack with leads

(x) Transilluminator

(xi) Other standard labware

PROCEDURE

All the buffers and λ / *EcoR*I digest were thawed and spun prior to use.

(i) Add 10µL of λ/ *Eco* I digest to the T_4 DNA ligase vial kept on ice

(ii) To the above, add 10µL of ligase assay buffer and gently mix by tapping.

(iii) Incubate the ligation mix at 16°C for 2 hours in a pre-set water bath.

(iv) After the completion of ligation, check the ligated samples on a 1% agarose gel.

(v) Mix 10µL of the ligated samples with 3µL of gel loading dye and load to the wells.

(vi) Mix 10µL of λ/ *Eco R*I digest with 3µL of gel loading buffer and load as control.

(vii) Run electrophoresis at 50 volts for 1 – 2 ½ hours.

(viii) Visualize and document the gel under an UV transilluminator.

OBSERVATION

The ligated sample appears as a single band as against 6 bands of λ / *Eco*RI digest (Fig.1).

Fig. 1. λ / ***EcoR*** I digest ligated with T_4 DNA Ligase

Lane : 1 & 3	: Ligated Product
Lane : 2	: λ / ***EcoR*** I digest
Run Voltage	: 50V
Run Buffer	: 1X TBE
Run Time	: 2 – 2 ½ hours

TRANSFORMATION OF *E.coli*

INTRODUCTION

Transformation is the process by which foreign DNA is introduced into a cell. Transformation of bacteria with plasmids is important not only for studies in bacteria but also because bacteria are used as the means for both storing and replicating plasmids. Because of this, nearly all plasmids (even those designed for mammalian cell expression) carry both a bacterial origin of replication and an antibiotic resistance gene for use as a selectable marker in bacteria.

Scientists have made many genetic modifications to create bacterial strains that can be more easily transformed and that will help to maintain the plasmid without rearrangement of the plasmid DNA. Additionally, specific treatments have been discovered that increase the transformation efficiency and make bacteria more susceptible to either chemical or electrical based transformation, generating what are commonly referred to as 'competent cells. 'Many companies sell competent cells, which come frozen and are prepared for optimal transformation efficiencies upon thawing.

REAGENTS

- LB agar plate (with appropriate antibiotic)
- LB or SOC media
- Competent cells
- DNA you'd like to transform

PROCEDURE:

1. Take competent cells out of -80°C and thaw on ice (approximately 20-30 mins).

2. Remove agar plates (containing the appropriate antibiotic) from storage at 4°C and let warm up to room temperature and then (optional) incubate in 37°C incubator.

3. Mix 1 - 5 µl of DNA (usually 10 pg - 100 ng) into 20-50 µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.

4. Incubate the competent cell/DNA mixture on ice for 20-30 mins.

5. Heat shock each transformation tube by placing the bottom 1/2 to 2/3 of the tube into a 42°C water bath for 30-60 secs (45 secs is usually ideal, but this varies depending on the competent cells you are using).

6. Put the tubes back on ice for 2 min.

7. Add 250-1,000 µl LB or SOC media (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 45 min.

8. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic (100µg/ml). Incubate overnight at 37°C.

9. Add 2µl of DNA (pBR322) to the competent cells.

OBSERVATION AND RESULT

BLUE WHITE SCREENING OF RECOMBINANTS

Blue-white screening is a rapid and efficient technique for the identification of recombinant bacteria. It relies on the activity of β-galactosidase, an enzyme occurring in *E. coli*, which cleaves lactose into glucose and galactose.

PRINCIPLE

For screening the clones containing recombinant DNA, a chromogenic substrate known as X-gal is added to the agar plate. If β-galactosidase is produced, X-gal is hydrolyzed to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerizes to produce an insoluble blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo. The colonies formed by non-recombinant cells, therefore appear blue in color while the recombinant ones appear white. The desired recombinant colonies can be easily picked and cultured.

Isopropyl β-D-1-thiogalactopyranoside (IPTG) is used along with X-gal for blue-white screening. IPTG is a non-metabolizable analog of galactose that induces the expression of lacZ gene. It should be noted that IPTG is not a substrate for β-galactosidase but only an inducer. For visual screening purposes, chromogenic substrate like X-gal is required.

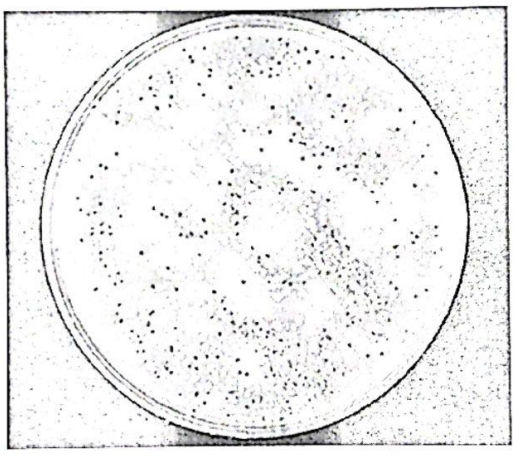
SCREENING

A Wide range of chromogenic substrates that aid screening of recombinant bacteria. Some products may be used to spread on LB agar plates (screening protocol 1), while the others are incorporated into the microbial medium (screening protocol 2). The products are used along with IPTG wherever required. The protocols for both the procedures are given below.

SCREENING PROTOCOL

Spread 40 µL or appropriate amount of stock solution of chromogenic substrate and 10 µL of IPTG solution on LB agar plates using a sterile spreader.

1. The plates should include those with appropriate antibiotic and without antibiotic as controls.
2. Leave the plates to dry in laminar flow chamber with lids slightly open.
3. Spread 10-100 µL of transformed *E. coli* cells onto the LB agar plates using sterile spreader.
4. Incubate the plates at 37°C for 24-48 hours.
5. Blue and white colonies appear on the agar surface. Select the recombinant cells in the white colonies to culture.



Blue-white color selection of recombinant bacteria using X-gal.

EXPT. NO. :

DATE :

SODIUM DODECYL SULPHATE – POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS - PAGE)

AIM

To separate the given mixture of protein sample obtained from crude *E.coli* cell extract by Sodium Deodecyl sulphate – Polyacrylamide gel Electrophoresis (SDS – PAGE) and to visualize the Protein band pattern by Coomassie Brilliant Blue Staining.

PRINCIPLE

Electrophoresis is the migration of charged molecules in solution in response to an electric field. Their rate of migration depends on the strength of the field, on the net charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used analytically to study the properties of a single charged species, and as a separation technique.

Proteins are amphoteric compounds; their net charge therefore is determined by the pH of the medium in which they are suspended. In a solution with a pH above its isoelectric point, a protein has a net negative charge and migrates towards the anode in an electrical field. Below its isoelectric point, the protein is positively charged and migrates towards the cathode. The net charge carried by a protein is in addition independent of its size – i.e.: the charge carried per unit mass (or length, given proteins and nucleic acids are linear macromolecules) of molecule differs from protein to protein. At a given pH therefore, and under non-denaturing conditions, the Electrophoretic separation of proteins is determined by both size and charge of the molecules.

Electrophoresis of proteins is generally carried out in gels made up of cross-linked polymer polyacrylamide. The polyacrylamide gel acts as a molecular sieve, slowing the migration of proteins approximately in proportion to their charge to mass ratio. In SDS – PAGE, the anionic detergent SDS is used. Sodium dodecyl sulphate (SDS) binds to most amino acids in amount roughly proportional to the molecular weight of proteins (about one

mole of SDS for every two amino acid residues). The bound SDS contributes a large net negative charge, rendering the intrinsic charge of the proteins, insignificant and conferring on each protein a similar charge to mass ratio. The native conformation of a protein is also altered when SDS is bound and most protein assumes a similar shape. Hence electrophoresis in the presence of SDS separates proteins almost exclusively on the basis of mass (mol. wt) with smaller polypeptides migrating more rapidly. It is also usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size. This is done with 2- mercaptoethanol or dithiothreitol.

There are two types of buffer systems in vertical gel electrophoresis, continuous and discontinuous. A continuous system has only a single separating gel and uses the same buffer in the tanks and the gel. In a discontinuous system, a non-restrictive large pore gel, called a stacking gel, is layered on top of a separating gel called a resolving gel. Each gel is made with a different buffer, and the tank buffers are different from the gel buffers. The resolution obtained in a discontinuous system is much greater than that obtained with a continuous system.

SDS – PAGE is a discontinuous buffer system. The stacking gel buffer has different pH (6.8), porosity and buffer composition from the resolving gel. In the stacking gel, chloride ionizes and run faster followed by the zwitterionic glycinate. In between Cl – and glycine the SDS-protein samples are stacked together and migrate in the same fashion. When they enter the resolving gel, they encounter a pH of 8.8.glycine becomes charged and moves ahead of the SDS – protein samples. The pore size is also small in the resolving gel. So proteins are left behind to separate according to their mass under the influence f electric field.

SDS – PAGE is used to separate proteins based on molecular mass with smaller polypeptides migrating more rapidly. It is also used to estimate the molecular weight of a protein by plotting the log Mr of the marker proteins versus relative migration of known samples during electrophoresis. A linear relationship exists between the logarithm of the molecular weight of an SDS – denatured polypeptide, or native nucleic acid, and it's *Rf.* The Rf is calculated as the ratio of the distance migrated by the molecule to that migrated by a marker dye-front.

REAGENTS REQUIRED

(i) 30% Acrylamide – Bis acrylamide solution : 30g of acrylamide and 0.8g of bis acrylamide were dissolved in 100mL of distilled water.

(ii) 10% Ammonium per sulphate

(iii) TEMED

(iv) Stacking gel buffer : 0.5M Tris HCl, pH 6.8

(v) Resolving gel buffer : 1.5M Tris HCl, pH 8.8

(vi) Resolving buffer : 3g Tris, 14.4g glycine and 1g SDS were dissolved in 1 L of distilled water, pH 8.3.

(vii) Staining Solution : 6.25% Comassive Brilliant Blue (CBB) in methanol – acetic acid and water (5 : 1 : 4) v/v/v

(viii) De-Staining Solution : Methanol : Acetic acid : Water (5 : 1 : 4) v/v/v

(ix) Sample solubilising buffer : Tris – 362mg, SDS – 600mg, glycerol – 5.2mL, BPB – 30μg, Beta Mercaptoethanol – 0.6μL.

(x) *E coli* Cell extract.

MATERIALS REQUIRED

(i) Vertical gel electrophoresis appartatus

(ii) Cooling centrifuge

(iii) Eppendorf tubes

(iv) Micropipettes

(v) Other standard labware

PROCEDURE

(i) Prepare 10% resolving gel and pour in between the glass plates.

(ii) Allow the gel to polymerize

(iii) Overlay the polymerized 10% resolving gel with 5% stacking gel.

(iv) Insert the comb without any air bubbles in between the plates containing stacking gel solution.

(v) After polymerization, remove the comb and the clean the wells formed.

(vi) 10µL of the sample was mixed with 3µL of the loading dye and loaded into the wells.

(vii) Run electrophoresis at 100 volts till the dye front migrates out of the gel.

(viii) After completion of the Electrophoretic run, remove the gel from the plates and place in the staining solution for three hours.

(ix) Remove the excess stain by washing in the de-staining solution till a clear background was obtained.

(x) Visualize and document the gel under white transilluminator.

OBSERVATION

The proteins from the crude cell extracts obtained from *E. coli* cells are observed as different bands according to their molecular masses. The lower molecular weight proteins migrates faster than the high molecular weight proteins (Fig.1).

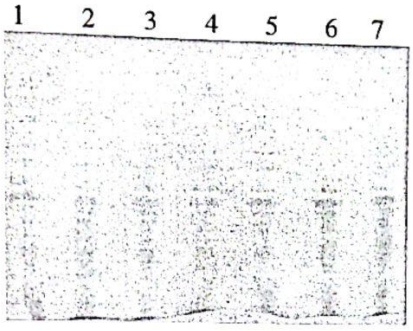


Fig. 1. SDS – Polyacrylamide gel electrophoregram of *E.coli* crude cell extract proteins visualized by Coomassie Brilliant Blue Staining

Lane : 1 – 7 : *E.coli* crude cell extract proteins
Run Voltage : 50V
Run Buffer : 1X Glycine
Run Time : 3 – 4 ½ hours

RESULT

The proteins present in the *crude E. coli* cell extract is separated by SDS – PAGE and visualized by Coomassie brilliant blue staining.

EXPT. NO. :

DATE :

WESTERN BLOTTING

AIM

To demonstrate Western blotting by a) Electrophoretically separating the given protein molecules by SDS - PAGE, b) Electrotransferring the separated protein molecules from SDS gel to nitrocellulose membrane, c) Immobilizzing the protein molecules to nitrocellulose membrane and d) Immune detection of the transferred protein (Blot development).

PRINCIPLE

Blotting describes a process that transfers molecules separated by electrophoretic procedures on agarose or polyacrylamide gels to a special membrane. Protein blots are called as western Blots. SDS – PAGE coupled with Western blotting (immunoblotting) is typically used to determine the presence and / or relative abundance of a given protein. Western blotting technique exploits the inherent specificity of antigen – antibody interaction to identify specific antigens by polyclonal or monoclonal antibodies.

In order for the proteins to migrate through the gel, they are first denatured and negatively charged by exposure to a detergent such as sodium dodecyl sulphate (SDS). The amount of bound SDS is relative to the size of the protein and the proteins have a similar charge to mass ratio. Bands in different lanes separate based on the individual component sizes. A molecular weight marker that produces band of known size is used to help identify proteins of interest.

After the protein components have been sufficiently separated by electrophoresis, they are transferred to a PVDF or nitrocellulose membrane. The electric current is applied at 90 degrees to the gel and the proteins migrate out of the gel onto the membrane. The transfer or blotting process is driven by hydrophobic interaction between the membrane and the protein countered by similar interactions between the gel and the protein.

After transferring the proteins, the membrane surface is deactivated or "blocked" by treatment with a bulk protein solution, usually consisting of a solution of milk powder. The membrane is subjected to immuno staining typically by applying an indirect ELISA protocol. One (or multiple) primary antibody is applied that is specific for the protein to be visualized on the membrane. Following several washing steps, a secondary conjugated antibody is applied that recognizes the constant region of the primary antibody. The conjugate is typically a marker enzyme such as Horseradish Peroxidase (HRP) or Alkaline phosphatase (AP). Alternatively, other markers such as Biotin or digitoxigenin (DIG), fluorescence markers or radiolabels can be employed. Marker enzymes do have the advantage of signal amplification, which provides additional sensitivity to the assay. The secondary antibody can be visualized by the application of the corresponding staining solution.

Western blotting allows determination of the presence and concentration of an antigen of interest in agiven sample also reveals data about the nature of the antigen detected, such as its molecular weight, isoelectric point, tertiary structure, and, in some cases, its biological activity.

In the present method, bacterial lysate having glutathione-S-transferase (GST) fusion protein is electorphoresed in duplicates along with a standard protein marker on a polyacrylamide gel. Following electrophoresis, the lysate and marker is stained to identify the electrophoretic mobility of the GST fusion protein, while the other electrophoresed lysate sample is transferred by electro blotting onto nitrocellulose membrane. The electro - blotted sample is then detected using anti GST IgG as primary antibody and secondary antibody labeled with Horse Radish Peoroxidase (HRP). HRP is then detected using hydrogen peroxide as a substrate and tertramethyl benzidine (TMB) as a chromogen. HRP acts on hydrogen peroxide to release oxygen, which oxidizes the TMB to TMB oxide. The TMB oxide is deposited wherever enzyme is present and appears as a blue band on the NC membrane.

REAGENTS REQUIRED

(i) SDS separating gel mix

(ii) SDS stacking gel

(iii) Ammonium Per Sulphate (APS)

(iv) 10 x Reservoir buffer : 25mL of 10 x reservoir buffer was added to 225mL of distilled water to get 250mL of 1 x reservoir buffer.

(v) Sample loading buffer

(vi) Protein marker

(vii) Protein samples : Resupend in 25µL of distilled water

(viii) 20 x blotting buffer, component A and B : 25mL each of blotting buffer component A and B were mixed with 450mL of distilled water.

(ix) Blocking agent : 300mg of blocking agent was suspended in 10mL of 1x diluent buffer (blocking buffer).

(x) 10 x diluent buffer : 1mL of 10 x diluent buffer was diluted to 10mL with distilled water just before use.

(xi) 10 x assay buffer : 2mL of 10 x assay buffer was added to 18mL of distilled water to get 20mL of 1 x assay buffer.

(xii) 25 x Wash buffer

(xiii) Primary antibody : An aliquot of primary antibody was resuspended in 1 x assay buffer, it was transferred to a test tube and made up the volume to 10 mL with 1 x assay buffer.

(xiv) 1000X HRP conjugate : 10µL of 1000X HRP conjugate was added to 9.90mL of 1 x assay buffer (1 x Secondary antibody).

(xv) 10 x TMB / H202

(xvi) Nitrocellulose (NC) membrane with filter paper

(xvii) Ezee blue stain

(xviii) Double distilled water

MATERIALS REQUIRED

(i) Gel rocker

(ii) Staining tray

(iii) Water bath

(iv) Eppendorf tubes

(v) Micro pipettes and tips

(vi) Other standard labware

PROCEDURE

All the steps were carried out by gentle rocking.

(i) Prepare the resolving gel and pour in between the glass plates.

(ii) Allow the gel to polymerize

(iii) Overlay the polymerized resolving gel with stacking gel.

(iv) Inset the comb without any air bubbles in between the plates containing stacking gel solution.

(v) After polymerization, remove the comb.

(vi) Mix 25µL of the sample-loading buffer to protein sample and 25µL of protein marker and keep in a boiling water bath for denaturation.

(vii) Load 30µL of protein marker in well 1, 40µL of protein sample in well 2 and 5µL of protein sample in well 4.

(viii) Run electrophoresis at 100 V till the dye front reached 0.5cm above the bottom of the gel. After completion of the electrophoretic run. Remove the gel from the plates and transfer to a tray containing water.

(ix) Wash the gel for 1 – 2 minutes at room temperature.

(x) Decant off the water and cut the gel along lane 3, the lane 4 i.e. protein samples and transfer to a petridish containing 10mL of blotting buffer.

(xi) Incubate at room temperature for 10 minutes.

(xii) Wash the gel pieces containing lanes 1 and 2 and stain with 20 mL of Ezee blue stain at room temperature for 1 – 2 hours (the gel was shaken intermittently every 10 to 15 minutes).

(xiii) Decant the staining solution, cover the gel with minimum quantity of water.

(xiv) Leave the gel overnight at room temperature.

(xv) Meanwhile assemble the blotting sandwich within the blotting cassette as followed: First place the filter paper on the cathode cassette cover followed by the cut gel (lane 4) and nitrocellulose membrane (the soft side of the nitrocellulose membrane is placed to the cut gel). Place the wet filter paper on the nitrocellulose membrane followed by anode cassette cover. Tightened the cassette using the screws provided (Ensure that no air bubbles are present between any of the layers of filter paper, cut gel and the nylon membrane).

(xvi) Insert the cassette into the apparatus filled with blotting buffer.

(xvii) Connect the blotting unit to the power supply as per the convention red : anode and black : cathode.

(xviii) Run electrophoresis at 50 volts for 2 hours for blotting to occur.

(xix) Gently remove the nitrocellulose membrane from the cassette and place in a Petri dish containing 10mL of freshly prepared blocking buffer and keep overnight at 4°C

(xx) After overnight incubation, discard the blocking buffer and immerse the blot in 10mL of primary antibody solution, mix gently for 30 minutes.

(xxi) Discard the primary antibody solution and wash the blot by immersing in 10mL wash buffer for 3 – 5 minutes..

(xxii) Repeat the wash twice, discarding the buffer each time.

(xxiii) Immerse the blot in 10mL of 1x HRP labeled antibody and mix gently for 30 minutes.

(xxiv) Discard the HRP labeled antibodies and wash the blot by immersing in 10mL wash buffer for 3 – 5 minutes.

(xxv) Repeat the wash twice, discarding the buffer each time.

(xxvi) Immerse the washed blot in 10 mL of substrate solution and shake gently for 5 – 10 minutes till the coloured band appear.

(xxvii) Wash the blot with distilled water and dry and compare the gel pattern with that of the SDS gel.

OBSERVATION

On staining SDSgel, different proteins appear as dark blue bands against a light blue background (Fig.1). On immunodetection, a single blue band of the GST fusion protein corresponding to 26 KD protein marker is observed on nitrocellulose membrane (Fig.2).

RESULT

A single blue band of GST fusion protein corresponding to 26 KD marker protein is detected by western blotting.