Semester – VI

17MBU613B MOLECULAR BIOLOGY - PRACTICAL (3H – 1C)

Instruction Hours / week: L: 0 T: 0 P: 3 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 6 Hours

SCOPE

This course explores technologies using molecular biology, embryo manipulation, cell and tissue culture to manipulate the genomes of animals for ways to improve the live stock for food production and biomedical purpose.

OBJECTIVE

- To provide an experience for the students in an interdisciplinary research program connecting animal genomics with animal reproduction and biotechnology.

EXPERIMENTS

1. Study of different types of DNA and RNA using micrographs and model / schematic representations.
2. Study of semi-conservative replication of DNA through micrographs / schematic representations.
3. Isolation of genomic DNA from *E. coli.*
4. Estimation of salmon sperm / calf thymus DNA using colorimeter (diphenylaminereagent) or UV spectrophotometer (A260 measurement).
5. Estimation of RNA using colorimeter (orcinol reagent) or UV spectrophotometer (A260 measurement).
6. Resolution and visualization of DNA by Agarose Gel Electrophoresis.
7. Resolution and visualization of proteins by Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SUGGESTED READINGS

1. Watson JD, Baker TA, Bell SP, Gann A, Levine M and Losick R (2008) Molecular Biology of the Gene, 6th edition, Cold Spring Harbour Lab. Press, Pearson Publication
2. Becker WM, Kleinsmith LJ, Hardin J and Bertoni GP (2009) The World of the Cell, 7th edition, Pearson Benjamin Cummings Publishing, San Francisco
3. De Robertis EDP and De Robertis EMF (2006) Cell and Molecular Biology, 8th edition. Lippincott Williams and Wilkins, Philadelphia
4. Karp G (2010) Cell and Molecular Biology: Concepts and Experiments, 6th edition, John Wiley & Sons. Inc.
5. Sambrook J and Russell DW. (2001). Molecular Cloning: A Laboratory Manual. 4th Edition, Cold Spring Harbour Laboratory press.
6. Krebs J, Goldstein E, Kilpatrick S (2013). Lewin's Essential Genes, 3rd Ed., Jones and Bartlett Learning.

EX.NO: 1

Study of different types of DNA and RNA using micrographs and model/schematic representations

Different forms of DNA:

The most common form of DNA which has right handed helix and proposed by Watson and Crick is called B-form of DNA or B-DNA. In addition, the DNA may be able to exist in other forms of double helical structure. These are A and C forms of double helix which vary from B- form in spacing between nucleotides and number of nucleotides per turn, rotation per base pair, vertical rise per base pair and helical diameter.

1. The B-Form of DNA (B-DNA):

Structure of B-form of DNA has been proposed by Watson and Crick. It is present in every cell at a very high relative humidity (92%) and low concentration of ions. It has antiparallel double helix, rotating clockwise (right hand) and made up of sugar- phosphate back bone combined with base pairs or purine-pyrimidine.The base pairs are perpendicular to longitudinal axis of the helix. The base pairs tilt to helix by 6.3°. The B-form of DNA is metabolically stable and undergo changes to A, C or D forms depending on sequence of nucleotides and concentration of excess salts.

2. The A-Form of DNA (A-DNA):

The A-form of DNA is found at 75% relative humidity in the presence of Na+, K+ or Cs+ ions. It contains eleven base pairs as compared to ten base pairs of B-DNA which tilt from the axis of helix by 20.2°. Due to this displacement the depth of major groove increases and that of minor groove decreases. The A-form is metastable and quickly turns to the D-form.

3. The C-Form DNA (C-DNA):

The C-form of DNA is found at 66% relative humidity in the presence of lithium (Lit+) ions. As compared to A-and B-DNA, in C-DNA the number of base pairs per turn is less i.e. 28/3 or 9 1/3. The base pairs show pronounced negative tilt by 7.8°.

4. The D-Form of DNA (D-DNA):

The D-form of DNA is found rarely as extreme vanants. Total number of base pairs per turn of helix is eight. Therefore, it shows eight-fold symmetry. This form is also called poly (dA-dT) and poly (dG-dC) form. There is pronounced negative tilt of base pairs by 16.7° as compared to C form i.e. the base pairs are displaced backwardly with respect to the axis of DNA helix.

5. The Z-Form of DNA (Z-DNA) or Left Handed DNA:

In 1979, Rich and coworkers at MIT (U.S.A.) obtained Z-DNA by artificially synthesizing d (C-G) 3 molecules in the form of crystals. They proposed a left handed (synistral) double helix model with zig-zag sugar-phosphate back bone running in antiparallel direction.

Therefore, this DNA has been termed as Z-DNA. The Z-DNA has been found in a large number of living organisms including mammals, protozoans and several plant species.

There are several similarities with B-DNA in having:

(i) Double helix,

(ii) Two antiparallel strands, and

(iii) Three hydrogen bonds between G-C pairing.

In addition, the Z-DNA differs from the B-DNA in the following ways:

(a) The Z-DNA has left handed helix, while the B-DNA has right handed helix.

(b) The Z-DNA contains zig-zag sugar phosphate back bone as compared to regular back bone of the B-DNA.

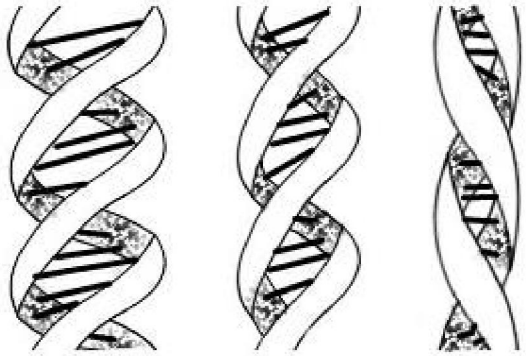
(c) The repeating unit in Z-DNA is a dinucleotide due to alternating orientation of sugar residues, whereas in B-DNA the repeating unit is a mononucleotide, and sugar molecules do not have the alternating orientation.

(d) In the Z-DNA one complete turn contains 12 base pairs of six repeating dinucleotide, while in B-DNA one full turn consists of 10 base pairs i.e. the 10 repeating units.

(e) Due to the presence of high number (12) of base pairs in one turn of Z-DNA, the angle of twist per repeating unit i.e. dinucleotide is 60° as compared to 36° of B-DNA molecule.

(f) In Z-DNA the distance of twist making one turn of 360° is 45Å as against 34Å in B-DNA.

(g) The Z-DNA has fewer diameters (18Å) as compared to the B-DNA (20Å diameter)



Form	A	B	Z
Helical Twist	right	right	left
base pairs per turn	11	10	12
occurrence	RNA, DNA	DNA	DNA

Types of RNA:

RNA molecules are single-stranded nucleic acids composed of nucleotides. RNA plays a major role in protein synthesis as it is involved in the transcription, decoding, and translation of the genetic code to produce proteins. RNA stands for ribonucleic acid and like DNA, RNA nucleotides contain three components:

- A Nitrogenous Base
- A Five-Carbon Sugar
- A Phosphate Group

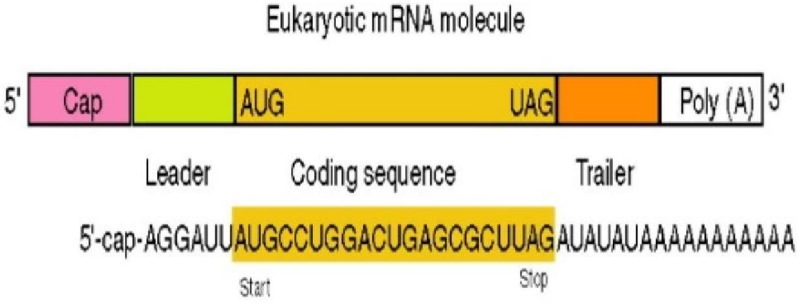
RNA nitrogenous bases include **adenine (A)**, **guanine (G)**, **cytosine (C)** and **uracil (U)**. The five-carbon (pentose) sugar in RNA is ribose. RNA molecules are polymers of nucleotides joined to one another by covalent bonds between the phosphate of one nucleotide and the sugar of another. These linkages are called phosphodiester linkages.

RNA molecules are produced in the nucleus of our cells and can also be found in the cytoplasm. The three primary types of RNA molecules are messenger RNA, transfer RNA and ribosomal RNA.

- **Messenger RNA (mRNA)** plays an important role in the transcriptionof DNA. Transcription is the process in protein synthesis that involves copying the genetic information contained within DNA into an RNA message. During transcription, certain proteins called transcription factors unwind the DNA strand and allow the enzyme RNA polymerase to transcribe only a single strand of DNA. DNA contains the four nucleotide bases adenine (A), guanine (G), cytosine (C) and thymine (T) which are

  paired together (A-T and C-G). When RNA polymerase transcribes the DNA into a mRNA molecule, adenine pairs with uracil and cytosine pairs with guanine (A-U and C-G). At the end of transcription, mRNA is transported to the cytoplasm for the completionof Protein.

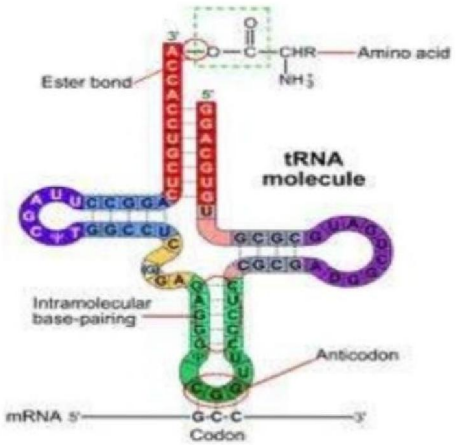
Structural Characteristics of m-RNA



5' cap and 3' tail impart stability to m RNA by protecting from specific exo nucleases.

- **Transfer RNA (tRNA)** plays an important role in the translation portion of protein synthesis. Its job is to translate the message within the nucleotide sequences of mRNA into specific amino acid sequences. The amino acid sequences are joined together to form a protein. Transfer RNA is shaped like a clover leaf with three hairpin loops. It contains an amino acid attachment site on one end and a special section in the middle loop called the anticodon site. The anticodon recognizes a specific area on mRNA called a codon. A codon consists of three continuous nucleotide bases that code for an amino acid or signal the end of translation. Transfer RNA along with ribosomes read the mRNA codons and produce a polypeptide chain. The polypeptide chain undergoes several modifications before becoming a fully functioning protein.

Secondary structure of t- RNA

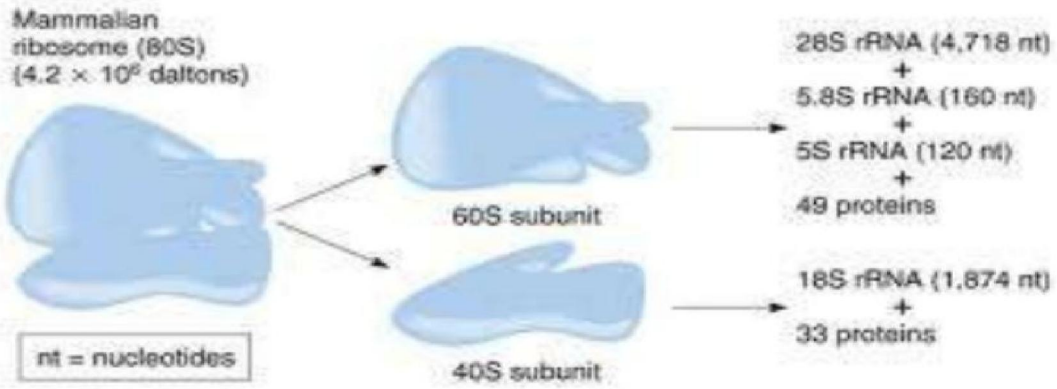


The carboxyl group of amino acid is attached to 3'OH group of Adenine nucleotide of the acceptor arm. The anticodon arm base pairs with the codon present on the m- RNA

Biochemistry For Medics 19

- **Ribosomal RNA (rRNA)** is a component of cell organelles called ribosomes. A ribosome consists of ribosomal proteins and rRNA. Ribosomes are typically composed of two subunits: a large subunit and a small subunit. Ribosomal subunits are synthesized in the nucleus by the nucleolus. Ribosomes contain a binding site for mRNA and two binding sites for tRNA located in the large ribosomal subunit. During translation, a small ribosomal subunit attaches to a mRNA molecule. At the same time, an initiator tRNA molecule recognizes and binds to a specific codon sequence on the same mRNA molecule. A large ribosomal subunit then joins the newly formed complex. Both ribosomal subunits travel along the mRNA molecule translating the codons on mRNA into a polypeptide chain as they go. Ribosomal RNA is responsible for creating the peptide bonds between the amino acids in the polypeptide chain. When a termination codon is reached on the mRNA molecule, the translation process ends. The polypeptide chain is released from the tRNA molecule and the ribosome splits back into large and small subunits.

Ribosomal RNA (rRNA)



Study of semi conservative replication of DNA through micrographs/schematic representations

DNA is the genetic material that defines every cell. Before a cell duplicates and is divided into new daughter cells through either mitosis or meiosis, biomolecules and organelles must be copied to be distributed among the cells. DNA, found within the nucleus, must be replicated in order to ensure that each new cell receives the correct number of chromosomes. The process of DNA duplication is called **DNA replication**. Replication follows several steps that involve multiple proteins called replication enzymes and RNA. In eukaryotic cells, such as animal cells and plant cells, DNA replication occurs in the S phase of interphase during the cell cycle. The process of DNA replication is vital for cell growth, repair, and reproduction in organisms.

Step 1: Replication Fork Formation

Before DNA can be replicated, the double stranded molecule must be "unzipped" into two single strands. DNA has four bases called **adenine (A)**, **thymine (T)**, **cytosine (C)** and **guanine (G)** that form pairs between the two strands. Adenine only pairs with thymine and cytosine only binds with guanine. In order to unwind DNA, these interactions between base pairs must be broken. This is performed by an enzyme known as DNA **helicase**. DNA helicase disrupts the hydrogen bonding between base pairs to separate the strands into a Y shape known as the **replication fork**. This area will be the template for replication to begin.

DNA is directional in both strands, signified by a 5' and 3' end. This notation signifies which side group is attached the DNA backbone. The **5' end** has a phosphate (P) group attached, while the **3' end** has a hydroxyl (OH) group attached. This directionality is important for replication as it only progresses in the 5' to 3' direction. However, the replication fork is bi-directional; one strand is oriented in the 3' to 5' direction **(leading strand)** while the other is oriented 5' to 3' **(lagging strand)**. The two sides are therefore replicated with two different processes to accommodate the directional difference.

Replication Begins

Step 2: Primer Binding

The leading strand is the simplest to replicate. Once the DNA strands have been separated, a short piece of RNAcalled a **primer** binds to the 3' end of the strand. The primer always binds as the starting point for replication. Primers are generated by the enzyme **DNA primase**.

Step 3: Elongation

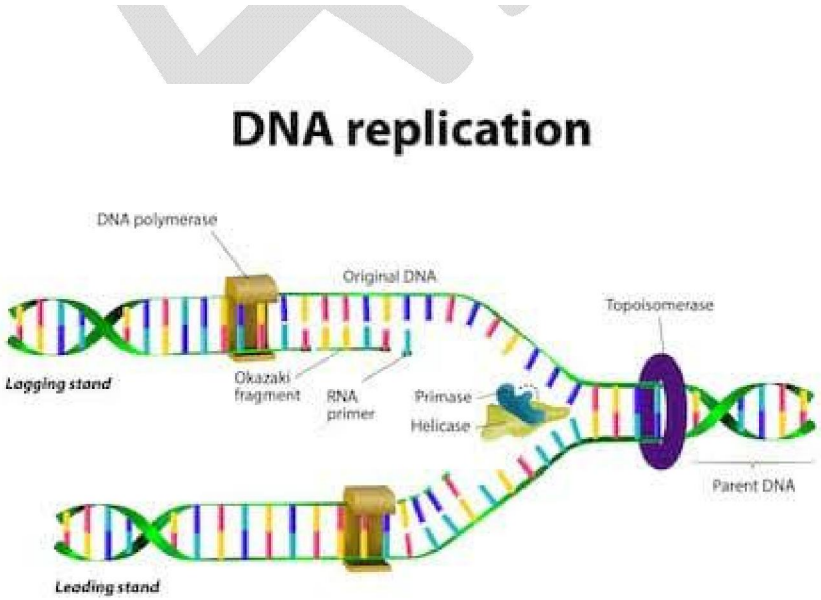
Enzymes known as **DNA polymerases**are responsible creating the new strand by a process called elongation. There are five different known types of DNA polymerases in bacteria and human cells. In bacteria such as *E.coli*, **polymerase III**is the main replication enzyme, while polymerase I, II, IV and V are

responsible for error checking and repair. DNA polymerase III binds to the strand at the site of the primer and begins adding new base pairs complementary to the strand during replication. In eukaryotic cells, polymerases alpha, delta, and epsilon are the primary polymerases involved in DNA replication. Because replication proceeds in the 5' to 3' direction on the leading strand, the newly formed strand is continuous.

The **lagging strand** begins replication by binding with multiple primers. Each primer is only several bases apart. DNA polymerase then adds pieces of DNA, called **Okazaki fragments**, to the strand between primers. This process of replication is discontinuous as the newly created fragments are disjointed.

Step 4: Termination

Once both the continuous and discontinuous strands are formed, an enzyme called **exonuclease** removes all RNA primers from the original strands. These primers are then replaced with appropriate bases. Another exonuclease "proofreads" the newly formed DNA to check, remove and replace any errors. Another enzyme called **DNA ligase** joins Okazaki fragments together forming a single unified strand. The ends of the linear DNA present a problem as DNA polymerase can only add nucleotides in the 5′ to 3′ direction. The ends of the parent strands consist of repeated DNA sequences called telomeres. Telomeres act as protective caps at the end of chromosomes to prevent nearby chromosomes from fusing. A special type of DNA polymerase enzyme called **telomerase** catalyzes the synthesis of telomere sequences at the ends of the DNA. Once completed, the parent strand and its complementary DNA strand coils into the familiar double helix shape. In the end, replication produces two DNA molecules, each with one strand from the parent molecule and one new strand.



EX.NO: 2

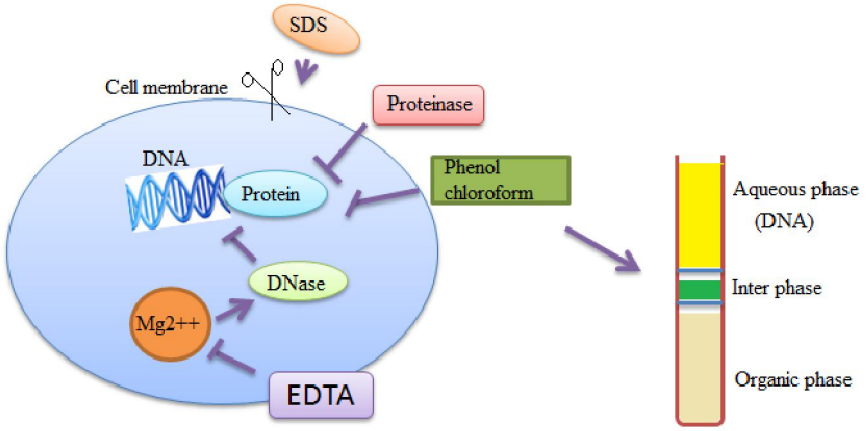
Isolation of Genomic DNA from E. coli

Aim: To isolate the genomic DNA from E .coli DH5α cells.

Principle:

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

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



Materials Required:

LB Broth, *E. coli* DH5α cells, TE buffer (pH 8.0),10% SDS, Proteinase K, Phenol-chloroform mixture, Isopropanol, 70% ethanol , Autoclaved distilled water, 1.5 ml Eppendorf tubes, Micropipette, Micro tips, Microfuge.

Reagent Preparation

Tris-Cl

To prepare a 1 M solution, dissolve 121.1 g of Tris base in 800 mL of H_2O. Adjust the pH to 8 by adding concentrated HCl. Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 L with H_2O. Dispense into aliquots and sterilize by autoclaving.

EDTA (ethylene diamene tetra acetic acid)

To prepare EDTA at 0.5 M (pH 8.0): Add 186.1 g of disodium EDTA•$2H_2O$ to 800 mL of H2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH.

Tris-EDTA buffer

To prepare Tris-EDTA buffer

Reagent	Amount to add	Final concentration
Tris-Cl (1 M, pH 8.0)	1 mL	10 mM
EDTA (0.5 M, pH 8.0)	200 µL	1 mM
Distilled H_2O	98.8 mL	

Procedure

1. Transfer 1.5 ml of the overnight *E. coli* culture grown in LB medium to a 1.5 ml eppendorf tube and centrifuge at max speed for 1min to pellet the cells. Discard the supernatant without disturbing the cell pellet.
2. Resuspend the cell pellet in 600 µl of lysis buffer and vortex to completely dissolve cell pellet, add 30 µl of Proteinase K, (20 mg/ml) and mix well by inverting the tube and incubate at 37 °C for 1 h.
3. After 1 h of incubation at 37 °C, add equal volume of phenol/chloroform and mix well by inverting the tube several times until the phases are completely mixed. *Do not vertex the tube it may shear the DNA.*

- ✓ Phenol is a very strong acid that causes severe burns. Chloroform is a carcinogen. Wear gloves, goggles and lab coat, and keep tubes capped tightly. To be safe, work in the hood if possible.

4. Centrifuge at 12,000 rpm for 5 min at room temperature. The upper aqueous phase alone was carefully transferred to a new 1.5 ml eppendorf tube without disturbing the other layers. The white layer in between the aqueous and organic interface is protein. Repeat the phenol/chloroform step until no *protein* is visible at the *interface*.
5. To the aqueous phase, add 2.5 ml volumes of ice cold absolute ethanol and mix well by inverting the tube several times. The DNA precipitation will be visible like a cotton thread.
6. Centrifuge at 12,000 rpm for 10 min at 4 °C and discard the supernatant without disturbing the DNA pellet.
7. To the DNA pellet 1 ml of 70% ethanol (stored at room temperature) was added and gently rinsed by inverting the tube several times and centrifuged at 12,000 rpm for 10 min at room temperature.
8. Carefully discard the supernatant and the DNA pellet was air-dried in a sterile environment for 10-15 min to remove traces of residual ethanol.
9. Re-suspend the DNA pellet in 50 µl of TE buffer or nuclease free water (NFW).
10. Measure optical density of DNA in a Nano drop Spectrophotometer at 260/280nM and 260/230 nm wavelength absorbance.

260/280 Ratio

- ✓ The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as "pure" for DNA. If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

260/230 Ratio

- ✓ This ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants (EDTA, carbohydrates and phenol) which absorb at 230 nm.

11. After quantification run a 1% agarose gel to check the quality of the DNA.

Observation

The DNA was visible like a white cotton thread during ice cold absolute ethanol precipitate step. The absorbance of the DNA was ~1.83 at 260/280 nm.

Result

The genomic DNA from *E .coli* was isolated, loaded and visualization on agarose gel electrophoresis.

EX.NO: 3

Resolution and visualization of DNA by agarose gel electrophoresis

Aim

To separate and purify genomic DNA using agarose gel electrophoresis.

Principle

Agarose is a polysaccharide derived from seaweeds, which forms a gel when boiled in water and cooled. The pore size of the gel depends upon the concentration of agarose used. Higher the percentage of agarose smaller is the pores size and greater is the resolution. Lower percentage of agarose must be used, when larger DNA molecules are to be separated. During electrophoresis, DNA being a negatively charged molecule moves towards the positive electrode (anode) when constant electric field is applied. Migration of DNA through the agarose gel matrix depends upon the size and conformation of the DNA. This allows separation of DNA based on their molecular weight. The rate of movement of DNA is inversely proportional to its size. Larger DNA fragments migrate slowly due to their size, which is larger than the pore size. Smaller fragments move faster and form a band farther from the loading well than the larger fragments.

Equipments

Casting tray, well combs, voltage source, gel box, UV light source and Microwave

Reagents

TAE Buffer (Tris-acetate-EDTA)

To make 1x TAE Buffer

Tris-base: 4.84 g
Acetate (100% acetic acid): 1.142 ml
EDTA: 2 ml 0.5M sodium EDTA
Add dH2O up to one litre.

DNA loading buffer (6X)

30% (v/v) glycerol
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF

Store at 4°C.

TAE Buffer, agarose, ethidum bromide (stock concentration of 10 mg/mL)

Procedure

1. The open side of the gel casting plate was sealed using cellotape.
2. Measure 1 g of agarose powder in 100 mL 1xTAE in a microwavable flask and microwave for 1-3 min until the agarose is completely dissolved to get a clear solution.

   Agarose gels are commonly used in concentrations of 0.7% to 2% depending on the size of bands needed to be separated.

3. Let agarose solution cool down to about 50 °C, add ethidium bromide (EtBr) to a final concentration of approximately 0.2-0.5 µg/mL (usually about 2-3 µl of stock solution per 100 mL gel). EtBr binds to the DNA and allows you to visualize the DNA under ultraviolet (UV) light.

   CAUTION: EtBr is a known mutagen. Wear a lab coat, eye protection and gloves when working with this chemical.

4. Pour the agarose into a gel tray with the well comb in place. Pour slowly to avoid bubbles which will disrupt the gel. Any bubbles can be pushed away from the well comb or towards the sides/edges of the gel with a pipette tip.
5. Place newly poured gel at 4 °C for 10-15 mins or let sit at room temperature for 20-30 mins, until it has completely solidified.

Loading Samples and Running an Agarose Gel

1. Add 1X loading buffer to each of your DNA samples.

   Note: Loading buffer serves two purposes: 1) it provides a visible dye that helps with gel loading and allows you to gauge how far the DNA has migrated; 2) it contains a high percentage of glycerol that increases the density of your DNA sample causing it settle to the bottom of the gel well, instead of diffusing in the buffer.

2. Once the agarose gel solidified, place the agarose gel into the gel box (electrophoresis unit) and fill gel box with 1xTAE until the gel is covered.
3. Carefully load a molecular weight ladder into the first lane of the gel.
4. Carefully load your samples into the additional wells of the gel.
5. Run the gel at 80-150 V until the dye line is approximately 75-80% of the way down the gel. A typical run time is about 1-1.5 hours, depending on the gel concentration and voltage.

   Note: Black is negative, red is positive. The DNA is negatively charged and will run towards the positive electrode. Always Run to Red.

6. Turn off power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
7. Using an UV transilluminator device visualize your DNA fragments. The fragments of DNA are usually referred to as ‘bands’ due to their appearance on the gel.

   Note: When using UV light, protect your skin by wearing safety goggles or a face shield, gloves and a lab coat.

Analyzing Your Gel:

Using the DNA ladder in the first lane as a guide (the manufacturer's instruction will tell you the size of each band), you can infer the size of the DNA in your sample lanes.

Observation

The DNA samples appeared as single bands in the gel, relative molecular weight of the DNA ladder.

Result

The isolated DNA was visualized and detected by agarose gel electrophoresis.

EX.NO: 4

ESTIMATION OF DNA BY DIPHENYL AMINE METHOD

Aim:

To estimate the amount of DNA present in the given solution by Diphenyl Amine method.

Principle:

The deoxy ribose of DNA in the presence of acid forms Hydroxyl Laevolinic Aldehyde which reacts with the colouring reagent Diphenyl amine to give a blue colour complex which is read at 640 nm.

Reagents required:

1. Stock standard solution:

About 200 mg of DNA is dissolved in 100 ml of 5% TCA solution.

Concentration = 2 mg/ml; 2000 µg/ml

2. Working standard solution:

About 10 ml of stock solution is diluted to 100 ml using 5% TCA solution.

Concentration = 200 µg/ml

3. Diphenyl amine reagent:

To prepare fresh reagent dissolve 1 gm of diphenyl amine in 100 ml of glacial acetic acid and add 2.5 ml of concentrated sulphuric acid.

Procedure:

The standard tubes are marked as S1-S5 and take 1 to 5 ml of working standard DNA solution in a series of standard test tubes. Then 1 ml and 2 ml of unknown solution is taken in the U1 and U2 test tubes. All the test tubes are made up to 5 ml using distilled water. The 5 ml of diphenylamine reagent is added to all the test tubes and shaken thoroughly and heated in a boiling water bath for 10 minutes. The blue colour complex developed is measured colorimetrically at 640 nm.

The calibration curve is plotted by taking the amount of DNA in X-axis and the corresponding optical density on Y-axis. From this experiment the amount of DNA in the sample is calculated.

Result:

The amount of DNA present in the given unknown solution was found to be

1. Theoretical value : 30 mg/dl
2. Graphical value : 30 mg/dl

ESTIMATION OF DNA BY DIPHENYL AMINE METHOD

Stock standard solution **Working standard solution**

200 mg/100 ml of 5% TCA 10 ml of Stock/100 ml of 5% TCA

2 mg/ml 20,000 µg/100 ml

2000 µg/ml 200 µg/ml

S.No.	Particulars	Working standard solution						Unknown	
		B	S1	S2	S3	S4	S5	U1	U2
1.	Volume of working standard solution (ml)	-	1.0	2.0	3.0	4.0	5.0	-	-
2.	Concentration (µg)	-	200	400	600	800	1000	-	-
3.	Volume of unknown solution (ml)	-	-	-	-	-	-	1.0	2.0
4.	Volume of distilled water (ml)	5.0	4.0	3.0	2.0	1.0	-	4.0	3.0
5.	Volume of diphenyl amine reagent (ml)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Mixed well and tubes are kept in a boiling water bath for 10 minutes									
6.	Optical density at 640 nm	0.00	0.2	0.4	0.6	0.8	1.0	0.3	0.6

Colour developed : Blue

Filter used : Red filter

CALCULATION

Theoretical value:

The amount of DNA present in the whole unknown solution } $\frac{\text{Unknown optical density}}{\text{Standard optical density}}$ xStd. Conc.

1.0 ml of unknown solution contain = 0.3 / 0.2 x 200

= 300µg of DNA

∴100ml of unknown solution = 300 / 1.0 x 100 = 30000µg of DNA

= 30000 /1000

= 30 mg of DNA per 100 ml unknown solution.

Graphical value:

1.0 ml of unknown solution corresponds to 0.3 Optical Density

0.3 OpticalDensity corresponds to 300µg of DNA

1.0 ml of unknown solution contains 300 µg of DNA

100ml of unknown solution contains = 300 /1.0 x 100 = 30000 µg of DNA

= 30000 /1000

= 30 mg of DNAper 100 ml unknown solution.

EX.NO: 5

ESTIMATION OF RNA BY ORCINOL METHOD

Aim:

To estimate the amount of RNA present in the given unknown solution.

Principle:

The ribose moiety condenses with Orcinol to yield a green colour solution that can be measured at 620 nm.

Reagents required:

1. Stock RNA solution:

About 100 mg of yeast RNA is dissolved in 100 ml of 10% TCA solution.

Concentration = mg/ml; 1000 µg/ml

2. Working RNA solution:

About 10 ml of stock solution is diluted into 100 ml using 10% TCA solution.

Concentration =10 mg/100 ml; 100 µg/ml

3. Unknown solution:

The given unknown solution was made up to 100 ml using 10% TCA solution.

4.Orcinol reagent:

About 300 g of orcinol was weighed and dissolved in 5 ml of ethanol. From this solution, take 3.5 ml and made up to 100 ml using 0.1% ferric chloride in Conc. HCl.

Procedure:

The standard tubes are marked as S1-S5 and take 0.2 to 1.0 ml of working standard RNA solution in a series of standard test tubes. Then 0.2 ml and 0.4 ml of unknown solution is taken in the U1 and U2 test tubes. All the test tubes are made up to 2.5 ml using distilled water. About 3 ml of orcinol reagent is added

to all the test tubes and shaken well thoroughly and heated in a boiling water bath for 10 minutes. The green colour complex developed is measured colorimetrically at 620 nm.

The calibration curve is plotted by taking the amount of RNA in X-axis and the corresponding optical density on Y-axis. From this experiment the amount of RNA in the sample is calculated.

Result:

The amount of RNA present in the given unknown solution was found to be

1. Theoretical value : mg/dl
2. Graphical value : mg/dl

ESTIMATION OF RNA BY ORCINOL METHOD

Stock standard solution

100 mg/100 ml of 10% TCA

1 mg/ml

1000 µg/ml

Working solution

10 ml/100 ml of 10% TCA

100 µg/ml

S.No.	Particulars	Working standard solution						Unknown	
		B	S1	S2	S3	S4	S5	U1	U2
1.	Volume of working standard Solution (ml)	-	0.2	0.4	0.6	0.8	1.0	-	-
2.	Concentration (µg)	-	20	40	60	80	100	-	-
3.	Volume of unknown solution (ml)	-	-	-	-	-	-	0.2	0.4
4.	Volume of distilled water (ml)	2.5	2.3	2.1	1.9	1.7	1.5	2.3	2.1
5.	Volume of Orcinol reagent (ml)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
All the tubes are kept in a boiling water bath for 10 minutes									
6.	Optical density at 620 nm	0.00	0.3	0.6	0.9	1.3	1.5	0.3	0.6

Colour developed : Green

Filter used : Red

CALCULATION

Theoretical value:

The amount of RNA present in the whole unknown solution } $\frac{\text{Unknown optical density}}{\text{Standard optical density}}$ xStd. Conc.

0.2 ml of unknown solution contain = / x

= ----------µg of RNA

∴100ml of unknown solution = / 0.2 x 100 = ----------µg of RNA

= /1000

= ----------mg of RNA

Graphical value:

0.2 ml of unknown solution corresponds to _____ optical density

_____ Optical density corresponds to _____ µg of RNA

0.2 ml of unknown solution contains _____ µg of RNA

100ml of unknown solution contains = /0.2 x 100 = ----------µg of RNA

= /1000

= ----------mg of RNA

= mg of RNA

= mg of RNA

EX.NO: 6

Resolution and visualization of protein by Sodium Dodocyle Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Aim: To determine the molecular weight of protein by Sodium Dodocyle Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Principle:

SDS-PAGE stands for Sodium Dodocyle Sulfate-Polyacrylamide Gel Electrophoresis. It is an electrophoresis technique used to separate proteins based on their mass. SDS is a detergent and used to give a negative charge to the denatured protein. One molecule of SDS binds every 2 amino acids of the protein. The proteins are denatured, and thus the gel used is called denaturing gel. There are two different gels used in SDS-PAGE. One is called separating gel or resolving gel, and other is called stacking gel. As the name suggests, the resolving gel is dedicated to the separation of the proteins, and the stacking gel is meant to stack the proteins in one band.

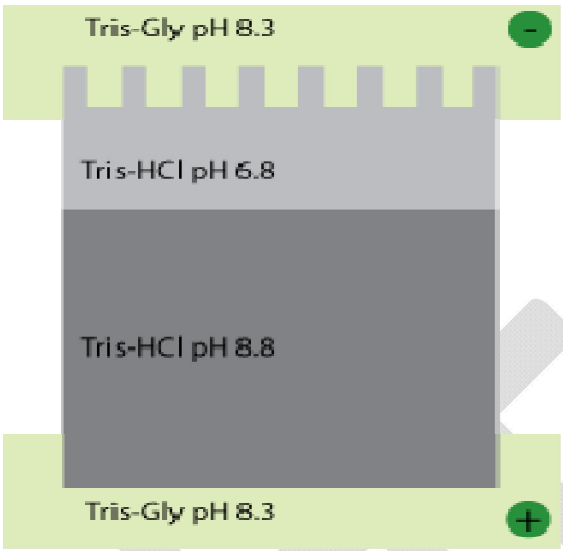
The gel is prepared from Acrylamide and Bis-acrylamide. Acrylamide is the monomeric subunit for the Polyacrylamide and the Bis-acrylamide is used for cross-linking between the polymer. Thus, Bis-acrylamide is used for producing the pores. The ratio of bisacrylamide to acrylamide can be varied for special purposes, but is generally about 1 part in 35. Greater the concentration of the Bis-acrylamide, smaller would be the pore size. Other components required for preparing the gel are TEMED (Tetra methyl ethylene diamine), APS (Ammonium per Sulfate) and Buffer. APS is used for generating the acrylamide free radical so that free radical polymerization can be initiated. Riboflavin can also be used as the free radical source. TEMED is a free radical stabilizer and added to promote polymerization.

Stacking Gel:

The buffer used is 1M Tris-Hcl (pH-6.8), the pH of the gel is 6.8. This lower ionic strength implies higher electrical resistance and consequently a higher electric field, provoking the faster movement of the proteins and of every other charged particle in the gel. Such a high electric field coupled with the glycine in the running buffer (that will not go into the resolving gel due to the pH), helps to clean the sample from the Cl- ions from the Tris-HCl buffer. The HCl provide Cl- ions and Glycine at this pH have net neutral charge. The glycine moves slowly as compared to the protein whereas the Cl- ions move faster than proteins. Thus, in the stacking gel, the proteins are sandwiched between the Cl- ions and Glycine.

Separating gel or Resolving Gel:

The buffer used is 1.5 M Tris-Hcl (pH-8.8), the pH of the gel is 8.8. When the stacking gel stacks the proteins and reaches the resolving gel, the pH changes. At this pH, the glycine would have net negative charge and moves faster and leaves the Cl- ion, and protein behind. Now, the protein would move in the gel based on their mass.



Protein sample preparation

The protein samples need to be denatured.

To denature the protein sample,

1. DTT (Dithiothreitol)
2. Beta-mercaptoethanol

These reagents would reduce the disulfide linkage between the cysteine in the proteins. Apart from this, EDTA and buffer are also added. EDTA will chelate the Ca2+ and Mg2+ ions which are co-factor for activation of proteases activity. The sample is also heated for denaturing it.

The SDS-PAGE electrophoretic unit is vertical. The two gels are prepared between two glass plates which are separated by a 1 mm spacer. First, resolving gel is poured and allowed to polymerize, and then the stacking gel is poured. The wells are created by putting a comb between the plates before pouring the stacking gel.

The sample concentration should be 2 mg/ml. In one well, 10 µl of the sample can be loaded. To ensure that the sample sinks to the bottom of the gel, sample buffer is supplemented with additives that increase the density of the sample. For such purpose, sucrose and glycerol solution is used.

Once the sample has been loaded, the electric field is applied. The applied voltage is between 100–150 volts. After the run (time depends on the voltage), the protein bands are fixed by addition of methanol and

acetic acid. Coomassie dye is used to stain the protein. After that, destaining is also carried out by increasing the methanol and acetic acid. Other staining method includes Zinc staining and Silver staining.

Materials

Equipment

1. SDS-PAGE gel apparatus.
2. Power pack.

Reagents

Acrylamide/Bis Acrylamide

Acrylamide - 29 g in 40 ml

N N Bis-Methylene-Acrylamide - 1 g in 20 ml

Make up to 100 ml with distilled water

Filter and store at 4º C in the dark

10 % (W/V) SDS (100 ml)

SDS 10.00 g in 50 ml

Make up to 100 ml with distilled water

1M Tris-Hcl Solution (pH-6.8)

Tris 6.057 g in water 30 ml of DH_2O

Adjust the pH to 6.8 and make up 50 ml with distilled water

1.5 M Tris-Hcl Solution (pH-8.8)

Tris 9.085 g in water 30 ml of DH_2O

Adjust the pH to 8.8 and make up 50 ml with distilled water

10 % (W/V) APS (Freshly prepared)

Ammonium persulfate – 100 mg/ml

Laemmli 2X buffer/Sample Loading Buffer (2X, 8 ml) (pH-6.8)

62.5mM Tris-Hcl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol blue

1 mM Tris-Hcl, pH 6.8 - 0.5 ml

25% Glycerol - 2.0 ml

1 % Bromophenol blue - 0.08 ml

10% SDS - 1.6 ml

DH_2O - 3.42 ml

Store as 1-2 ml aliquots at -70 C and β-Mercaptoethanol (0.4 ml) or 3% DTT (Dithiothreitol) immediately before use.

10X running buffer/(Tris-Glycine/SDS) (pH-8.3)

Dissolve 30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1000 ml of H_2O. The pH of the buffer should be 8.3 and no pH adjustment is required. Store the running buffer at room temperature and dilute to 1X before use.

Procedure

Pouring SDS-polyacrylamide Gels

1. Assemble the glass plates according to the kit manual.

2. Determine the volume of the gel mold provided by the manufacturer. In a 15 ml falcon tube, prepare appropriate volume and desired concentration of acrylamide for the resolving gel or separating gel, using the concentration provided below in the table, after adding the TEMED and APS to the separation gel solution, the gel will polymerize quickly, so add these two reagents when ready to pour. Pour the acrylamide solution into the gap between the glass plates. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm)

TABLE A8-9 Solutions for Preparing Resolving Gels for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis

⇓ Components / Gel Volume ⇒	Volume (ml) of Components Required to Cast Gels of Indicated Volumes and Concentrations							
	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
10% gel								
H_2O	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% acrylamide mix <!>	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate <!>	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED <!>	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

Modified from Harlow and Lane (1988).

3. Layer the top of the separating gel with isopropanol, the overlay prevents oxygen from diffusing into the gel and inhibiting polymerization, remove bubbles and will also keep the polymerized gel from drying out.
4. After polymerization is complete (30 minutes), pour off the isopropanol and wash the top of the gel several times with deionized H_2O to remove any unpolymerized acrylamide and then remove remaining H_2O with the edge of a paper towel.
5. Prepare the stacking gel in a disposable 15 ml falcon tube. Mix the components in the order shown in the table below. Polymerization will begin as soon as the TEMED has been added. Without delay, swirl the mixture rapidly.

TABLE A8-10 Solutions for Preparing 5% Stacking Gels for Tris-glycine SDS-polyacrylamide Gel Electrophoresis

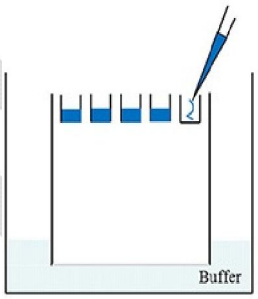
Components / Gel Volume ⇒	Volume (ml) of Components Required to Cast Gels of Indicated Volumes							
	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	8 ml	10 ml
H_2O	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% acrylamide mix <!>	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% ammonium persulfate <!>	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED <!>	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

Modified from Harlow and Lane (1988).

6. Pour the stacking gel solution directly onto the surface of the polymerized resolving gel. Teflon combs should be cleaned with H_2O and dried with ethanol just before use. Immediately insert a clean Teflon comb into the stacking gel solution, being careful to avoid trapping air bubbles.

Preparation of samples and running the gel

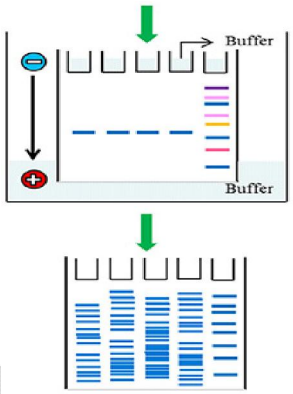
7. While the stacking gel is polymerizing, prepare the samples in appropriate volume of 1X sample solubilisation buffer and heat the samples at 100º c for 10–15 min to denature the proteins. Solubilise sample at 1 mg/mL and run 1–2 µL/lane (1–2 µg/lane).
8. After polymerization is complete (30 minutes), remove the Teflon comb carefully. Use a squirt bottle to wash the wells immediately with deionized H_2O to remove unpolymerized acrylamide. Mount the gel in the electrophoresis apparatus. Add Tris-glycine electrophoresis buffer to the top and bottom reservoirs. Remove bubbles that become trapped at the bottom of the gel between the glass plates.
9. Load up to 15 µl of each samples and 6 µl protein marker into the bottom of the wells. This is done with a micro pipette. Load an equal volume of 1X SDS gel loading buffer into wells that are unused.



Protein samples and marker loaded in vertical SDS-PAGE system

10. Attach the electrophoresis apparatus to an electric power supply (the positive electrode should be connected to the bottom buffer reservoir). Apply a voltage of 50 V to the gel. After the dye front has moved into the resolving gel, increase the voltage to 100 V and run the gel until the bromophenol blue reaches the bottom of the resolving gel approximately 4 hours.

11. When the dye front reaches the bottom of the gel, turn off the power, disassemble the gel apparatus, and place the gel in 200 - 300 ml of fixer/destainer. Gently shake for 16 h pour off spent fixer/destainer and add CBB. Gently shake for 30 min. Destain the gel in several changes of fixer/destainer untill the background is almost clear. Then place the gel in dH_2O, and gently mix untill the background is completely clear. The peptide bands will become a deep purple-blue. The gel can now be photographed. To store the gel wet, soak the gel in 7% glacial acetic acid for 1 h, and seal in a plastic bag.



Direction of migration of samples in vertical SDS-PAGE system

SDS-PAGE gel after Coomassie blue staining

Observation

After staining with coomassie blue proteins appeared as discrete bands in the gel. The relative molecular weights of the protein with respect to their bands were observed in Kilo Daltons.

Result

The protein samples were separated by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis.