PRACTICAL SYLLABUS

1. Verification of Chargaff's rule by paper chromatography.
2. Ultraviolet absorption spectrum of DNA and RNA.
3. Determination of DNA and RNA concentration by A_{260nm}.
4. Determination of the melting temperature and GC content of DNA.
5. A study on the viscosity of DNA solutions.
6. Isolation of chromosomal DNA from *E. coli* cells.

EXPERIMENT 1 - Verification of Chargaff's rule by paper chromatography.

Aim: To verify the Chargaff's rule by paper chromatography.

Principle:

Paper chromatography is based on the principle of partition of solute between two immiscible phases. Since solute in the sample-mixture will have different solubility or partition coefficient between two phases, multiple partitioning processes will result in their separation from each other. In Paper chromatography, as mobile phase containing sample-mixture passed over the immobilised stationary phase by capillary action, the sample is easily resolved from mixture by their Rf value. The Rf value is defined as the relation of the distance travelled by a sample to that of solvent front.

$$Rf = \frac{\text{distance travelled by sample from origin}}{\text{distance travelled by solvent from origin}}$$

The feasibility of separating nucleic acid derivatives by paper chromatography was demonstrated by Marshak and Vogel (1950). This involves separation of the purines and the pyrimidines: the former are precipitated on methanolysis with dry hydrogen chloride, and liberation of the latter is completed by perchloric acid. The two groups are then estimated on separate chromatograms.

Materials required:

1. Whatmann No.1 filter paper sheet
2. Developing solvent: Mix *isopropanol*, *Water* and *Concetrated HCl* in the ratio 130:37:33.
3. Sample containing mixture of Nucleic acids (DNA, RNA).
4. Micropipette, UV chamber.

Procedure:

1. Cut suitable length of filter paper (12 cm), slightly longer than the glass chamber.

2. At one end of the filter paper mark the base line at about 1 cm.
3. The sample was prepared by dissolving 100 mg of nucleic acid with 1 ml of 72% perchloric acid and heat on boiling water bath for 1 hour. Cover the tube with marbles to prevent evaporation. Then cool the sample and add 1 ml of water and centrifuge them at 3000 rpm for 10 minutes to remove any sediment. The supernatant is used as sample to spot on filter paper.
4. With the help of micropipette apply 2µl of the sample on the centre of the base line drawn in the filter paper.
5. Place the filter paper in a chromatographic chamber which has previously been saturated with the solvent system.
6. Close the tank with air tight lid and allow sufficient time for the paper to get fully hydrated.
7. Remove the paper from chamber and allow it to air dry at room temperature.
8. The spots were detected by exposing the dried paper to ultraviolet light.
10. The area containing nucleic acid appears as dark spots.
11. Finally, the Rf value was calculated for the sample and compared with the Rf value of the reference standards.

Result: The Rf values of nucleic acid was found to be _____

EXPERIMENT 2 - Ultraviolet absorption spectrum of DNA and RNA.

Aim: To determine the UV absorption spectrum of DNA and RNA

Principle:

UV- spectrophotometer is used for enumeration of DNA/RNA. The DNA/RNA has maximum and minimum absorption at 260 and 234 nm, respectively. The λmax is used to assess purity of the DNA/RNA and concentration present in the sample. DNA/RNA absorbs UV more or less strongly depending upon the wavelength. By using a UV-Visble spectrophotometer, which takes measurements at wavelengths of 260 and 280 nm, and in addition determines an absorption spectrum from 220 – 350 nm.

Materials Required:

1. DNA and RNA sample
2. UV spectrophotometer
3. Quartz cuvette
4. Distilled water
5. Wash bottle

Procedure:

1. Make the dilution of the given DNA sample in the ratio 1:20.

2. Take the absorbance from 220 to 350 nm.

3. Note the readings and plot the graph of absorbance against the wavelength and make the conclusions.

Result:

Nucleic acids DNA/RNA absorbs maximally at 260 nm. Hence, maximum absorbance at 260 nm indicates presence of nucleic acids.

Table: UV absorption spectrum of DNA and RNA

Si.No.	Absorption Spectrum (nm)	O.D values
1	220	2.0
2	230	1.5
3	240	1.8
4	250	2.0
5	260	2.5
6	270	2.0
7	280	1.5
8	290	0.5
9	300	0.1
10	310	0.0
11	320	0.0
12	330	0.0
13	340	0.0
14	350	0.0

EXPERIMENT 3 - Determination of DNA and RNA concentration by A260nm.

Aim: To determine the absorption spectrum of DNA and RNA at O.D 260 nm

Principle:

Once the contamination has been removed from DNA preparation, the concentration of the DNA in the solution can be determined. The method most commonly used to determine DNA concentration involves the use of UV absorption spectroscopy. Just as all organic compounds have characteristic absorption spectra, the nitrogenous bases of dsDNA exhibit strong absorption maxima at a wavelength of 260 nm. At this wavelength, the extinction coefficient of DNA, E260

= 20, indicates that DNA at a concentration of 1 mg/mL will have an absorption (A260) = 20.

As the relationship between DNA concentration and A_{260} is linear to an A_{260} = 2, the concentration of DNA in a solution can be determined. For example, A_{260} = 0.5 corresponds to

25 μg/mL, A_{260} = 0.1 corresponds to 5 μg/mL, and so on. Use of the conversion factor, 50 μg/Ml = 1 A_{260} unit, enables the concentration of most DNA solutions to be determining easily. However, this relationship only applies to purify dsDNA with a G + C content of 50%. The presence of RNA, proteins, detergents, and organic solvents will also contribute to absorbance at this wavelength. Since the absorption maxima for DNA and protein are 260 nm and 280 nm, respectively, an approximate measure of the purity of the isolated DNA can be obtained by determining the A_{260}/A_{280} ratio. Pure *E. coli* DNA has A260/A280 = 1.95. This ratio, however, is dependent on the overall base composition of the DNA and will vary with different organisms. UV-spectrophotometer is used for enumeration of DNA. The DNA concentration can be calculated by the formula DNA conc. (μg/mL) = *A*260/∈260.

Where, A260 is absorbance at 260 nm and ε260 is the DNA extinction coefficient. For dsDNA, the ε260 is 0.02 (μg/mL)−1 cm −1. Thus, an absorbance of 1 at 260 nm gives a DNA concentration of 50 μg mL−1 (1/0.2 = 50), the ε260 is 0.027 (μg/mL)−1 cm−1 for ssDNA at absorbance of 1 (1/0.027 = 37).

The purity of DNA can be determined by the formula = the ratio of A 260 nm/A 280 nm.

The concentration can be determined by DNA conc. (g/mL) = OD 260 nm × 50 × dilution factor (i.e., 20).

Materials Required:

1. DNA sample
2. UV spectrophotometer
3. Quartz cuvette
4. Distilled water
5. Wash bottle

Procedure:

1. Make the dilution of the given DNA sample in the ratio 1:20.

2. Take the optical density (OD) at 260 nm and 280 nm.

3. Note the readings and make the conclusions.

Result:

The purity of DNA was found to be = 1.5/0.85 = 1.8

The concentration of DNA conc. (g/mL) was found to be = 1,500

EXPERIMENT 4 - Determination of the melting temperature and GC content of DNA.

Aim: To determine the melting temperature and GC content of DNA.

Principle: A double-helical DNA is made up of two strands that run antiparallel to each other. Each adenine (A) in one strand is paired with a thymine (T) on the other; similarly, each guanine (G) on one strand is paired with a cytosine (C) on the other. A–T and G–C are said to constitute the complementary base pairs. This pairing is achieved through stacking interactions and hydrogen bonding between the bases and is the basis of the double stranded DNA structure and its stability. Heating disrupts these non-covalent interactions between the bases; this could unwind the two strands separating the two strands apart. Separation of the two DNA strands is termed as denaturation or melting of DNA. In the double-helical structure, guanine forms three hydrogen bonds with cytosine while adenine forms two hydrogen bonds with thymine. It is therefore evident that the amount of heat required for denaturing the DNA would depend on its nucleotide composition. The temperature at which 50% of the DNA gets denatured is termed as its melting temperature (Tm).

Nucleic acids absorb very strongly in the near UV region. The absorbance is attributed to the heterocyclic rings present in the nucleotides. At neutral pH, DNA would typically absorb with an absorption maximum around 260 nm. Denaturation of DNA leads to higher absorption of ultraviolet radiation (**hyperchromicity**). The melting temperature of DNA can therefore be determined simply by monitoring its absorbance at 260 nm while heating it.

Experimentally, the absorbance of the DNA molecule remains fairly constant at lower temperatures giving a plateau. As the temperature increases, the AT rich regions start melting thereby causing an increase in absorbance. Further increase in temperature causes steep rise in the absorbance followed by another plateau as the DNA gets completely denatured at these temperatures.

Materials Required:

Equipments:

1. UV/Visible spectrophotometer
2. Water baths

Glassware and plasticware:

1. Pipettes
2. Pipette tips
3. Parafilm
4. 1.5 ml microfuge tubes
5. Distilled water
6. 1 ml Quartz cuvettes (Note 1)
7. The DNA solution was prepared using Sodium citrate saline buffer (SSC) [Add 175.3g Sodium chloride and 88.2g trisodium citrate in 1 liter of distilled water and the pH was adjusted to 7 with few drops of 1M HCl].

Procedure:

1. Prepare sufficient volume (at least 15 ml) of the DNA sample in the given buffer so as to obtain an absorbance between 0.1 – 0.4.
2. Take fourteen 1.5 ml microfuge tubes and label them 1 – 14.
3. Add 1 ml of DNA solution into each of the microfuge tubes.
4. Tightly seal all the tubes with parafilm.
5. Label the three water baths as I, II, and III.
6. We shall be measuring absorbance at 20, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, and 95 degrees Celsius i.e. at 14 different temperatures.
7. Set water baths I, II, and III at 20 °C, 30 °C, and 40 °C temperatures, respectively.
8. Place the tubes 1, 2, and 3 in water baths I, II, and III, respectively and incubate at least for 10 minutes.

9. Take tube 1 out and immediately measure its absorbance at 260 nm against the buffer blank.

10. Set the water bath I to 45 °C and place tube 4 in it once the specified temperature is achieved.

11. Meanwhile, take out tube 2 and measure its absorbance.

12. Set the water bath II to 50 °C and place tube 5 in it once the specified temperature is achieved.

13. Meanwhile, take out tube 3 and measure its absorbance.

14. Set the water bath III to 55 °C and place tube 6 in it once the specified temperature is achieved.

15. This cycle is to be followed until the absorbance is recorded for all the 14 tubes.

16. Record the measurements in the observation table shown below.

Observation table:

Table 1. Observation table for DNA melting curve

Temperature`	**A_{260}**
20°C	
95°C	

Analysis:

1. Plot the Absorbance (A260) against the temperature.

2. Determine the mid-point of the curve i.e. , where is the maximum change in absorbance during thermal denaturation.

3. The temperature corresponding to is the melting temperature.

EXPERIMENT 5 - A study on the viscosity of DNA solutions

Aim: To determine the viscosity of DNA solutions.

Principle:

The separation of complementary strand of DNA involves the change from the rigid linear double helix to a random coil formation and leads to a drop in viscosity.

Materials Required:

1. 0.1 ml pipette
2. 0.1 M Acetate buffer (15 g of sodium acetate was dissolved in 250 ml of distilled water.

Exactly 1.5 ml of glacial acetic acid was added very slowly into the sodium acetate aqueous solution. Finally, distilled water was added into the solution to fill the volume.

Procedure:

1. Gently dissolve around 200 mg of DNA in 7.5 ml of 0.1M Acetate buffer to obtain a viscous solution and transfer to a test tube.
2. In another tube take only the acetate buffer of the same volume.
3. Using 0.1 ml pipette draw the acetate buffer solution to the zero mark.
4. Keep the tip ends of the pipette touching the buffer solution and now open the closed end, simultaneously starting the stopwatch.
5. Stop the watch as soon as the level drops to a fixed point in the pipette, say 0.05 ml.
6. Repeat a few times to obtain the average points.
7. The same procedure is repeated with the prepared DNA solutions preferably using the same pipette.
8. The relative viscosity of the DNA sample is roughly calculated by the time taken for the DNA solution divided by the time taken for the buffer to fall through the same distance.

Relative Viscosity = t_1/t_0 where t_1 – time taken by the test solution, t_0 – time taken by the buffer

Result: The relative viscosity of the given DNA sample was found to be

EXPERIMENT 6 - Isolation of chromosomal DNA from *E. coli* cells.

Aim: To isolate chromosomal DNA from the *E.coli* cells and visualizing the same DNA by gel electrophoresis.

Principle:

Genomic DNA preparation differs from the plasmid DNA preparation. Genomic DNA is extracted from bacterial cells by immediate and complete lysis whereas plasmid DNA is isolated by slow-cell lysis to form a sphaeroplast.

The procedure of genomic DNA extraction can be divided into 4 stages:

1. A culture of bacterial cell is grown and harvested.

2. The cells are broken open to release their contents.

3. The cells extracted are treated to remove all components except the DNA.

4. The resulting DNA is then purified.

Materials required:

1. TE buffer (pH 8.0)
2. 10% (w/v) sodium dodecyl sulfate (SDS)
3. 20 mg/mL proteinase K
4. Phenol/chloroform (50:50)
5. Isopropanol
6. 70% ethanol
7. 3M sodium acetate pH 5.2

Procedure:

1) *E. coli* culture was grown overnight in Luria broth.
2) The overnight culture was transfered into 1.5 mL to a microcentrifuge tube and centrifuged at 10000 rpm for 5 min. The supernatant was decanted and repeated with another 1.5 mL of cells.

3) The bacterial pellet was resuspended in 467 µL TE buffer by repeated pipetting. 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K was added, mixed, and incubated for 1 hr at 37°C.

4) Equal volume of buffer saturated phenol: chloroform: isoamyl alcohol (25:24:1) or chloroform: Isoamyl alcohol (24:1) was added and mixed well by inverting the tube until the phases are completely mixed. (Caution: Phenol causes severe burns. Wear gloves, goggles, and a lab coat, and keep tubes capped tightly)

5) Centrifuged at 12000 rpm for 5 min.

6) The upper aqueous layer phase was transferred to a new 1.5 ml eppendorf tube and equal volume of chloroform:Isoamyl alcohol (24:1) was added, Again mixed well by inverting and transfered to a new tube and centrifuged at 12000 rpm for 5 minutes.

7) The upper aqueous phase was again transferred to a new 1.5 ml eppendorf tube.

8) To the aqueous phase, 1/10 volume of 3M sodium acetate (pH 5.2) was added.

9) Then 0.6 volume of isopropanol was added and mixed gently until the DNA gets precipitates.

10) Centrifuged at 12000 rpm for 10 min and the supernatant was discarded.

11) The DNA pellet was washed by adding 200 µL of 70% ethanol (Centrifuge at 10000 rpm for 3 min)

12) Finally the DNA pellet was suspended in a 100–200 µL TE buffer. Complete resuspension may take several days.

13) After DNA has dissolved, the purity of the DNA was checked by electrophoresis and spectrophotometric analysis.

14) 0.8 % Agarose gel was prepared using 1X TAE buffer.

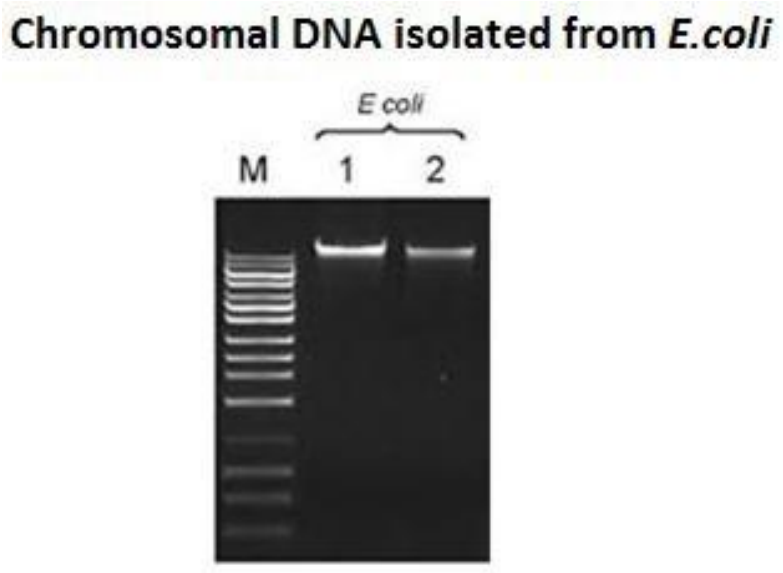
15) Isolated DNA samples were loaded into the wells, recording which samples are loaded into which wells as lane 1, 2, etc. The power supply was started after sample loading, with the voltage set to 50 V.

16) Ran the gel until the second dye from the well has reached 3/4 th of the gel.

17) After the run was completed, the gel was observed under UV trans-illuminator.

18) Then DNA was stored at 4°C short term, –20°C or –80°C long term

Results: A sharp band of chromosomal DNA was observed.



Lanes 1 and 2 – Chromosomal DNA of Gram negative bacteria *E. coli*, Lane M is 1kb DNA Ladder

Semester IV

17BCU411 GENE ORGANISATION, REPLICATION AND REPAIR 4H-2C PRACTICAL

Instruction hours/week: L:0 T:0 P:4 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 3 Hours

Course objectives

- The main aim is to impart practical skill on DNA and RNA.
- Both qualitative and quantitative analysis of DNA and RNA are determined.
- The physical properties these biomolecules possess are also studied.

Course outcomes (CO's)

- Better understanding and their importance of these molecules are the outcome of the study that student will come to know.

1. Isolation of chromosomal DNA from *E. coli* cells.
2. Isolation of chromosomal RNA from *E. coli* cells.
3. Ultraviolet absorption spectrum of DNA and RNA.
4. Determination of DNA and RNA concentration by A_{260nm}.
5. Determination of the melting temperature and GC content of DNA.
6. A study on the viscosity of DNA solutions.
7. Verification of Chargaff's rule by paper chromatography.

REFERENCES:

Watson, J.D., Baker, T.A., Bell, S.P., Gann, A., Levine, M. and Losick, R., (2008). Molecular Biology of the Gene 6th ed., Cold Spring Harbor Laboratory Press, Cold spring Harbor (New York), ISBN:0-321-50781 / ISBN:978-0-321-50781-5.

Nelson, D.L., and Cox, M.M., (2013). Lehninger: Principles of Biochemistry 6th ed., W. H. Freeman & Company (New York), ISBN:13: 978-1-4292-3414-6 / ISBN:10-14641-0962-1.

Snustad, D.P., and Simmons, M.J., (2010). Principles of Genetics 5th ed., John Wiley & Sons Asia, ISBN:978-0-470-39842-5.