Indira Gandhi
National Open University
School of Sciences

BBCCL-124
GENE EXPRESSION AND REGULATION (PRACTICAL)

GENE EXPRESSION AND REGULATION

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GENE EXPRESSION AND REGULATION

Welcome to the laboratory course on **Gene expression and regulation**, **BBCL-124**. In this laboratory course, you will perform experiments to isolate nucleic acids and the estimations, along with determining purity. This laboratory course is connected with the core course BBCCT-123. The basics of these experiments will help you for better performance in the experiments in upcoming courses.

The first experiment of this course demonstrates how to isolate total nucleic acids from plant tissue. The following experiment is on isolating nucleic acids from the onion bulb. In the third experiment, you will isolate mRNA using the TRIzol reagent. In the fourth experiment, you will quantify the RNA using the Orcinol method. The last experiment is the determination of the purity of nucleic acids by spectrophotometry.
Learners are advised to follow the standard operating procedures and instructions given by the respective counsellors and instructors.

Expected Learning Outcomes:

After performing these experiments you shall be able to:

- isolate total nucleic acids from plant tissue;
- isolate mRNA using TRIzol reagent;
- describe the importance of reagents used in the isolation of RNA;
- quantitatively estimate the amount of nucleic acids by Orcinol method; and
- perform determination of purity of nucleic acids by spectrometry.

We hope you will find these experiments quite interesting while performing.

Our best wishes!

EXPERIMENT 1

EXTRACTION OF TOTAL NUCLEIC ACIDS FROM PLANT TISSUE

Structure

1.1 INTRODUCTION

The extraction of DNA from plant materials is one of the most challenging processes. In order to extract tissues effectively, the tissues must be properly prepared. All plant DNA extraction protocols comprise of the basic steps of disruption of the cell wall, cell membrane and nuclear membrane to release the DNA into solution followed by precipitation of DNA, while ensuring removal of the contaminating biomolecules such as the proteins, polysaccharides, lipids, phenols and other secondary metabolites. Cauliflower is taken as an example for the plant tissue for the purpose of understanding. However, you can explore and perform the experiment with different plant materials (seedlings, leaves, cotyledons, seeds, endosperm, tissue culture callus, roots etc.,). This method is popularly known as **CTAB** method as Cetyl Trimethyl Ammonium Bromide (CTAB) is used as a detergent in this experiment.

Expected Learning Outcomes

After performing this experiment, you should be able to:

- explain the principle behind the nucleic acid extraction;
- perform the steps involved in nucleic acid extraction;
- describe the role of various reagents in nucleic acid extraction; and
- enlist the materials required for extraction of nucleic acids from plant tissue.

1.2 PRINCIPLE

The broad principle operation behind the extraction of nucleic acids form plant tissue involves the use of high concentration of salt (hypertonic solution) that, enables exomosis thereby helps cell contents to come out of the cell. Anionic detergents like CTAB or Sodium Dodecyl Sulphate (SDS) denatures proteins and solubilizes membrane lipids (lysis of cell membrane). The organic solvent chloroform ($CHCl_3$) dissolves the solutes present in the solution and brings them into the organic layer. Ethanol is dehydrating agent which helps in precipitating the DNA.

1.3 MATERIALS REQUIRED

Plant Material: Cauliflower or leaves of any plant

Reagents and Buffers: Saturated phenol pH 8.0, chloroform: isoamylalcohol (in 24:1 ratio) mix, TE Buffer = Tris: EDTA (10mM : 1mM) pH 8.0, 70% ethanol,

i. RNase A (10 mg/mL):

a. Dissolve RNase A in 10 mMTris-Cl, pH 7.5, 15 mM NaCl. Heat at 100 °C in water bath for 2-5 mins.

b. Cool to room temperature. Store as aliquots at -20 °C.

ii. Extraction (CTAB) Buffer:

Note: Learners are advised to follow the instructions and precautions learnt in the lab course of BBCL-102 while preparing the buffers and handling the pH meter.

Extraction Buffer A (EBA)	**Per 10 mL**
10% (w/v) Cetyl Trimethyl Ammonium Bromide (CTAB)	3mL
Tris (pH 8.0) (Use 1 M stock)	1mL
EDTA (Use 0.5 M stock, pH 8.0)	0.4 mL
5 M NaCl	2.8mL
4% (w/v) polyvinylpyrrolidone (PVP)	0.3 g
β-mercaptoethanol (BME)* (Use 14.3 M stock)	0.02 mL
Double Distilled water or MilliQ water	2.48mL
TE Buffer	**Per 100 mL**
10 mM Tris (pH 8.0) (Use 1 M stock)	10 mL
1 mM EDTA (Use 0.5 M stock)	50 µL

Other Required Reagents	
20% (w/v) sodium dodecyl sulphate (SDS) 5 M potassium acetate (Stored at-20°C) 3 M sodium acetate (pH 5.2) 70% ethanol (stored at -20°C) Absoulte isopropanol (stored at -20°C)	

The role of various components of DNA extraction protocol is as follows: (Adopted from DBT life Sciences protocol Manual):

a) The extraction/Lysis buffer:

This includes a detergent such as Cetyl Trimethyl Ammonium Bromide (CTAB) or SDS which disrupts the membranes, a reducing agent such as β-mercaptoethanol which helps in denaturing proteins by breaking the disulphide bonds between the cysteine residues and for removing the tannins and polyphenols present in the crude extract, a chelating agent such as EDTA which chelates the magnesium ions required for DNase activity, a buffer which is almost always Tris at pH 8 and a salt such as sodium chloride which aids in precipitation by neutralizing the negative charges on the DNA so that the molecules can come together. These denaturing conditions efficiently solubilized the nucleic acid and generally do not adversely affect them and lyse the cell by destabilizing the cell wall.

b) Phenol chloroform extraction:- Protein Precipitation:

Nucleic acid solutions commonly contain undesirable contaminants that are mainly contributed by proteins and polysaccharides. A classic method of removing these contaminants is phenol – chloroform extraction in which the nucleic acid solution is extracted by successively washing with a volume of phenol(pH 8.0); a volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) and chloroform: isoamyl alcohol (24:1). Centrifugation is performed and the upper aqueous phase is transferred to a new tube while avoiding the interphase. The contaminants are denatured and accumulated in the organic phase or in the marginal layer between the two phases and the nucleic acids are preserved in the aqueous phase. Another way of removing proteins is by using the enzyme proteinase K which however again is denatured by phenol via phenol chloroform extraction.

c) Precipitation of nucleic acids:

Alcohol precipitation is the most commonly used method for nucleic acid precipitation. This requires diluting the nucleic acid with a monovalent salt, adding alcohol to it and mixing gently. The nucleic acids get precipitated spontaneously and can be pelleted by centrifugation. The salts and alcohol remnants are removed by washing with 70% alcohol. The most commonly used salts include sodium acetate pH 5.2(final volume 0.3M), sodium chloride

(final concentration 0.2M), ammonium acetate (2-2.5M), lithium chloride (0.8M) and potassium chloride. Ethanol (twice the volume) or isopropanol (two thirds volume) are the standard alcohols used for nucleic acid precipitation.

d) Resuspending DNA:

The nucleic acid pellet can be resuspended in either sterile distilled water or TE (10 mMTris: 1mM EDTA).

e) Purification of DNA:

The DNA is purified by incubating the nucleic acid solution with RNaseA (10 mg/mL) at 37°C and reprecipitation following phenol: chloroform extraction to remove the RNase.

1.4 PROCEDURE

Extraction Protocol

1. Weight out 0.3 g of plant tissue (example: cauliflower).
2. Place tissue on a clean glass slide. Chop the tissue into a paste using a clean single edge razor blade. If available tissue homogenizer can be used.
3. Immediately transfer tissue to a 1.5 mL microcentrifuge tube and (optional) further grind tissue with a tube pestle.
4. Once the sample is prepared add 1200 µL EBA and 100 µL SDS.
5. Vortex and incubate at 65^{o}C for 10 min.
6. Place tube on ice and add 410µL cold potassium acetate. Mix by inversion and place tube back on ice for 3 min.
7. Centrifuge at 13,200 rpm for 15 min. (if possible, use a refrigerated microcentrifuge set to 4^{o}C).
8. Transfer the supernatant in new microcentrifuge tube.
9. Add equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1).
10. Centrifuge at 10,200 rpm for 10 min.
11. Transfer the upper aqueous phase to a new microcentrifuge tube while avoiding the interphase.
12. Add equal volume of chloroform: isoamyl alcohol (24:1).
13. Centrifuge at 10,200 rpm for 10 min.
14. Transfer the upper aqueous phase to a new microcentrifuge tube while avoiding the interphase.
15. Add 600µL of ice cold absolute isopropanol, and incubate in ice for 20min.

16. Centrifuge at 10,200 rpm for 10 min. discard the supernatant. Wash the pellet once in 500 µL 70% ethanol and let dry.

17. Resuspend the dry pellet in 600 µL of TE. Add 60 µL 3M sodium acetate (pH) 5.2 and 360 µL ice cold absolute isopropanol. Incubate on ice for 20 min.

18. Repeat Steps 9-11 twice.

19. Resuspend the pellet in 50 µL TE and carry out agarose gel electrophoresis.

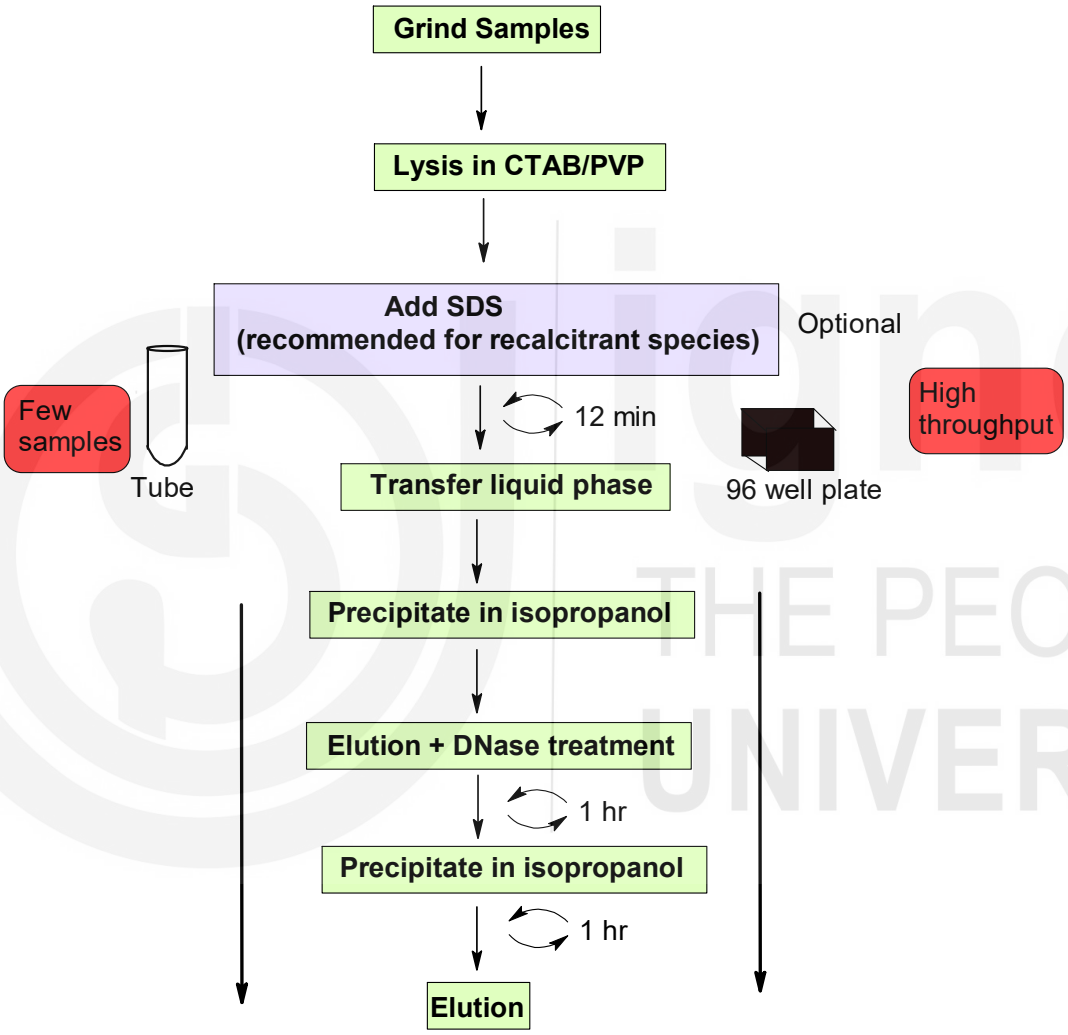


Fig. 1.1: Flow chart showing DNA extraction protocol.

Agarose Gel electrophoresis

1. Cast a 1.0% (w/v) regular agarose gel in 1X TBE.

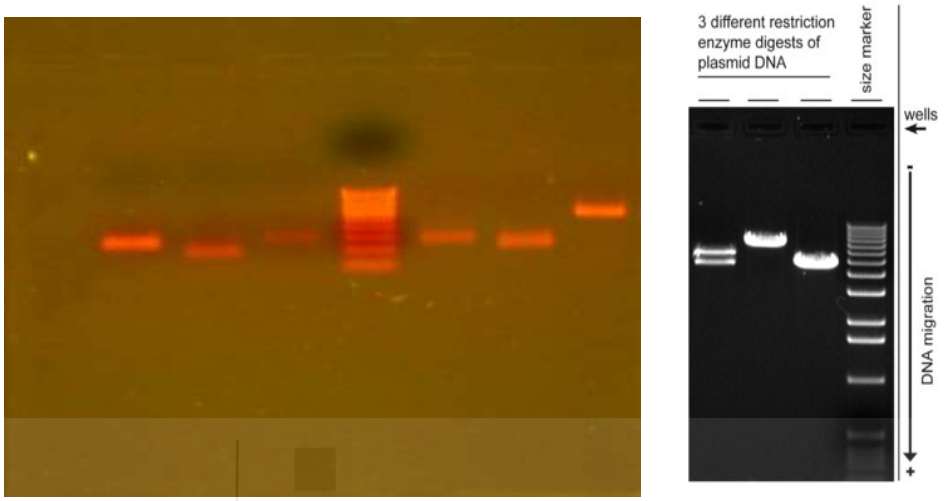
2. Place 5 µL of extracted DNA and 5 µL sterile water in a 0.2 mL microcentrifuge tube along with 2 µL of gel tracking dye.

3. Run the gel for 20 min. at 100V.

4. Stain the gel with EtBr and view result.

Observation:

The gel was observed under UV radiation (Fig.1.2) for the presence or the absence of DNA bands. DNA marker also loaded in middle lane to confirm size of DNA.



(A) (B)

Fig. 1.2: A. Visualization of DNA bands (sample images).

B. Plasmid DNA (https://en.wikipedia.org/wiki/Agarose_gel_electrophoresis)

1.5 RESULTS

The DNA band at the top of the gel suggest that they are of high molecular weight (higher than all the DNA fragments of Marker DNA hence they are genomic DNA. The intensity and the brightness DNA band directly proportionate to the concentration of DNA. The presence of clear or straight DNA band indicates the intactness of the genomic DNA.

1.6 PRECAUTIONS

1. All procedural steps of the experiment should be conducted in a very clean laboratory working area.

2. It is recommended to use plasticware and reagents free of DNase. Tubes and tips should only be used if they are clean, dry, and autoclaved.

3. Precipitated reagents should be examined. The reagent must be warmed to 55-65 °C until the precipitate gets dissolved, then allowed to cool down to room temperature (15-25 °C) before use. If the precipitate forms, take it warm at 55-65 °C until it dissolves and then let it cool down to room temperature (15-25 °C).

4. To obtain pure DNA, repeat the phenol chloroform extraction based on the sample source.

5. Handle ethidium bromide stained agarose gels with gloves (ethidium bromide is carcinogenic).

6. When handling ethanol, take precautions due to its high flammability.

EXPERIMENT 2

ISOLATION OF DNA FROM ONION BULBS

Structure

2.1 INTRODUCTION

In the previous experiment you have performed the extraction of total nucleic acids from plant tissue. In this experiment you will be isolating the DNA present in onion bulbs. The major objective of this experiment is to give the learners an understanding of the various methods that are available for the isolation of DNA based on the nature of the tissue sample. This is a simple grinding experiment where the principle of exoosmosis is applied. You will also learn the importance of reagents and detergents used in this experiment. Follow the instructions given in the experiment for the successful completion of the experiment.

Expected Learning Outcomes

After performing this experiment, you should be able to:

- ❖ isolate DNA from the onion tissue;
- ❖ explain the importance of reagents used in this experiment; and
- ❖ describe the steps involved in the isolation of DNA from onion bulb.

2.2 PRINCIPLE

High concentration of salt enables exomosis thereby helps cell contents to be come out of the cell. Anionic detergents like sodium dodecyl sulfate (SDS) denatures proteins and solubilizes membrane lipids (denaturation of cell membrane). The organic solvent chloroform ($CHCl_3$) dissolves the solution into an organic layer containing all contents and aqueous layer of DNA. Ethanol is dehydrating agent which helps in precipitating of DNA.

2.3 MATERIALS REQUIRED

Onions, table salt, 10% sodium dodecyl sulfate, chloroform, ethanol, ice cubes, mortar and pestle, funnel, filter paper, pipettes, glass rod, beakers and water bath.

2.4 PROCEDURE

Cut onion into small pieces and blend it by adding water to prepare slurry. Take slurry into a fresh beaker and add 1 table spoon of salt and add 10 mL of SDS and stir it thoroughly. Incubate it at 60^{o} C for 15 minutes. Then cool the solution on ice for 5 min then filter the solution. To the filtrate slowly add 2 mL of chloroform. You can observe the formation two distinct layers (Top organic and bottom aqueous layer). Separate the top layer and add ice cold 95% ethanol to it. Incubate it on ice for 10 minutes.

You are advised to watch the YouTube video available at the given link to get an idea how to perform the experiment:
https://www.youtube.com/watch?v=MBTxC89tniY

2.5 RESULT

While adding ice cold ethanol a fine thread like DNA appeared like a thread has been isolated.

EXPERIMENT 3

RNA ISOLATION BY TRIZOL METHOD

Structure

3.1 INTRODUCTION

In the previous experiments, you have performed the extraction of total nucleic acids from plant tissue. In this experiment, You Will be performing the isolation of RNA using TRIzol method. This is one of the most widely used methods in the research laboratories. In this method, TRIzol reagent is used which is commercially available and supplied by different manufacturers.

Isolation of RNA is a crucial step while conducting GENE expression studies. While studying BBCCT-123 course you must have observed the various factors regulating the expression of genes inside a living cell. Here it is important to re-collect the concepts of transcription and translation. The success of this method depends on maintaining the standard operating procedures in the laboratory along with following the instructions provided by the manufacturers of the regent. The RNA isolated in this experiment can be used to perform the next two experiments of this lab course.

Expected Learning Outcomes

After performing this experiment, you should be able to:

- separate RNA from biological sample;
- enlist the reagents required for performing RNA isolation; and
- explain the working principle of this method.

3.2 PRINCIPLE

TRIzol reagent is a ready to use reagent for the isolation of RNA from biological samples, especially from cells and tissues. The RNA isolated from this method can be used to perform polymerase chain reaction (PCR). TRIzol reagent is made up off phenol and guanidine isothiocyanate. This Regent plays an important role in maintaining the integrity of RNA present in sample while performing homogenisation and dissolving the cells or tissue components. In the later stage of this experiment chloroform is used to generate aqueous and organic phases where RNA remains in the aqueous phase. Later RNA is recovered by precipitation by the addition of isopropanol. The precipitated RNA, is re-dissolved in nuclease free water.

3.3 MATERIALS REQUIRED

1. Chemicals and reagents:

DEPC-treated water, TRIzol Reagent, Ice cold Phosphate buffered saline (PBS), Cell scraper, 70% ethanol, Isopropyl alcohol

2. Basic equipment and accessories:

Refrigerated centrifuge, Microcentrifuge, Micropipettors, Aerosol-barrier tips, Vortex mixer, Powder free gloves, Centrifuge tubes

3.4 PROCEDURE

Sample preparation:

i. Tissue Sample

Take 50-100 mg of tissue sample in 1 mL of TRIzol reagent and homogenize using a glass-Teflon or power homogenizer (refer Unit-2, BBCCT-105 PROTEINS).

ii. Cell lines

Following procedure is used in case cells grown in a culture medium are source for isolating the RNA.

1-2 x 106 cells were dislodged from culture dish, washed with PBS and lysed in 1 mL of TRIzol reagent and allowed to stand for 5 minutes at room temperature (RT).

The following will remain same for either tissue or cell homogenate:

PHASE SEPERATION

200 µL of distilled chloroform was added, mixed vigorously by vortexing and kept at RT for 15 minutes. The resulting mixture was centrifuged at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase was carefully transferred to another tube and RNA was precipitated by the addition of 500 µL isopropanol followed by thorough mixing and incubation at RT for 10 minutes (Fig. 3.1).

RNA WASH

RNA was pelleted by centrifuging at 12,000 x g for 10minutes at 4°C and washed twice with 75% ethanol. The RNA pellet obtained was allowed to air dry to remove traces of ethanol and resuspended in 30 µL of DEPC treated water.

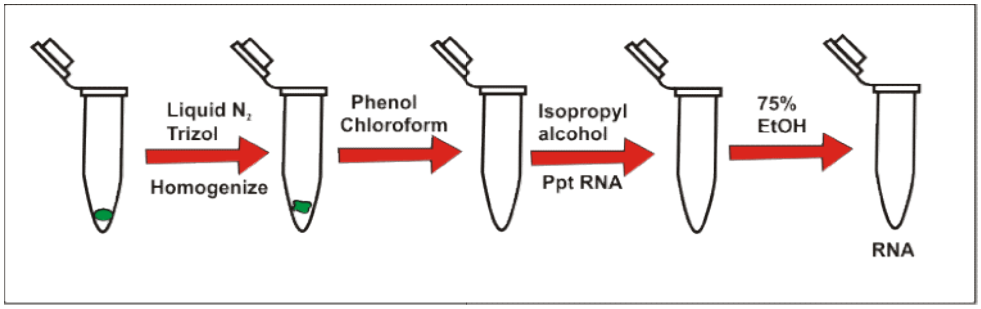


Fig. 3.1: Schematic representation of Trizol method.

SPECTROPHOTOMETRIC ANALYSIS

The concentration of RNA was determined by using Nanodrop or UV spectrometer by taking absorbance of 1:500 dilution of the RNA at 260 nm and calculated by using the formula: Concentration of RNA in µg/ml = A260 x 40 x dilution factor. Incubate the homogenized sample for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Centrifuge to remove cell debris. Transfer the supernatant to new tube.

3.5 RESULTS

RNA successfully isolated by using TRIzol method.

Note: The isolated RNA sample is used to perform quantitative estimation by Orcinal method (experiment-5) or used to determine the purity by spectrophotometer (experiment-6).

3.6 PRECAUTIONS

1. Use Standard Precautions when handling all body fluids, tissues and cell cultures. Refer to the Specimen Collection and Handling, GEN-1, for guidelines specific for the Molecular Genetics Laboratory and samples.
2. TRIzol reagent is toxic when in contact with skin and if swallowed. It will cause burns. Be sure to wear a lab coat, gloves and safety glasses when working with TRIzol reagent. If in contact with skin, wash immediately with plenty of soap and water. Work in a chemical fume hood.
3. To prevent RNase contamination, always wear gloves and change them frequently. Also, use sterile, disposable plasticware and pipettes dedicated strictly to RNA work to prevent cross-contamination with RNases from shared equipment.

4. RNA is extremely susceptible to degradation by ribonucleases that are ubiquitous in the environment. To ensure preservation of target RNA or RNA probes, special precautions are needed. Records are maintained to show that RNasefree conditions (i.e. wiping the lab areas with RNaseZAP) are met, with corrective action if conditions are not met.

EXPERIMENT 4

ESTIMATION OF RNA BY ORCINOL METHOD

Structure

4.1 INTRODUCTION

In the previous experiment you have performed isolation of RNA using TRIzol method. In this experiment, you will estimate the quantity of RNA usingorcinol method. This method is sensitive towards oxyribose (pentose sugar) present in RNA molecule. There are different methodsavailable for estimating pentose sugars, howeverthis method is widely used due to its easy detection ability using colorimeter.

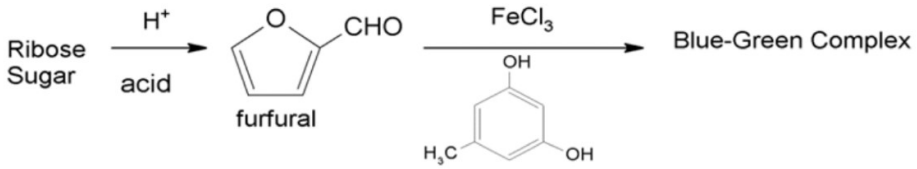
Expected Learning Outcomes

After performing this experiment, you should be able to:

- explain the principle of orcinol method;
- quantify the RNA present in a sample;
- prepare the various reagent used in the experiment; and
- plot the standard graph.

4.2 PRINCIPLE

This is a general reaction for pentoses and depends on the formation of furfural when the pentose is heated with concentrated hydrochloric acid. Orcinol reacts with the furfural in the presence of ferric chloride as a catalyst to give a green colour, which can be measured at 665 nm.



Reaction showing formation of blue-green color complex

4.3 MATERIALS REQUIRED

1. **Chemicals and Reagents:** 5% perchloric acid, commercial RNA sample, sodium chloride, sodium citrate.
2. **Equipments and Glass ware:** colorimeter or spectrophotometer, boiling water bath, pipettes, test tubes, beakers, measuring cylinders test tube stands and holders.
3. Prepare standard RNA solution by dissolving 200 µg/ml of RNA in 1 N perchloric acid or buffered saline.
4. Prepare orcinolreagent by dissolving 0.1 g of ferric chloride in 100 ml of concentrated HCllater add 3.5 ml of 6% w/v orcinol in alcohol.
5. Prepare buffered saline by dissolving 0.5 mol/litreNaCl; 0.015 mol/litre sodium citrate, and adjust the pH to 7.

4.4 PROCEDURE

1. Take 6 clean and dry test tubes and label them as “standard” 1to5.
2. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of RNA working standard in to the above labeled test tubes respectively (Table 4.1).
3. Take a clean and dry test tube label as “test or unknown”, pipette out 1 ml of the given test sample in into this tube.
4. Make up the final volume to 1 ml in all the test tubes.
5. Take a clean and dry test tube and label as “blank”, pipette out 1 ml of distilled water into this tube. This tube serves as blank.
6. To all the above seven test tubes add 2 ml of orcinol reagent.
7. Carefully mix the contents of the test tubes by vortexing/shaking the tubes. Incubate all thetubes in a boiling water bath at 100°c for 20 min.
8. After incubation time cool the contents and record the absorbance using colorimeter at 665 nm against blank.
9. Then plot the standard curve by taking concentration of RNA standards on X-axis and absorbance valuesat 665 nm on Y-axis.
10. Calculate the concentration of RNA in the given sample using this standard curve.

4.5 RESULTS

The given unknown sample contains---- µg RNA/ml.

Table 4.1: Observation table

S.No	Volume of standard (ml)	Volume of distilled water (ml)	Concentration of RNA (µg/ml)	Volume of Orcinol reagent (ml)	Incubate in boiling water bath for 20 min. Cool the tube before taking absorbance values	Absorbance values at 665nm
Blank	0.0	1.0	00	2		0.00
Standar 1	0.2	0.8	40	2		
Standar 2	0.4	0.6	80	2		
Standar 3	0.6	0.4	120	2		
Standar 4	0.8	0.2	160	2		
Standar 5	1.0	0.0	200	2		
Test/ Unknown	1.0	0.0	To be estimated (?)	2		

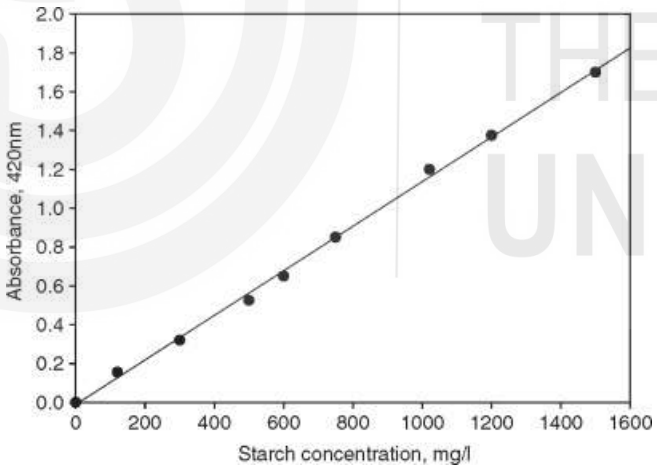


Fig. 4.1: Standard curve for RNA estimation by Orcinol reaction.

(Source: https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/orcinol)

4.6 PRECAUTIONS

1. Follow standard operating procedures while handling acid solution
2. Use proper safety measures while boiling the solutions
3. Use pipettes and holders avoid direct contact.

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EXPERIMENT 5

DETERMINATION OF PURITY OF NUCLEIC ACID (DNA/RNA) BY SPECTROPHOTOMETRY

Structure

5.1 INTRODUCTION

In the previous experiment you have performed the estimation of quantitative analysis of RNA by orcinol method. In this experiment you will study about spectrophotometric method that is based on U.V. absorption. This is a widely used method in molecular biology research to determine the purity of either DNA or RNA.

For better understanding of this experiment it is advised to go through Units-13 &14 of course BBCCT-101. We all know that nucleic acids act as genetic material and are made up of nitrogenous bases like purines and pyrimidines. Due to the presence of conjugate structures these bases show absorption maxima at 260nm. On the other side proteins with aromatic amino acids shows absorption maxima at 280nm. That's why this method has gained significance in terms of both purity i.e., quality and quantitative determination of nucleic acids.

In this method we are not going to observe any colour change either in the substrates or in the products. But it is the ability of spectrophotometer that makes it possible to read the changes in the intensity of U.V. light. However, you'll know more about this technique in skill enhancement (BBCS-183) i.e., Tools and techniques in Biochemistry. However in this experiment we'll be focusing more on determining the purity of DNA.

Expected Learning Outcomes

After performing this experiment, you should be able to:

- ❖ explain the principle behind the determination of purity of nucleic acids;
- ❖ identify specific absorption maxima of nucleic acids and proteins;
- ❖ determine the purity of nucleic acid; and
- ❖ know how to operate spectrophotometer.

5.2 PRINCIPLE

Principle: The presence of conjugate structures in the nucleic acids shows absorption maxima at 260nm. This property is typically used in determining nucleic acids purity through U.V absorption spectrophotometric method. The ratio between absorbance values at 260 nm and 280 nm (OD260:OD280) provides an estimate of the purity of the nucleic acid (Fig. 5.1). Pure preparation of DNA and RNA have (OD260:OD280) value of 1.8 and 2.0, respectively. If there is any protein or phenol contamination, the sample ratio value will be less than 1.8 and 2.0. Due to this significance, the absorption ratio at 260 nm and 280 nm has been used to measure the purity of isolated nucleic acids.

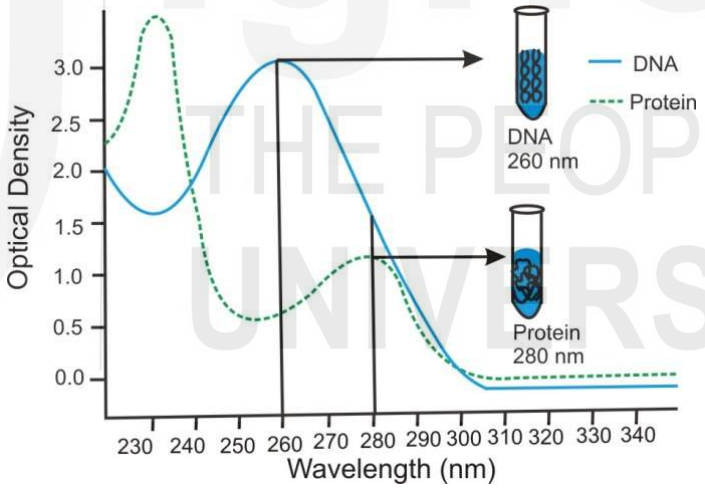

Fig. 5.1: Absorption of U.V. light by DNA and protein.

5.3 MATERIALS REQUIRED

Glassware: Cuvette,Volumetric conical flask, Pipette, Burette, Burette stand and Standard flask.

Chemicals: Commercial DNA/RNA sample, Ethylene diaminetetraacetic acid (EDTA), Sodium phosphate, Sodium chloride, Tris-HCl

Equipments: U.V. Spectrophotometor

Preparation of stock solution (100µg/ mL**)**: Weigh 500mg of commercially available DNA/RNA into a 100 mL volumetric flask and dissolve in little volume

of 20 mM sodium phosphate, *p*H 7.0 or 0.1 M sodium chloride and make up the volume to 100 mL with 20 mM sodium phosphate, *p*H 7.0 or 0.1 M sodium chloride.

Preparation of DNA/RNA working standard solution (50µg/ mL**):** Dilute 1mL of the stock solution to 100 mL, with 20 mM sodium phosphate, *p*H 7.0 or 0.1 M sodium chloride in a 100 mL volumetric flask.

Preparation of 1 M Tris- HCL:

Preparation of 0.5 EDTA:

Preparation of Tris-EDTA (TE) buffer:

Take 10 mL of 1M Tris-HCl (*p*H 8.0) buffer and 2 mL of 0.5 M EDTA solution, mix both the solutions with distilled water and make up the volume to 1000 mL in a graduated measuring cylinder.

Note: Test Sample: If available DNA sample isolated from biological material can be used

5.4 PROCEDURE FOR DETERMINING THE PURITY OF NUCLEIC ACIDS

A. To measure the purity of nucleic acids in the given sample:

i. Standardize the UV-Visible spectrophotometer with Tris-EDTA buffer as blank at both 260 and 280 nm

ii. Dissolve the DNA/RNA sample in appropriate volume (either 1:50 of 1:100 mL) of same buffer and measure the absorbance at both 260 and 280 nm.

iii. Calculate the concentration of DNA/RNA by using following formula

Purity of DNA= OD at 260 nm / OD at 280 nm

Prior to start the experiment it is advised to go through the instruction manual provided along with the spectrophotometer.

However for routine practice in the laboratory learners can use commercially available DNA powder and prepare the DNA sample as discussed in the above section 8.2.

5.5 RESULTS

A. Absorbance values of the DNA/RNA sample at 260and 280 nm using UV-VIS Spectrophotometer are

A260 - _____ OD; A280 - _____ OD and the ratio of absorption is OD at 260 nm / OD at 280 nm i.e, _____

Reference values:

Standard reference values for various nucleic acid solutions with absorption at 260 nm =1, the following approximations are valid:

1 A_{260} unit* of double stranded DNA = 50 µg/ mL

1 A_{260} unit* of single stranded DNA = 37 µg/ mL

1 A_{260} unit* of single stranded RNA = 40 µg/ mL

1 A_{260} unit* of oligonucleotide = 20 to 33 µg/ mL

*__Unit definition:__ The concentration of nucleic acid dissolved in 1 mL buffer (20 mM sodium phosphate, *p*H 7.0 or 0.1 M NaCl), which has an absorbance of 1 (at A_{260}). The spectrophotometric measurement is made in a 1 cm cuvette, at 20^{o} C.

5.6 PRECAUTIONS

1. Wore gloves to avoid contamination
2. Use separate pipettes to avoid contamination.

EXPERIMENT 6

DIAUXIC GROWTH CURVE

Structure

6.1 INTRODUCTION

This experiment is to demonstrate that glucose is the preferred carbon source for growth of *E. coli*. You will observe that only glucose is used by bacterial cells even when an alternative carbon source lactose is available in the culture medium. Experimentally, it is demonstrated by depletion of glucose as the cells multiply in culture, while the amount of lactose remains constant. As all the glucose molecules are exhausted, the cells are forced to use the lactose as the alternative source. So a second lag phase in the bacterial growth curve is observed after all the glucose molecules present in the medium are consumed by the cells.

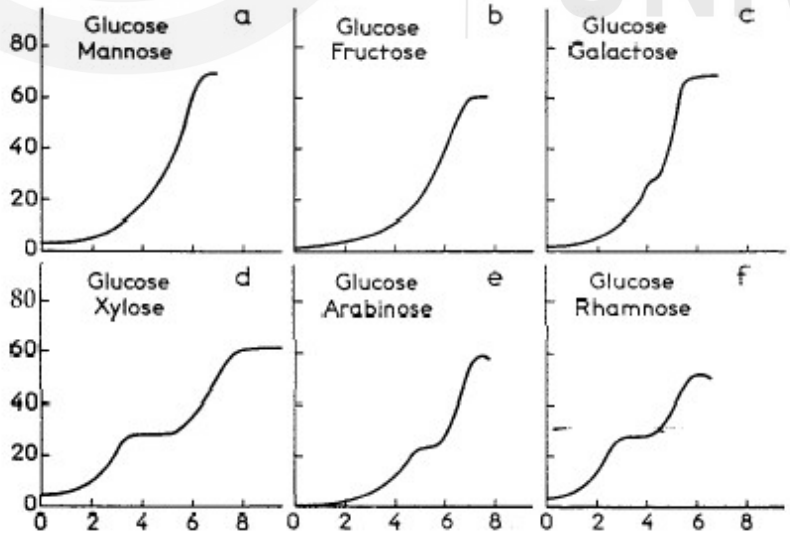

Fig. 6.1: Growth of *Escherichia coli* in the presence of different carbohydrate sources (https://en.wikipedia.org/wiki/Diauxic_growth).

During this lag phase, the cells adapt to the new conditions in which glucose is absent and lactose is present, and prepare themselves to utilize lactose molecules by expressing the genes of *lac* operon. Learners can practice quantitative colorimetric assays with the following experiment (refer the web link for more details: https://en.wikipedia.org/wiki/Diauxic_growth). You are advised to watch the video provided at the YouTube link: https://youtu.be/4_dIGV76rtQ

This experiment is divided in to two phases. Where, a liquid medium containing glucose and lactose is used to culture *Escherichia coli* during the first phase. According to Monod and Audureau, the diauxic growth pattern (Fig. 6.1) is observed when culture turbidity is measured over time.

It is important to remember that, when bacteria is utilizing the glucose as carbon source, the enzymes required for the catabolism of other carbon sources are repressed (not expressed).

Anthrone assays for hexose are performed during the second phase. In order to obtain the concentration of lactose, the glucose contribution to the anthrone reaction must be calculated and added to the anthrone result.

Expected Learning Outcomes

After performing this experiment, you should be able to:

- perform *E.coli* culture;
- explain the effect of metabolites on enzyme repression; and
- describe the significance of this experiment in understanding gene expression.

6.2 MATERIALS REQUIRED

Bacterial source: Strain of *E. coli* without mutations in the lac operon or in catabolite repression could be used. 20 ml culture medium was taken into a clean and dry 125ml culture flask. The culture is supplemented with 0.6 mM glucose and 0.6 mM lactose (carbon source).

Culture Media: Culture media suitable for the growth of selected strain may be used. However, here are some of the commonly used media supplied by authorized manufacturers are: Nutrient agar, Nutrient broth. For more details on different culture media's and their preparation refer courses BBCET-143 and BBCEL-144 of programme BSCBCH.

Glass ware and equipments:

Culture flasks, Pipettes, Beakers, Measuring cylinders, Photometer, Spectrophotometer.

6.3 PROCEDURE

Phase-I

Counselor or demonstrator will provide inoculum to the learners. Growing culture of *E.coli* is sedimented by centrifugation at 500 x g for 10 min in a sterile centrifuge tube. The pellet is resuspended in culture medium containing 1.5 mM glucose. Bacterial density of 2.7 mg dry weight per milliliter is measured using spectrophotometer (A_{600}=2.0).

Growth study and preparation of culture filtrates

Pipette out 1.0 ml initial sample from culture flask, dilute the culture with 1.0 ml distilled water and place it in a freezer. Now inoculate with 1.0 ml of the concentrated *E.coli* suspension in the culture flask. Note this time point as zero and immediately measure the culture turbidity (A_{600}). Later put the flask in a shaking water bath at 37^{o}C. At regular intervals of 10 or 20 minutes measure the turbidity of the bacterial culture.

After every 20 min, prepares a culture filtrate by taking 1ml aliquot of the culture using a sterile pipette and filter through a membrane. The membrane filter is rinsed with 1 ml of Medium. The volume of the combined filtrate is adjusted to 2.0 ml. Place the filtrate inside freezing chamber of the refrigerator. Repeat the experiment as above until the culture turbidity levels come down for the second time (see Fig. 6.1).

Phase-II

In this phase estimate the amount of glucose and lactose present in the culture filtrates using procedure provided by commercially availed anthrone reagent. A standard curve for the glucose is prepared against glucose standard with 5.0x10^{-4} M concentration. Finally the glucose concentration in the culture filtrates is calculated.

The different colour yields of glucose and lactose complicate interpretation of the anthrone assay results. 0-1.0 ml aliquots of the 5.0 x 10-4 M standard solutions are analyzed by the students to prepare standard curves for each sugar. Culture filtrates in one ml aliquots are analyzed. It is possible to estimate the absorbance due to glucose in the anthrone reaction of a culture filtrate based on the concentration of glucose in each sample and the glucose standard curve for the anthrone reaction. In order to arrive at the total absorbance value, this value must be subtracted from it. In this case, lactose is assumed to account for the rest of the absorbance. To determine lactose concentration in culture filtrate, the lactose standard curve is used. Glucose and lactose concentrations are plotted versus time after correction for the two-fold dilution of culture medium during preparation of the culture filtrates (Fig. 6.2).

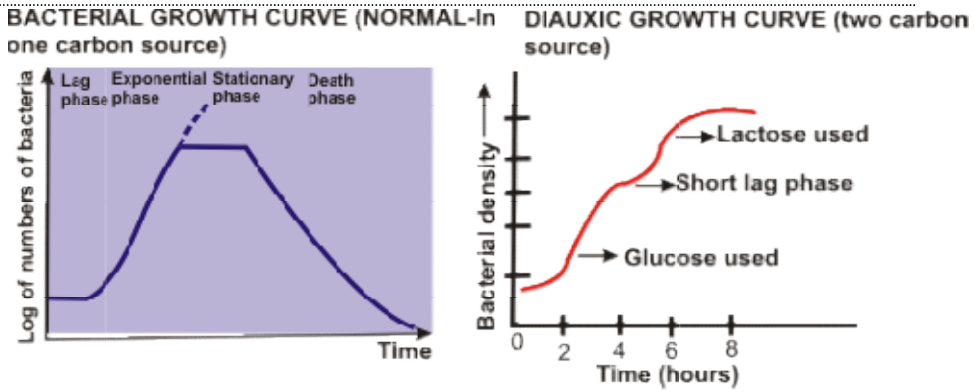

Fig. 6.2: Microbial growth curve.

Observations and Results: As illustrated in Figure 6.2, turbidity measurements reveal that bacterial growth occurs in two distinct phases separated by a lag period. At the beginning of the growth phase, glucose is consumed by the cells. A slow decrease in lactose concentration occurs during the diauxic lag phase, which is followed by a rapid decrease in lactose concentration during the second growth phase.