DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF ENGINNERING AND TECHNOLOGY

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A project report submitted in fulfilment of the summer internship programme 2022-23

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ACKNOWLEDGEMENT

A Summer Internship is a golden opportunity for learning and self-development. I consider myself very lucky and honoured to have so many wonderful people lead us through in the completion of this internship.

First and foremost, I would like to thank **Dr. Abhimanyu Kumar Jha**, HOD, Department of Biotechnology who gave us an opportunity for undertaking this internship.

My grateful thanks to all the teachers for their guidance in this internship who, in spite of being extraordinarily busy with academics, took time out to hear, guide, and keep us on the correct path. The biotechnology department monitored our progress and arranged all facilities to make life easier. I choose this moment to acknowledge their contribution gratefully.

Name and signature of Student

Afan Ahmed (2019007969)

CERTIFICATE

This is to certify that the AFAN AHMED successfully completed with the SUMMER INTERNSHIP REPORT . The work was completed under the supervision of **Dr. Sunita Sharma , Assistant professor , Department of Biotechnology , Sharda University .**

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Experiment 1

Objective: Designing and executing a biotechnology research experiment

Principle: When we are involved in conducting a research project, we generally go through the steps described below, either formally or informally. Some of these are directly involved in designing the experiment to test the hypotheses required by the project.

Steps to initiate and run a research experiment:

1. Review pertinent literature to learn what has been done in the field and to become familiar enough with the field to allow you to discuss it with others. The best ideas often cross disciplines and species, so a broad approach is important. For example, recent research in controlling odours in swine waste has exciting implications for fly and nematode control.

2. Define objectives and the hypotheses that you are going to test. You can't be vague. You must be specific. A good hypothesis is: a. Clear enough to be tested b. Adequate to explain the phenomenon c. Good enough to permit further prediction d. As simple as possible

3. Specify the population on which research is to be conducted. For example, specify whether you are going to determine the P requirements of papaya on the Kauai Branch Station (a Typic Gibbsihumox), or the P requirements of papaya throughout the State, or the P requirements of papaya in sand or solution culture. The types of experiments required to solve these problems vary greatly in scope and complexity and also in resource requirements.

4. Evaluate the feasibility of testing the hypothesis. One should be relatively certain that an experiment can be set up to adequately test the hypotheses with the available resources. Therefore, a list should be made of the costs, materials, personnel, equipment, etc., to be sure that adequate resources are available to carry out the research. If not, modifications will have to be made to design the research to fit the available resources.

5. Select Research Procedure:

a) Selection of treatment design is very crucial and can make the difference between success or failure in achieving the objectives. Should seek help of a statistical resource person (statistician) or of others more experienced in the field. Statistical help should be sought when planning an experiment rather than afterward when a statistician is expected to extract meaningful conclusions from a poorly designed experiment.

b) Selection of the sampling or experimental design and number of replicates. This is the major topic of this course so this will not be discussed further other than to comment that in general one should choose the simplest design that will provide the precision you require.

c) Selection of measurements to be taken. With the computer it is now possible to analyze large quantities of data and thus the researcher can gain considerably more Information about the crop, etc. than just the effects of the imposed variables on yield. For example, with corn, are you going to measure just the yield of grain, or of ears, or of grain plus stover? What about days to tasseling and silking? Height of ears, kernel depth, kernel weight, etc. What about nutrient levels at tasseling, or weather conditions, especially if there are similar experiments at other locations having different climates? With animal experiments, you can measure just the increase in weight or also total food intake, components of blood, food digestibility etc.

d) Selection of the unit of observation, i.e., the individual plant, one row, ora whole plot, etc? One animal or a group of animals?

e) Control of border effects or effects of adjacent units on each other or "competition". Proper use of border rows or plants and randomization of treatments to the experimental units helps minimize border effects. Proper randomization of treatments to the experimental unit is also required by statistical theory so be sure this is properly done.

f) Probable results: Make an outline of pertinent summary tables and probable results. Using information gained in the literature review write out the results you expect. Essentially perform the experiment in theory and predict the results expected.

g) Make an outline of statistical analyses to be performed. Before you plant the first pot or plot or feed the first animal, you should have set up an outline of the statistical analysis of your experiment to determine whether or not you are able to test the factors you wish with the precision you desire. One of the best ways to do this is to write out the analysis of variance table (source of variation and df) and determine the appropriate error terms for testing the effects of interest

6. Selection of suitable measuring instruments and control of bias in data collection: Measuring instruments should be sufficiently accurate for the precision required. Don't want a gram balance (scale) to weigh watermelons or sugarcane. Experimental procedure should be free of personal bias, i.e., if treatment effects must be graded (subjective evaluation) such as in herbicide, or disease control experiments, the treatments should be randomized and the grader should not know what treatment he is grading until after he has graded it. Have two people do the data collection, one grade and the other record.

7. Install experiment: Care should be taken in measuring treatment materials (fertilizers, herbicides, or other chemicals, food rations, etc.) and the application of treatments to the experimental units. Errors here can have disastrous effects on the experimental results. In field experiments, you should personally check the bags of fertilizer or seed of varieties which should be placed on each plot, to be certain that the correct fertilizers or variety will be applied to the

correct plot before any fertilizer is applied or any seed planted. Once fertilizer is applied to a plot, it generally cannot be removed easily.

8. Collect Data: Careful measurements should be made with the appropriate instruments. It is better to collect too much data than not enough. Data should also be recorded properly in a permanent notebook. In many studies data collection can be quite rapid and before you know it you have data scattered in 6 notebooks, 3 folders, and 2 packs of paper towels!! When it is time to analyze the data, it is a formidable task, especially if someone has used the paper towels to dry their hands. Thus a little thought early in the experiment will save a lot of time and grief later. Avoid recording data on loose sheets at all costs as this is one good way to prolong your stay here by having to repeat experiments because the data were lost.

9. Make a complete analysis of the data: Be sure to have a plan of analysis, e.g., which analysis and in what order will they be done? Interpret the results in the light of the experimental conditions and hypotheses tested. Statistics do not prove anything and there is always the possibility that your conclusions may be wrong. The experimental data should be checked very carefully if this occurs, as the results must make sense!

10. Finally, prepare a complete, correct, and readable report of the experiment. This may be a report to the farmer or researcher or an extension publication. There is no such thing as a negative result. If the null hypothesis is not rejected, it is positive evidence that there may be no real difference among the treatments tested.

In summary, we should remember the 3 R's of experimentation:

1. Replicate: This provides a measure of variation (an error term) which is used in evaluating the effects observed in the experiment. This is the only way that the validity of you conclusions from the experiment can be measured.

2. Randomize: Statistical theory requires the assignment of treatments to the experimental units in a purely random manner. This prevents bias.

3. Request Help: Ask for help when in doubt about how to design, execute or analyze your experiment. Not everyone is a statistician, but should know the important principles of scientific experimentation. Be on guard against common pitfalls and ask for help when you need it. Do this when planning an experiment, not after it is completed

Experiment 2

Aim: To isolate and estimate the concentration of total cell protein in *Datura stramonium* and compare with different plant species in a given geographical area.

Objectives:

(1) To Select and collect of leaves of *Datura stramonium.*
(2) To isolate the total cell protein in *D.stramonium* and its estimation using Lowry's Method.
(3) To find out Absorbance of every plant sample isolated using UV-spectroscopy at specific wavelength of light and analyze about the concentrations of the different samples in comparison with *D. stramonium* to conclude about the differing protein content of different leaf samples.

Theory:

The isolation of total cell protein involves a series of experiments and data analysis which in return gives us how much protein does our plant sample have. Some plants are rich in nutrient content whereas some lack such properties. The varying nutrient content gives the species their unique properties to be used as food, fodder, medicine etc. In our experiment we have used different plant species, 12 to be in specific and checked the concentration of protein in them using Lowry's Method of for estimation of protein in a sample. The analysis using different plants facilitates us to identify the presence of varying amount of proteins in different species of plants.

1.1 Selection of the Plant Species

The demand for plant-based products in the field of medicine and food industry is on hike these days. Therefore we selected a very common medicinal weed *Datura stramonium*, which has been seen to have various pharmacological and medicinal properties. Rather than the basic property of *Datura sp.* to be poisonous, *D.stramonium*is seen to have anti-allergic and anti-inflammatory properties as well.

The plant is native to Southeast Asian countries, Europe, India, and America. It is a common offering being made to Lord Shiva in the Indian subcontinent along with *Cannabis sp*. The plant is herbaceous, and branched in nature [**Figure 1**]. The plant grows annually with all year round flowering. It has hairy, soft and pale greenish leaves [**figure 2**] which act as a protective layer against stress in the leaves. The flowers are white in colour with a light pungent smell and bloom at night [**figure 3**]. The fruits are seen to have a hard covering with lots of thorns on its surface.

Due to this reason often the fruit is known as “thorn apple”. The seeds of the Datura plant are highly narcotic in nature and can even be fatal on high consumption due to its high poison content.

Talking about its medicinal properties, D.stramonium is one of the old folklore plant based drug used for the treatment of lung-related disorders like asthma and bronchitis. It is done by inhalation of the fumes of the burning leaf. European remedies for treatment of haemorrhoid included, steaming the part over boiling water with the leaf of D.stramonium in it. The juice of the fruit is used a remedy against hair fall and dandruff issues. It is also used as a halucinergic drug to treat the problems related to insomnia. Scopolamine which is found in the plant, is an effective cholinergic hallucinogen which is used to treat schizoid patients.

1.2 Lowry’s Method for the estimation of total cell protein

Total cell protein is defined as the total protein content of a particular mass of cells, for instance in 100ml of a cell suspension. D.stramonium is noted to have 16.20% of protein in 100ml of cell suspension. After the use of extraction buffer for lysis of cells and releasing its contents in a solution we use centrifugation technique to separate cell debri from proteins.

There are various different methods that we use to estimate the total cell protein:

1. Bradford Assay Method: In this method, we use Coomasie Brilliant blue stain which imparts Blue colour once mixed to proteins and the intensity increases with increase of protein concentration in a sample. Then the absorbance is taken out for calculating the concentration of protein using UV- Spectrophotometer at 570nm.
2. UV-Spectrometry: This method is only used if the protein sample is completely pure and contains Aromatic amino acid residues.
3. Ninhydrin test: This method is used for quantification of individual amino acids of a protein sample.
4. Lowry’s Assay Method: This method is in use if the protein sample is not completely pure as the reagents used would give colour on reacting with proteins.

   The Lowry’s Assay involves two reagents:

   1. Analytical Reagent: It contains the following compounds:
      - (I) 1% Copper sulphate solution (1g in 100ml of H_2O),
      - (II) 2% Sodium Potassium Tartarate (2g in 100ml of H_2O),
      - (III) Alkaline Disodium Carbonate (1g of NaOH+5g of Na_2CO_3 in 250 ml of H_2O).

      These compounds are mixed in the proportion of 196 ml(III)+2ml(I)+2ml(II) to give a 200ml of Analytical reagent solution.

   2. Folin’s Reagent: It contains Sodium Potassium Molybdenate. It is prepared by mixing 25 mL of Folin’s solution with 25ml of H_2O in a 1:1 ratio to make a 50ml solution.

   The Assay is based on two different reactions:

- Copper of Copper sulphate solution reacts with peptide bond of amino acids under alkaline conditions thereby getting reduced and forming a complex.
- The complex combines with sodium potassium molybdenate to give bluish-purple colour.

The absorbance is noted from the range of 650-750nm or more specifically at 720nm for best results.

We used Lowry' Assay Method for the estimation of protein in our protein sample.

MATERIALS USED:

The materials used for the purpose of experimentation are being listed under two subheadings

(1) Chemicals used:

- Freshly prepared or commercially Available Phosphate Buffer Saline(PBS Solution)
- 1% Copper sulphate solution (1g in 100ml of H_2O),
- 2% Sodium Potassium Tartarate(2g in 100ml of H_2O),
- Alkaline Disodium Carbonate (1g of NaOH+5g of Na_2CO_3 in 250 mL of H_2O).
- Sodium Potassium Molybdenate
- Protease free H_2O
- 70% ethanol

(2) Instruments Used:

- Refrigeration Centrifuge
- UV-Spectrophotometer
- Mortar and Pestle
- Glass wares
- Eppendorf tubes
- Micropipette(1000µl/1ml)

METHOD OF EXPERIMENTATION

Preparation of 1L PBS Solution

Constituent with molar mass (g)	**Molarity (mM)**	**Weight to be taken for 1L solution (g)**
Sodium Chloride (58.5 g)	137	8.006
Potassium chloride(74.55)	2.7	0.201
Sodium Phosphate Dibasic (144.96)	10	1.440
Potassium Phosphate Dibasic (136.09)	1.8	0.245

Table 1: Constituents of PBS Solution

(1) Weigh the constituents in the given proportion using analytical balance.
(2) Mix the constituents in 700 ml of distilled water with continuous stirring.
(3) Make up the volume to 1L using 300 ml of distilled water.
(4) Check for confirming the pH to be 7.4, the ideal pH of PBS.

Preparation of Cell Extract:

(1) Grind the leaf using Mortar and Pestle.
(2) Add 10mL of PBS to the mashed leaf.
(3) Prepare a suspension of leaf mash and PBS solution.
(4) Transfer the suspension into a clean test tube and let it to rest for 15-20 minutes for cell debri to settle down.
(5) Transfer the upper aqueous layer to Eppendorf tube (measurement 1ml) using micropipette.
(6) Centrifuge the solution for 30 minutes at 5000 RCF and 4 degree C.

Preparation of Lowry's Assay Reagent:

(1)Analytical Reagent:

Constituent	**Proportion for 200ml Solution**	**Proportion for 100ml solution**
1% Cupric Sulphate(I)	1g in 100ml water	0.5g in 50ml of water
2% Sodium Potassium Tartarate(II)	2g in 100ml water	1g in 50ml of water
Alkaline Disodium Carbonate(III)	1g NaOH+5g Na_2CO_3 in 250ml water	0.5g NaOH+2.5g Na_2CO_3 in 125ml water

Table 2: Constituents of Analytical Reagent of Lowry's Assay

Mix the constituents in the proportion of 98ml(III) +1ml(I) +1ml(II) giving the resultant concentration of 100mL.

(2)Folin's Reagent:

Mix 11mL of Folin's Reagent with 11ml of distilled water in the ratio of 1:1 to make a solution of 22ml of Folin's Reagent.

Estimation of Protein using Lowry's Assay method

(1) Pipette out the supernatant of the solution and discard the pellet.
(2) Put the extracted supernatant (1ml) in a clean test tube.
(3) Add 2.5ml of Analytical reagent into the Solution and incubate it at optimum room temperature for 10 minutes.
(4) After 10 minutes of incubation add 0.5ml of Folin's Reagent to the solution and incubate for 30 minutes at room temperature.
(5) Add 1mL of Distilled water into the solution to dilute the mixture.

Analyzing the Absorbance of sample using UV-Spectroscopy

(1) Prepare a Blank using 2mL of PBS solution.
(2) Clean the transparent side of the cuvette to avoid any particles to stay which may deviate the path of light.
(3) Add 2mL of sample solution into the cuvette using a micropipette.

(4) Set the Spectrophotometer at 720nm wavelength and first take the reading of the blank.
(5) Proceed to take 3 consecutive readings of the sample for better accuracy and find the average of them to detect the final OD value

Preparation of Standard Curve using given standards

(1) Plot a graph on the basis of the given standards.
(2) Define the trend line to identify the errors.
(3) Find out the standard deviation and m and c value of the equation: Y=mx+c

Concentration of BSA (µg/mL)	**Absorbance**
100	0.08
200	0.20
400	0.40
600	0.60
900	0.80
1200	1.10
1500	1.20

Table 3: Entries for standard curve

OBSERVATIONS AND RESULTS:

[*] *shows experimental error* **Table 4: Observation table showing different OD value for different plant species and their concentrations derived from the standard curve drawn on the basis of given absorbance**

Serial No.	Plant	PBS buffer	Analytical buffer	Incubate at room temperature for 10 minutes	Folin's reagent	Incubate at room temperature for 30 minutes	OD value at 720nm	Concentration of protein in ug/ml
1	*Ficus benghalensis*(Banyan)	1ml	2.5ml	10min	0.5ml	30min	1.32	1320
2	*Syzygiumcumini* (Java)	1ml	2.5ml	10min	0.5ml	30min	1.96	1960
3	*Syzygiumcumini*(Jamun)	1ml	2.5ml	10min	0.5ml	30min	1.89	1890
4	*Azadirachta indica* (Neem)[*]	1ml	2.5ml	10min	0.5ml	30min	0.51	510
5	*Datura stramonium* (White Dhatura)	1ml	2.5ml	10min	0.5ml	30min	1.89	1890
6	*Azadirachta indica* (Neem)	1ml	2.5ml	10min	0.5ml	30min	0.79	790
7	*Rosa* (Rose)	1ml	2.5ml	10min	0.5ml	30min	1.1	1100

8	*Spinacia oleracae*(Spinach)*	1ml	2.5ml	10min	0.5ml	30min	0.523	523
9	*Saracaasoca*(Ashoka)	1ml	2.5ml	10min	0.5ml	30min	1.96	1960
10	*Cannabis sativa* (Bhang)	1ml	2.5ml	10min	0.5ml	30min	0.81	810
11	*Punica granatum* (Pomegranate)	1ml	2.5ml	10min	0.5ml	30min	1.66	1660
12	*Epipremnumaurem* (Money Plant)	1ml	2.5ml	10min	0.5ml	30min	1.29	1290

CALCULATIONS:

From Table 3, *y1*=0.4*; y2*=0.6 *x1*=400;*x2*=600

According to the formula, we have,

m=(y2-y1)/(x2-x1)

- *m*=(0.6-0.4)/(600-400)
- ***m*=0.2/200= 0.001**

Now,

y=mx+c

- 0.4=0.001(400)+*c*
- ***c*=0.4-0.4=00**

Considering the absorbance of D.stramonium we have, y=1.89 now we have, y =mx+c

- 1.89=0.001(x)+0

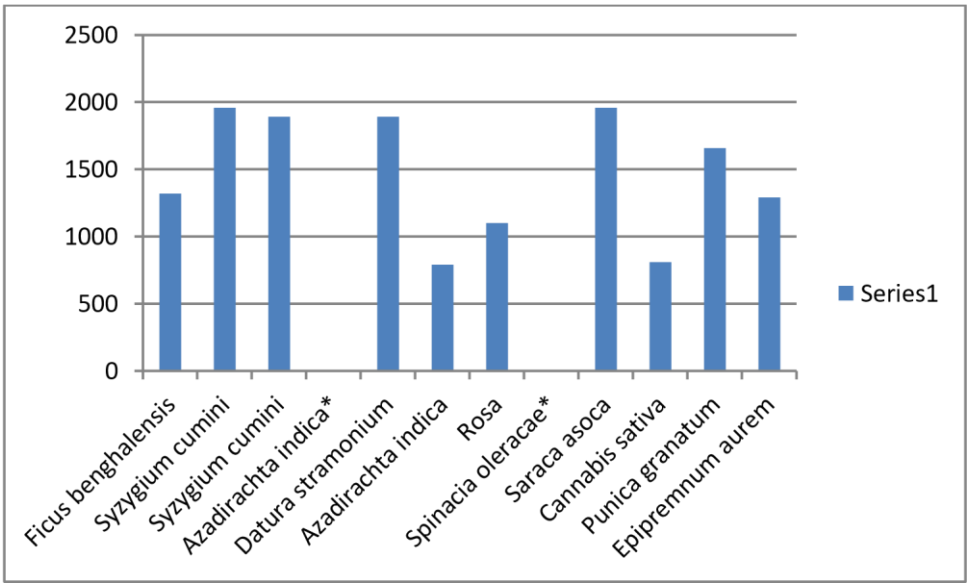
x(Concentration)=1890µg/mL

DATA ANALYSIS

The data that we collected and the experimental outcomes that we get, we are able to see that the concentration of protein *in D.stramonium* is around 1890µg/ml at the OD value of 1.89 at wavelength of 720nm. This shows a potential amount of protein lies inside the *D.stramonium* species of plants. From literature study we have got that the protein content of *D.stramonium* species is around 16.20% in 100 mL leaf extract, greater than other species of Datura plant.

The standard curve was prepared with the help of standard values of concentration od BSA giving OD value at 720nm. The Graph 1 shows that certain points deviate from the trend line, which shows certain standard deviations in the graph which may have aroused due to experimental errors. The 'm' and 'c' value are calculated taking a set of values from 'y' and 'x' axis and then taking into consideration the absorbance *of D.stramonium* which was being revealed by the spectrophotometric analysis we were able to calculate its protein concentration.

Now, proceeding towards the comparison between different plant species on the basis of their protein concentrations we plotted a histogram (Graph 2) taking plant species on X-axis and the protein concentrations on Y-axis. Unfortunately, due to certain experimental errors sample 4 and 8(from observation table4), could not be considered for comparative study. The resultant histogram statistically reveals how the different plant species growing in a specific geographical plot or biome has different protein contents and hence different utilizations.



Graph 2: Comparison of Concentrations of protein in different plant samples (* *shows experimental error not considered for data analysis*)

OUTCOMES AND CONCLUSION:

(1) Hierarchy of *Datura stramonium.*
Kingdom: Plantae
Phyllum: Tracheophytes
Class: Angiosperms
Subclass: Eudicots
Subclass: Asterids
Order: Solanales
Family: Solaneccae
Genus: Datura
Species: stramonium

(2) The theoretical literature study showed the presence of 16.20% of protein in 100 ml cell solution

(3) Using Lowry's Method, we saw that the solution on addition of Feling's reagent containing sodium potassium molybdenate, turned bluish-purple in color, concluding the presence of protein in the sample.

(4) The OD value for D.*stramonium* is 1.885 and the resultant concentration of protein in the sample is 1890µg/mL. On comparison with other concentration it revealed that plants like neem, rose& money plant are less in protein content than D.*stramonium*.

FIGURES:

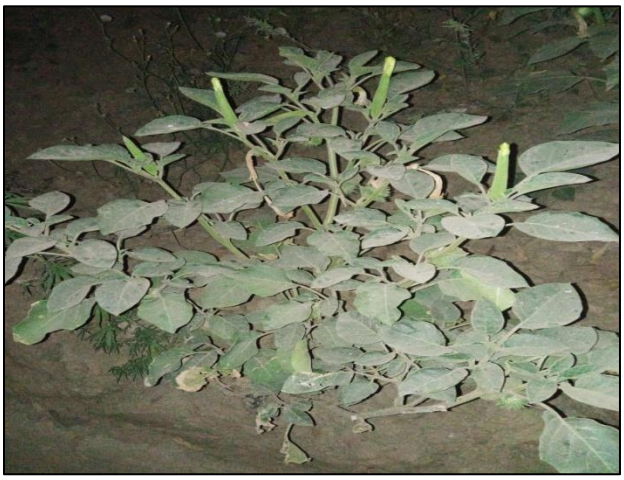


Figure 1 *Datura stramonium* plant flower

Figure 2 *D.stramonium*

Figure 3 *D.stramonium* fruit

Experiment 3

Aim: To isolate genomic DNA from blood sample using Triton-X(salting out method).

Theory and Principle

DNA was first isolated by Friedrich Miescher who discovered a substance called "nuclein" in 1869. Blood is the main source of DNA for genotype-related studies in humans. A rapid, efficient, and cost effective method for the isolation of genomic DNA from whole blood is needed for screening a large number of samples. There are many published protocols [1-3]. DNA was isolated from the 201 blood samples by a rapid non-enzymatic method by salting outcellular proteins with saturated solution and precipitation by dehydration [4]. Salting out method is one of the simplest of all the published methods. Following this procedure, it takes anywhere from 3 to 4 hrs to isolate DNA for large number of samples with the yield ranging (6 to10ug) good quality of DNA from 300uL whole blood. From our experience we suggest salting out method is less time consuming and not cost effective as well as gives better concentration of DNA which required for the genotype studies with large sample size. MATERIALS AND METHODS Standard chemicals: This method uses standard chemicals that can be obtained from major suppliers; we used chemicals supplied by Sigma & Himedia. Materials Tris-HCl, Potassium Chloride, Magnesium Chloride, Blood collection EDTA, Sodium Chloride, Sodium dodecyl sulphate(SDS), Isopropanol, Ethanol, Triton-X, 1.5ml eppendorf tubes and micro centrifuge . Blood collected in EDTA-containing vacutainer tubes. As will all body fluids, blood represents a potential biohazard, thus care should be taken in all steps requiring handling of blood. If the subject is from a known high-risk category, additional precautions may be required. Blood samples stored at room temperature for DNA extraction within the same working day & also at refrigerator for later uses.

Preparation of Reagents

The reagents were prepared as described below:

a. TKM 1 Buffer / Low salt buffer (500 ml): 0.605 g of TrisHCl (10mM) pH 7.6, 0.372 g of KCl (10 mM), 1.016 g of MgCl2

b. Triton-X (10ml): Added 0.1 ml of 100 % Triton-X to 9.9ml of distilled water. (10 mM), 0.372g of EDTA (2mM) was dissolved in 500ml of distilled water

c. TKM 2 Buffer / High salt buffer(100 ml): 0.121 g of TrisHCl (10mM) pH 7.6, 0.074 g of KCl (10 mM), 1.203 g of MgCl2

d. SDS: One gram of sodium dodecyl sulphate was dissolved in 10ml distilled water. (10 mM), 0.074 g EDTA (2mM), 0.467 g of NaCl (0.4 M) was dissolved in 100ml of distilled water

e. 6M NaCl : 8.765 g of NaCl was dissolved in 25 ml of distilled water.

f. TE Buffer: 0.030 g of TrisHCl (10mM) pH 8.0, 0.009 g of EDTA (1mM) was dissolved in 100ml of distilled water

DNA extraction Protocol

RBC Lysis

1. 900 µl of TKM 1 and 50 µl of 1x Triton-X were added to 300 µl of heparinised blood in an autoclaved 1.5 ml eppendorf.

2. Incubated at 37degree Celsius.

3. Cells were centrifuged at 10000 rpm for 5 minutes and the supernatant was discarded. see for 5 minutes to lyse the RBCs.

4. This step was repeated 2-3 times with decreasing amount of 1x Triton-X till RBC lysis was complete and a white pellet of WBCs was obtained.

Cell Lysis

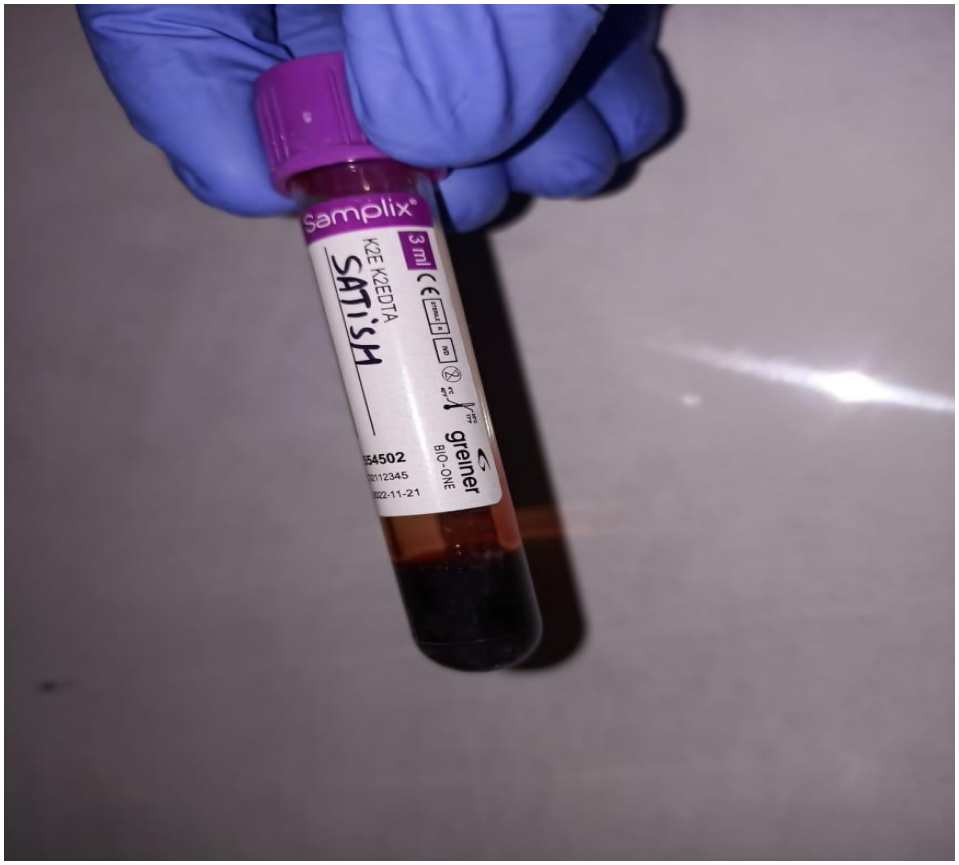
- To the cell pellet, 300 µl of TKM 2 and 40 µl of 10% SDS were added,
- Mixed thoroughly and incubated at 37degree Celsius for 5 minutes.
- At the end of incubation, 100 µl of 6M NaCl was added and vortexed to precipitate the proteins.
- Cells were centrifuged at 10000 rpm for 5 minutes.

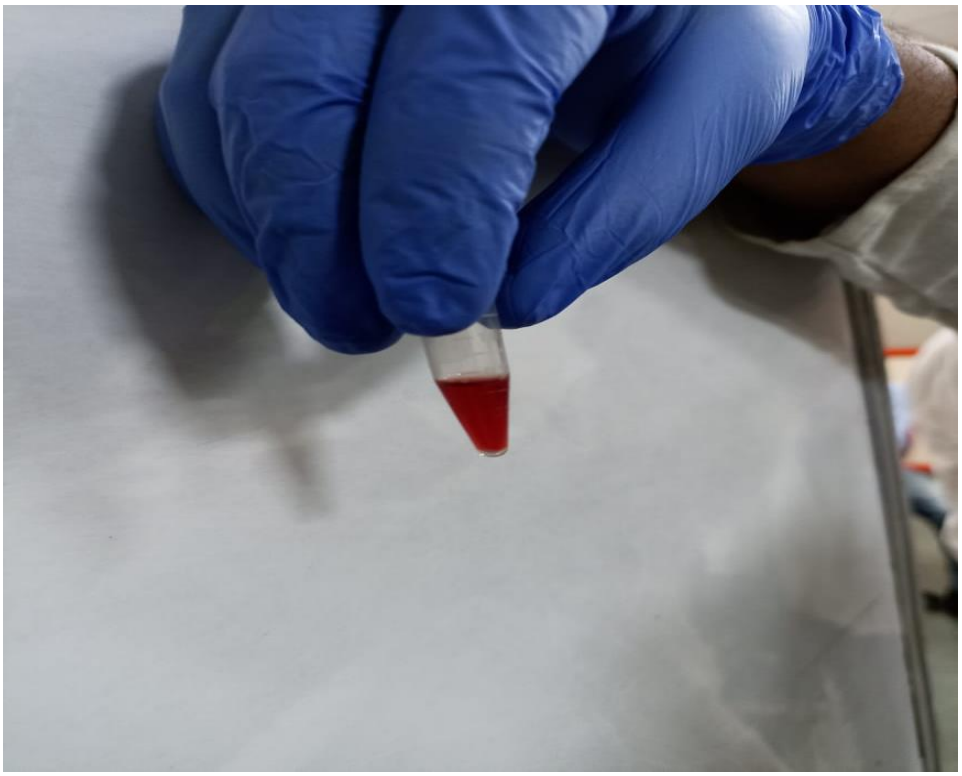
Precipitation of DNA

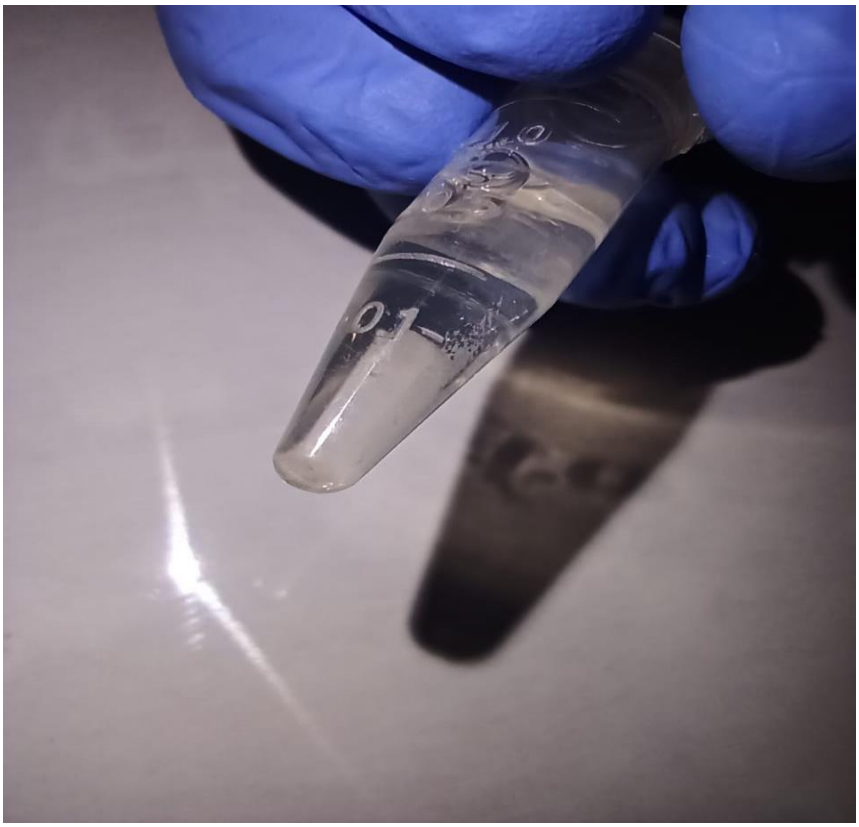
- The supernatant was transferred into a new eppendorf tube containing 300 µl of isopropanol.
- DNA was precipitated by inverting the eppendorf slowly.
- Further, the eppendorfs were centrifuged at 10000 rpm for 10 minutes to pellet down the DNA.
- Supernatant was discarded, 70% ethanol was added and mixed slowly to remove any excess salts.
- Finally, the tubes were centrifuged at 10000 rpm for 5 minutes to pellet down the DNA.
- Supernatant was discarded and DNA air-dried.
- After thorough drying, 20 µl of TE buffer was added to dissolve the DNA.

RESULTS

Agarose gel electrophoresis used to check and Remaining DNA was stored at 4-8 C for downstream applications.We routinely use about 1-2µl per PCR reaction without adverse effects. DNA quantified by Spectrophotometric method and diluted to a working concentration at this point or simply use 1-2 µl per PCR reaction







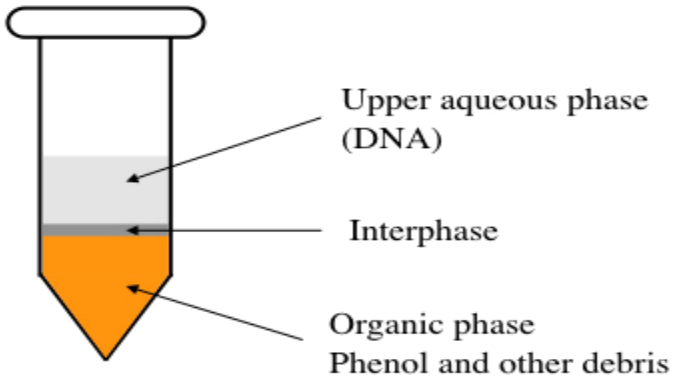
DISCUSSION

Extracted genomic DNA from human whole blood samples is to be use for diagnostic & for genotype studies which will help in personalized medicine. The physical as well as chemical treatments involved in DNA extraction can affect both the quantity and quality of the DNA obtained [5].In our method, genomic DNA extraction consumes less time and with high quality and quantity by using simple materials and equipments. Not only was high quality DNA extracted from blood that was stored at 4ºC, -20ºC In a workday, This study results one person can isolate DNA from more than 50 blood samples using this method. This method has been routinely used to extract DNA from whole blood of human for PCR based applications in our laboratory. It has several advantages such as; economical spending, no need to the specialized and expensive equipments, spending little time, no need to the experimented and experienced staff and more important, DNA extraction from whole blood stored at usual fridges for long time. In this method, genomic DNA with high quality and quantity can be acquired from whole blood . Time of extraction of genomic DNA in our method is 3-4 hrs for 20 samples so within one working day 50 isolations can be done.

Experiment 4

Objective: To isolate genomic DNA from blood sample using phenol-chloroform-isoamyl alcohol (pci) method

Principle: DNA isolation is a process of purification of DNA from sample using a combination of physical and chemical methods. The first isolation of DNA was done in 1869 by Friedrich Miescher. Currently it is a routine procedure in molecular biology or forensic analyses**. Phenol-chloroform isoamyl alcohol relies on the principle of liquid-liquid extraction of bio molecules. It denatures the protein part and separates the genomic DNA into a soluble phase.**



A pictorial illustration of PCI-based DNA extraction.

To understand it precisely, we need to look inside the tube, let dive into the tube.

Suppose the tube is filled with phenol, chloroform, isoamyl alcohol and cell suspension. The phenol is less-polar while the watery part (containing chloroform) is polar in nature. Also, note that phenol is denser than water so remained at the bottom of the tube.

DNA is a polar molecule having a negative charge. The principle of the polarity of biomolecules says that the polar molecules dissolve in the polar solvent and the non-polar molecules in the non-polar solvents.

Henceforth, water (present in the solution) dissolve DNA but not protein while phenol can't dissolve the DNA. Due to the higher density of phenol, it remains at

the bottom. So the genomic DNA remains in the upper watery-soluble part while the cell debris remains below.

DNA extraction from blood by phenol-chloroform method

Solutions and reagents used for DNA isolation-

• Saline Sodium Citrate (SSC) 20X: 20 ml SSC is prepared by dissolving 3.56 g NaCl (3M) and 1.764 g of sodium citrate (0.3M) in 20 ml DW followed by autoclaving.

• Sodium Dodecyl Sulphate (SDS) 10%: 1 g of SDS is dissolved in 8.0 ml DW and final volume was set to 10 ml with DW.

• 0.2 M Sodium Acetate (SAc): 0.164 g of Sodium acetate is dissolved in 10 ml DW and pH is adjusted to 7.0 by glacial acetic acid followed by autoclaving.

• TE (Tris –EDTA): 10 mM Tris-Cl 1 mM EDTA (0.5 M EDTA is prepared as stock by adjusting the pH to 7.8 with NaOH. 1 M Tris-Cl is prepared as stock and pH is adjusted to 7.6. For preparing 10 ml of TE, 20 µl (0.5 M EDTA) and 100 µl (1 M Tris-Cl) is added to 9.88 DW. Final pH is adjusted to 8.0)

Protocol

1. Add 1.0 ml SSC (1X) to 1.0 ml blood sample (without anticoagulant) and centrifuge at 10000 rpm for 5 min at 40C. Discard the upper aqueous phase.

2. Wash with 1.0 ml SSC (1X) and discard the upper layer. Repeat this step 3 times.

3. Add 0.2 M SAc (375 µl), 10% SDS (18 µl) and 10 mg/ml proteinase-K (6 µl) to the pellet and incubate overnight at 550C.

4. Add Phenol (60 µl), Chloroform (57.6 µl) and Isoamyl alcohol (2.4 µl) to the sample and centrifuge at 10000 rpm for 5 min at 40C. Transfer the upper aqueous phase to a fresh micro-centrifuge tube.

5. Add 115.2 µl Chloroform and 4.8 µl Isoamyl alcohol and centrifuge at 10000 rpm for 5 min at 40C and then transfer upper aqueous phase to a fresh micro-centrifuge tube.

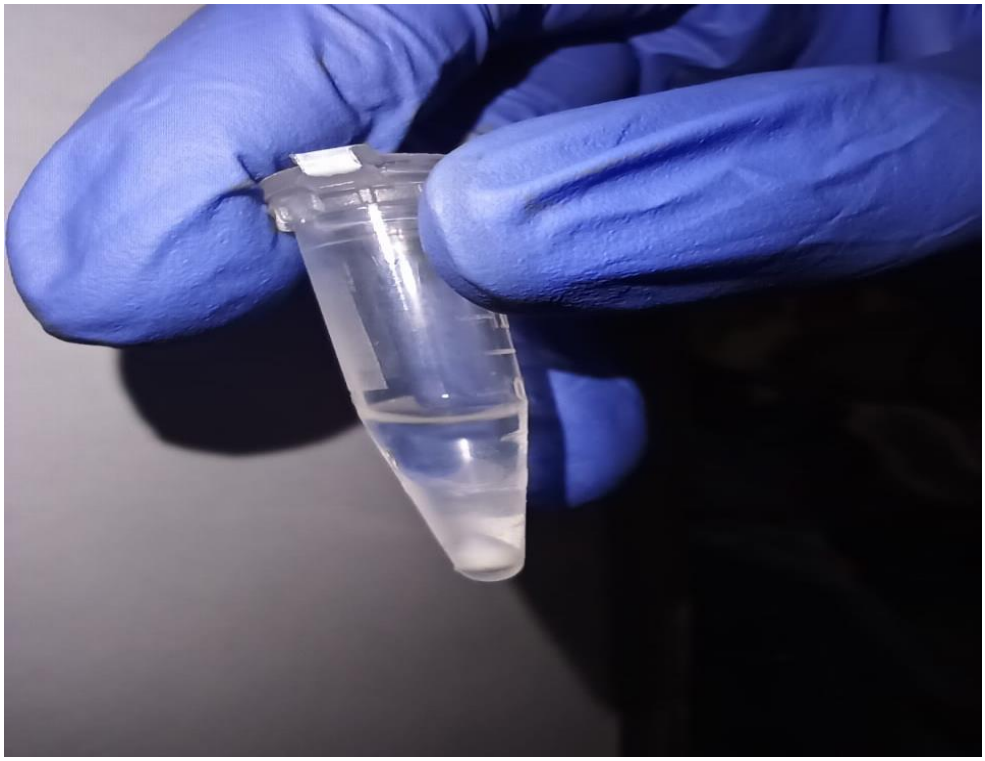
6. Add 1.0 ml chilled ethanol (95%) to precipitate DNA and keep at -20ºC for overnight.

7. Centrifuge the sample at 5000rpm for 5 minutes at 4 0 C, and discard the upper aqueous phase.

8. Wash the pellet with 1.0 ml of 70% ethanol, followed by centrifugation at 7000 rpm for 7 min at 40C. Discard the upper aqueous phase.

9. Leave the pellet to dry and add 80 µl TE to dissolve DNA which was stored at -20ºC. 10. Measure the DNA concentration by a spectrophotometer and analyze on 0.8% agarose gel in TAE buffer (pH 8.0).

Results and Interpretation-



Experiment 5

Objective: To run a Methylation-specific polymerase chain reaction (MS-PCR) for amplification of a selected DNA fragment

Theory and Principle: Methylation Specific PCR (MSP) is a bisulphite conversion based PCR technique for the study of DNA CpG methylation. For MSP experiment, two pairs of primers are needed with one pair specific for methylated DNA (M) and the other for unmethylated DNA (UM). To achieve discrimination for methylated and unmethylated DNA, in each primer (or at least one of the pair) sequence, one or more CpG sites are included. First, DNA is modified with sodium bisulfite and purified. Then, two PCR reactions are performed using Methylated primer pair and Unmethylated primer pair. Successful amplification from Methylated pair and Unmethylated pair indicated methylation and unmethylation respectively.

Primers used:

Primers should contain at least one CpG site within their sequence, and the CpG site should preferably be located in the very 3' –end of their sequence to discriminate maximally methylated DNA against unmethylated DNA.

Dilution of Primers:

Working solution is prepared from stock solution by adding 10 µl of stock solution of primer to 40 µl of TE buffer (5 times dilution).

Reaction Mixture in PCR Tube (Master Mix)(unmethylated – 91bp)	**Quantity**
Taq BUFFER	2.0µl
dNTP	0.6 µl
Unmethylated Forward Primer	0.6 µl
Unmethylated Reverse Primer	0.6 µl
DNA	1.0 µl
Taq Polymerase	1.0 µl
Double Distilled water	12.2 µl
Mgcl2	2 µl

Reaction Mixture in PCR Tube (Master Mix)(methylated – 79bp)	**Quantity**
Taq BUFFER (200 mM MgCl2)	2.0µl
dNTP	0.6 µl
Mgcl2	2 µl
Methylated Forward Primer	0.6 µl
Methylated Reverse Primer	0.6 µl
DNA	1.0 µl
Taq Polymerase	1.0 µl
Double Distilled water	12.2 µl

Procedure-

➢ Master mix is prepared for 17.8 µl, with Taq buffer, dNTPs, DNA sample & nuclease free water.

➢ Primers (forward & reverse) are added to it, the volume of the reaction mixture would be 19.0 µl. (Taq polymerase is added later on i.e., Hot start)

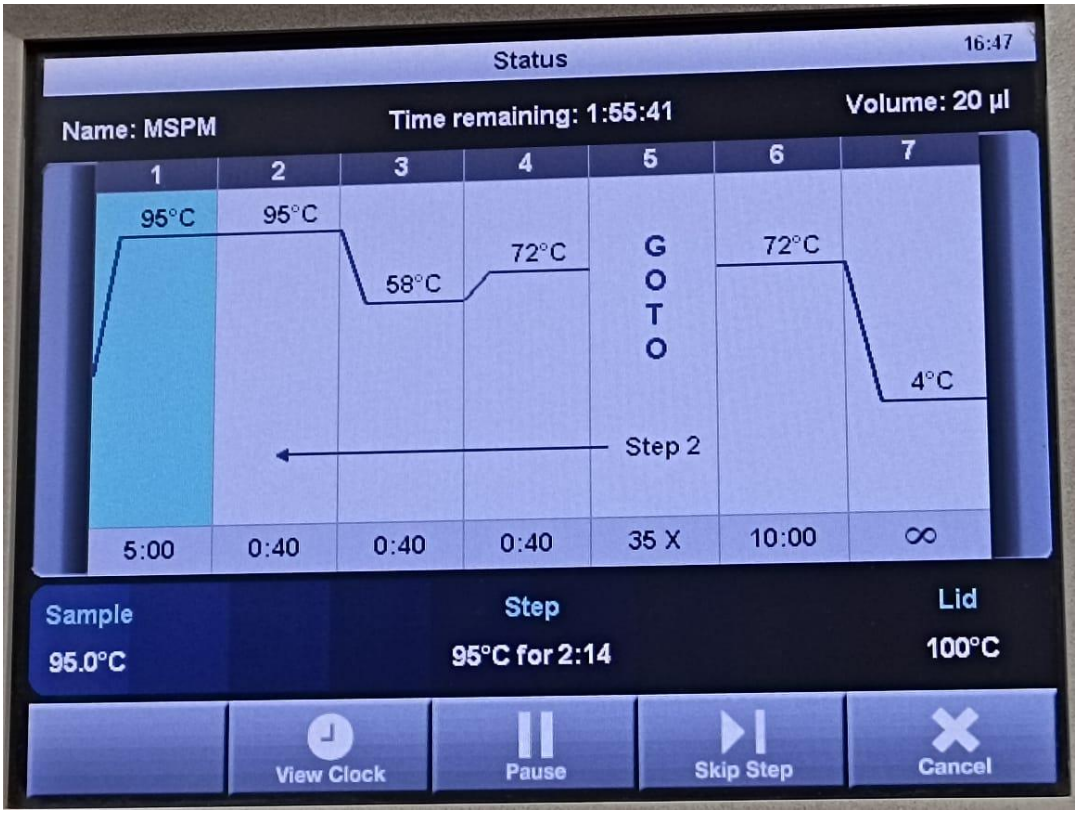
➢ The samples are placed at defined PCR conditions to obtain PCR products. ➢ PCR is started. After the Initialization, the PCR machine is paused for the Hotstart.

➢ The optimum annealing temperature is calculated for SOX-17 gene for methylated specific primer is 58○C and for unmethylated specific primer is 61○C.

➢ After that samples are loaded in agarose gel (2.5%). Methylated and unmethylated PCR products are loaded in adjacent wells. DNA Ladder is also loaded simultaneously.

➢ Band is observed in UV-transilluminator or Gel doc.

Results and Interpretation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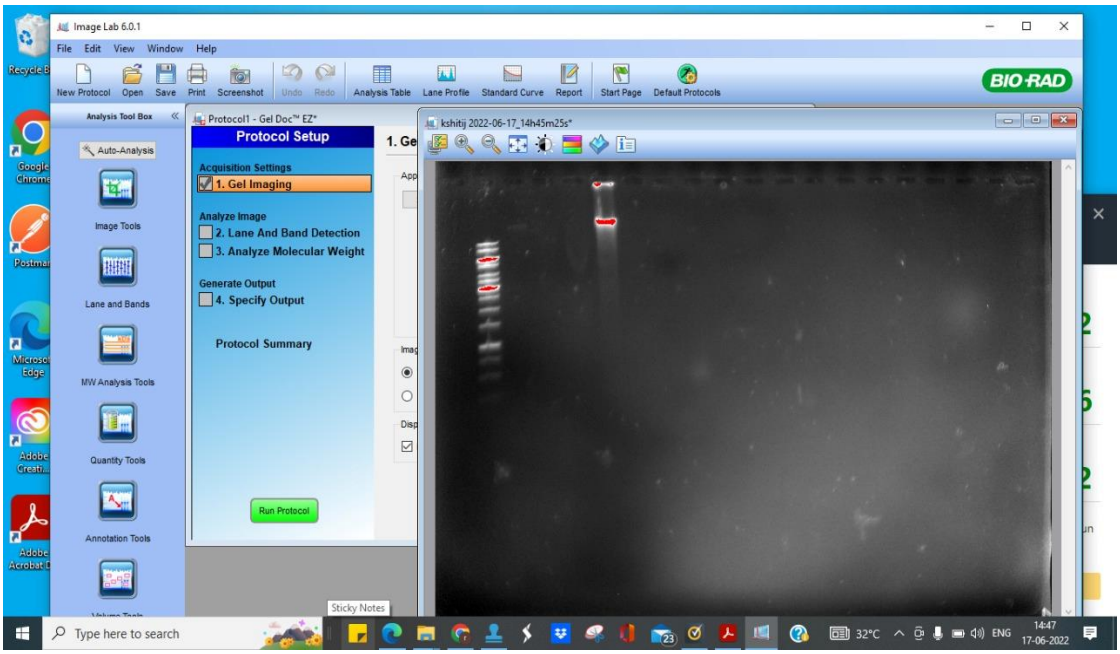
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