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DEPARTMENT OF SCIENCE AND MATHEMATICS

SLT 371 DNA FINGERPRINTING AND FORENSIC ANALYSIS LABORATORY MANUAL.

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By

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PREFACE

Modern scientific learning and practice involves appreciation of both **products** of science theories, concepts, laws, techniques, principles etc) and **processes** of science (manipulative skills of scientific instruments and making inferences) with utmost attention . What follows below is a brief summary of the **scientific** skills involved in laboratory science technology.

1 . Planning

• Defining the problem
Students should be able to use information provided about the aims of the investigation, or experiment, to identify the key variables. They should use their knowledge and understanding of the topic under consideration to make a quantitative, testable, prediction of the likely outcome of the experiment.

• Methods
The proposed experimental procedure should be workable. It should, given that the apparatus is assembled appropriately, allow data to be collected without undue difficulty. There should be a description, including diagrams, of how the experiment should be performed and how the key variables are to be controlled. Equipment, of a level of precision appropriate for the measurements to be made, and quantities to be used should be specified. The use of control experiments should be considered.

• Risk assessment
Candidates should be able to carry out a simple risk assessment of their plan, identifying areas of risk and suggesting suitable safety precautions to be taken.

• Planning for analysis, conclusions and evaluation
Students should be able to describe the main steps by which their results would be analyzed in order that those valid conclusions might be drawn. This may well include the generation of a results table and the proposal of graphical methods to analyze data. Also, they should propose a scheme for the interpretation and evaluation of the results themselves, and of the experimental procedure employed in obtaining those results. There should be an indication of how the outcomes of the experiment would be compared with the original hypothesis.

2. Setting up / manipulating apparatus

It is important that students are allowed sufficient time and opportunity to develop their manipulative skills to the point where they are confident in their approach to experimental science. They must be able to follow instructions, whether given verbally, in writing or diagrammatically, and so be able to set up and use the apparatus for experiments correctly.

3. Making measurements and observations

• Measuring/observing
Whilst successfully manipulating the experimental apparatus, it is crucial that students are able to make measurements with accuracy and/or to make observations with clarity and discrimination. Accurate readings of meters or burettes and precise descriptions of colour changes and precipitates will make it much easier for students to students to draw valid conclusions, as well as scoring more highly in the test.

• Deciding on what measurements/observations to make
Time management is important, and so students should be able to make simple decisions on the number and the range of tests, measurements and observations that can be made in the time available. For example, if the results of the first two titrations are in good agreement, there is no need to carry out a third. Students need to be able to make informed decisions regarding the appropriate distribution of measurements within the selected range, which
may not always be uniform, and the timing of measurements made within the experimental cycle. They should also be able to identify when repeated measurements or observations are appropriate. The strategies required for identifying and dealing with results which appear
anomalous should be practiced.

4. Recording and presenting observations and data

An essential, but frequently undervalued, aspect of any experimental procedure is the communicating of the results of the procedure to others in a manner that is clear, complete and unambiguous. It is vital that students are well practiced in this area.

• The contents of the results table
The layout and contents of a results table, whether it is for recording numerical data or observations, should be decided before the experiment is performed. 'Making it up as you go along' often results in tables that are difficult to follow and don't make the best use of space. Space should be allocated within the table for any manipulation of the data that will be required.

• The column headings in a results table
The heading of each column must be clear and unambiguous. In columns which are to contain numerical data, the heading must include both the quantity being measured and the units in which the measurement is made. The manner in which this information is given should conform to 'accepted practice'.

• The level of precision of recorded data
It is important that all data in a given column is recorded to the same level of precision, and that this level of precision is appropriate for the measuring instrument being used.

• Display of calculations and reasoning

Where calculations are done as part of the analysis, all steps of the calculations must be displayed so that thought processes involved in reaching the conclusion are clear to a reader. Similarly, where conclusions are drawn from observational data, the key steps in reaching the conclusions should be reported and should be clear, sequential and easy to follow.

• Significant figures

Students should be aware that the number of significant figures to which the answer is expressed shows the precision of a measured quantity. Therefore, great care should be taken with regard to the number of significant figures quoted in a calculated value. The general rule is to use the same number of significant figures as (or at most one more than) that of the least precisely measured quantity.

• Data layout

Students should be able to make simple decisions concerning how best to present the data they have obtained, whether this is in the form of tabulated data or as a graph. When plotting graphs they should be able to follow best practice guidelines for choosing suitable axis scales, plotting points and drawing curves or lines of best fit. In drawing tables they should be able to construct a table to give adequate space for recording data or observations.

5. Analyzing data and drawing conclusions

This skill requires students to apply their understanding of underlying theory to an experimental situation. It is a higher-level skill and so makes a greater demand one student's basic understanding of the biology involved. Even when that understanding is present, however, many students still struggle. The presentation of a clear, lucid, watertight argument does not come naturally to most people and so much practice in this area is recommended.

• Interpretation of data or observations

Once data has been presented in the best form for analysis of the results of the experiment, the student should be able to describe and summaries any patterns or trends shown and the key points of a set of observations. Further values such as the gradient of a graph may be calculated or an unknown value found, for example from the intercept of a graph.

• Errors

Students should be used to looking at an experiment, assessing the relative importance of errors and where appropriate, expressing these numerically. Students should be aware of two kinds of error. The 'error' that is intrinsic in the use of a particular piece of
equipment. Although we refer to this as an equipment error, we really mean that there is a 'range of uncertainty' associated with measurements made with that piece of equipment. This uncertainty will be present no matter how skilled the operator might be. Experimental error, which is a direct consequence of the level of competence of the operator or of the effectiveness of the experimental procedure.

- **Concluding**

Students should learn to use evidence to support a given hypothesis, to draw conclusions from the interpretation of observations, data or calculated values and to make scientific explanations of their data, observations and conclusions. Whatever conclusions are drawn, they must be based firmly on the evidence obtained from the experiment. At the highest level, students should be able to make further predictions and ask appropriate questions based on their conclusions.

6. Evaluating procedures

Arguably, this is one of the most important, and probably one of the most difficult skills for a student to develop. In order for the evaluation to be effective, students must have a clear understanding of the aims and objectives of the exercise, otherwise they will not be able to judge the effectiveness of the procedures used. They must be able to evaluate whether the errors in the data obtained exceed those expected due to the equipment used. If this is the case, they then need to identify those parts of the procedure which have generated these excess errors, and suggest realistic changes to the procedure which will result in a more accurate outcome. Students should also be able to suggest modifications to a procedure to
answer a new question. The evaluation procedure may include:

- The identification of anomalous values, deducing possible causes of these anomalies and suggesting appropriate means of avoiding them,

- An assessment of the adequacy of the range of data obtained,
- An assessment of the effectiveness of the measures taken to control variables,
- Taking an informed judgment on the confidence with which conclusions may be drawn.

7. Evaluating conclusions

This is also a higher-level skill, which will demand of the student a thorough understanding of the basic theory that underpins the science involved. The conclusions drawn from a set of data may be judged on the basis of the strength or weakness of any support for or against the original hypothesis. Students should be able to use the detailed scientific knowledge and
Understanding they have gained in theory classes in order to make judgments about the reliability of the investigation and the validity of the conclusions they have drawn. Without practice in this area, students are likely to struggle. In order to increase the confidence in drawing conclusions, it is recommended that practical exercises, set within familiar contexts, be used to allow students the opportunity to draw conclusions, make evaluations of procedure and assess the validity of their conclusions. In the examination, students may be required to demonstrate their scientific knowledge and understanding by using it to justify their conclusions.

EXPERIMENT 1; ISOLATION OF MESSENGER RIBONUCLEIC ACID FROM MAMMALIAN CELLS.

INTRODUCTION;

According to the central dogma of molecular biology, genetic information flows from deoxy ribonucleic acid to ribonucleic acid and finally proteins. Messenger RNA can be isolated and converted to DNA in Polymerase Chain Reaction and the DNA can be analyzed for viral load detection, forensic analysis and many other scientific analyses. Even gene expression can be analyzed at RNA level by isolation of RNA and conversion to DNA and analyzed.

Principle

RNA extraction with Trizol is a common method of total RNA extraction from cells based on the research of (2440339). The main components of Trizol are guanidinium isothiocyanate (powerful protein denaturant) that inactivates RNases and acidic phenol/chloroform. Low pH is crucial since at neutral pH DNA not RNA partitions into the aqueous phase. RNA is stable in Trizol which deactivates only RNases. Hence Trizol mainly used to lyse cells without changing the integrity of RNA. After addition of Trizol, incubation at room temperature allows complete dissociation of nucleoprotein complexes. Isopropanol (IP) acts as precipitant that precipitates RNA in the suspension. Precipitation with ethanol removes DNA from the interphase, and an additional precipitation with IP removes proteins from the organic phase. Total RNA extracted by Trizol Reagent is free from the contamination of protein and DNA.

Procedure

- The media removed from the plates and each well is washed with PBS.
- 350µl of Trizol was added in each sample, and the cells were lysed directly on the culture dish. Trizol reagent was mixed properly with cells.
- The lysate was transferred to sterile micro centrifuge tube and kept at room temperature for 5 minutes, for complete dissociation of nucleoprotein complexes.
- 200µl of chloroform was added to each tube and the samples were shaken vigorously and allowed to stand for 2 minutes at room temperature.
- Trizol phases after chloroform addition.

- Colourless aqueous phase (RNA)
- Interphase (DNA)
- Red (organic) phenol-chloroform phase (proteins & lipids)
- The resulting mixture was centrifuged at 12,000g for 15 minutes at 4°C.
- The aqueous phase was transferred to a fresh tube and 300µl of IP was added and mixed gently by inverting 2-3 times and allowed the sample to stand for 5-10 minutes at room temperature.
- Centrifuged at 12,000g for 10 minutes at 4°C. The RNA forms a pellet which was washed with 75% ethanol (100µl).
- The samples were centrifuged at 7600g for 5 minutes at 4°C; the supernatant was decanted and the pellet was air dried.
- RNA pellet was re-suspended in 20µl of RNase free water and heated on thermo mixture at 55°C for 30 seconds for complete dissolution. Pure RNA should have an A260/A280 ratio of ≥ 1.7

Estimation of RNA

- First the blank was set by measuring absorbance in Nano-spectrophotometer of 2µl RNase free water as blank.
- 2µl of sample was used after mixing properly in nano-quvette to take absorbance.
- Concentration and A260/A280 ratio was noted.
- Normalization was done using above obtained values and 1µg of RNA is used for cDNA synthesis.

cDNA synthesis

Principle

Gene expression analysis has become an indispensable tool. Researchers are always keen to find out whether their gene of interest is expressed or not. For this, the mRNA (messenger RNA) is quantified in the given sample. mRNAs carry the information coded by DNA and, thus, further gets translated to produce respective proteins. RNAs are very unstable and fragile, and are very likely to degrade by the omnipresent RNases. The biological information encoded in mRNA are stored in more stable form of nucleic acid, i.e. cDNA, prepared from RNA. This conversion is

brought about by reverse transcriptase. Reverse transcriptase is a RNA-dependent DNA polymerase. Using mRNA as a template, reverse transcriptase produces its complementary DNA based on the pairing of RNA base pairs. The reverse transcriptase used (commonly used- Moloney Murine Leukaemia Virus (MMLV) RT) display terminal transferase activity on reaching at the end of the RNA template. It adds 3-5 residues (usually dC) to the 3′-terminal of the cDNA. A random primer cocktail is used to produce cDNA from the RNAs. The cDNAs produced are not full length. Random primer is extremely useful if production of the shorter cDNA fragments is desirable. Its use increases the probability of converting the entire 5′-end of the mRNA into the cDNA.

Protocol

- In 0.5 ml of micro centrifuge tubes, Master Mix for cDNA synthesis was prepared.

Component	**Amount(for20µl reaction)**
10X RT Buffer	4 µl
10mM dNTPs	2µl
Random primers (0.5µg/µl)	2 µl
Reverse transcriptase (1000U)	1µl
RNase free water	7 µl

- 16 µl of above mixture was distributed to the tubes.
- 4µl of RNA (1µg for 20µl reaction) was added to tubes containing Master mixture to make total volume of 20 µl and given a short spin.
- The tubes were put in PCR machine at 25°C for 10 mins, 42°C for 1 hour and 10 min at 60°C to stop the reaction. cDNA is ready for use in PCR reaction.

PCR Reaction

The generated cDNA was used for PCR analyses to assess mRNA expression and β-actin gene was used as an internal control. Preparation of master mixture:

Components	**10µl reaction**
Master mix (dNTPs, Buffer, Taq Polymerase, Loading Dye)	4 µl
Forward primer (10pM)	1 µl
Reverse primer (10pM)	1 µl
RNase free water	3 µl

- 9 µl of above mixture was distributed to 0.2 ml PCR tubes and 1 µl of cDNA was added to each tube.
- PCR reaction can be performed in gradient thermocycler and the PCR conditions.
- The syntesised DNA can be store at 4 degrees celcius for further analysis.

Rsults/Observations/ Discussion

EXPERIMENT 2: DNA DETECTION AND CHARACTERIZATION BY GEL ELECTROPHORESIS

INTRODUCTION:

Agarose gel electrophoresis is the most widely used technique for separating nucleic acid fragments because it is simple and nontoxic. It also offers a broad separation range. The size of the gel pore can be controlled by simply adjusting the agarose concentration in the preparation of the gels appropriate for separating a wide range of different sizes of nucleic acid molecules. The migration of nucleic acid molecules in the agarose gel is also affected by the voltage. Agarose is a linear polymer composed of alternating D and L galactose joined by α (1-3) and β (1-4) glycosidic linkages.

PRINCIPLE:

The electrophoretic resolution of DNA is carried out on a gel matrix of agarose or polyacrylamide. Agarose is used for horizontal electrophoretic procedure while polyacrylamide gel is used in vertical procedures. Agarose gels are used and cast by boiling agarose in a suitable dilute buffer, pouring into a mould and then allow it to solidify to form a matrix. The porosity of the formed matrix decreases with an increase in agarose concentration. When the DNA samples are loaded in an applied electric field in a gel, DNA molecules migrate toward positively charged electrode at rates determined by their molecular size and conformation. Smaller DNA molecules move faster than larger and bulkier ones.

The DNA resolved on an electrophoresed gel may be stained by planar aromatic compounds like ethidium bromide proflavine and acridine orange. These dyes bond to double stranded DNA by intercalating with base pairs. They have an intrinsic property of fluorescing under UV light. As little as 50ng of DNA may be detected in a gel by staining it with ethidium bromide.

REQUIREMENTS:

- Horizontal electrophoresis apparatus
- Micropipettes
- UV transilluminator
- Agarose (0.8%)
- Ethidium bromide 5× TAE buffer
- Gel loading buffer.

Preparations of reagents:

- 5× TAE buffer (pH 8.3):
- Tris base 242g
- Glacial acetic acid 5.71ml
- 0.5ml EDTA -10ml
- DW =1000ml

- Sterilize by autoclaving and store at R.T

5× Gel loading dye:

- 30% glycerol cv/v
- 0.2 % xylene cyanol w/v
- 0.25 bromophenol blue w/v

Gel staining dye

- Ethidium bromide (10mg/ml)
- Prepare 1g in 100ml distilled water
- Stir using a magnetic stirrer.

Agarose gel (0.8%)

- 0.8g agarose powder in 100ml of sterile distilled water and boil to complete dissolution of agarose.
- Pour into casting frame and add 4ml of EtBr solution prior to casting the gel.

PROCEDURE:

- Pour a suitable volume of 0.8% molten agarose solution into the gel mould and immediately inset the comb in the poured gel at a distance 1cm from one end of the plate such that its teeth do not touch the bottom glass plate.
- Allow the gel to solidify at room temperature and remove the comb carefully.
- Transfer the gel plate along with the gel in the electrophoretic tank. Position it in such a way that the wells lie toward cathode.
- Fill the tank with appropriate volume of 1× TAE buffer and load 10-20µl of the DNA marker in separate wells.
- Run the electrophoresis at 10-25mA till the dye front reaches the opposite edge of the gel
- Switch off the power supply and observe the gel under UV transilluminator.

Results/Observations/Discussion/Coclusion

EXPERIMENT 3; ISOLATION OF CHROMOSOMAL DNA FROM SACCHAROMYCES CEREVISAE:

INTRODUCTION:

Saccharomyces cerevisae is a species of yeast composed of 5-10µm round or avoid cells reproducing by budding. Total chromosomal DNA can be isolated by following a stringent protocol. The isolation and purification of DNA form yeast cells is the most common procedure in contemporary molecular biology and embodies a transition from cell biology to molecular biology. Different methods are available for isolation of DNA from yeast. The quality, quantity and required purity and molecular weight of DNA serves as the basis for the choice of extraction method and downstream processing.

PRINCIPLE:

The genomic DNA isolation needs to separate total DNA from RNA, protein and lipid. Initially the cell membrane has to be disrupted in order to release cell lysate into extraction buffer. Thus, is usually achieved through glass bead beating SDS. Endogenous nucleases tend to cause extensive DNA hydrolysis. This is avoided by chelating the DNA using EDTA.

Nucleoprotein interactions are disrupted with SDS/ proteinase, phenol and chloroform are used to denature and stabilize the rather unstable boundary between aqueous phase and pure phenol bilayer. The denatured proteins leave the aqueous phase and organic phenol phase and can be removed by aspiration and centrifugation. The DNA in the aqueous phase is precipitated by absolute ethanol.

REQUIREMENTS:

A. BUFFERS AND SOLUTIONS

- Ammonium acetate (10M)

Dissolve 770g of ammonium acetate in 800ml of H_2O, adjust volume 1 litre with H_2O. sterilise it by filter sterilization (0.22µm filter) and store in tightly sealed bottles at 4ºC or at room temperature.

- Ethanol 99%
- Phenol Chloroform (1:1 v/v)
- TE (Tris-EDTA pH 8) (10×)
- 100 mM Tris Cl^- (pH 8.0)
- 10Mm EDTA (pH 8.0)
- Sterilise these solutions by autoclaving and store at room temperatures.
- T.E. containing 20µg/ml RNase
- Triton × 100/SDS solutions.

B. MEDIA

- YPD (Yeast peptone dextrose)
- Yeast extract 7.2g
- Peptone 14.4g
- Dextrose 14.4g
- Distilled water 720ml
- pH 6.0
- Glass beads (Acid washed)
- Centrifuge and votor
- Microtubes
- Micro pipettes and sterile tips.

PROCEDURE

- GROWTH OF CELLS:
- Inoculate a yeast colony into YPA (100m/l) and incubate overnight at 30ºC with shaking at 120-150 rpm
- Collect the cells by centrifugation at 4000 rpm for 5min.
- Remove the supernatant, replace with 1ml of sterile H_2O resuspend the cells by tapping.
- Collect the cells by centrifugation as per earlier step (b)
- Remove the supernatant, resuspend the cells into 0.5ml of dd H_2O
- Transfer it to sterile 1.5ml micro centrifuge tube.
- Pellet down the cells by centrifugation at maximum speed (10,000 rpm) for 5 seconds.

EXTRATION OF DNA

- Add 0.2ml of triton/SDS solution to the cells and re-suspend the cells by tapping the side of the tube
- Add 0.2ml of phenol: chloroform and 0.3g of glass beads to the cells and vortex the cells suspension for 2min at rtp. Add 0.2ml of T.E (Ph 8.0) and mix the solution by vortexing briefly.
- Separate the organic and aqueous phase by centrifugation at maximum speed for 5min, in microfuge. Transfer the aqueous upper layer to a fresh microfuge tube taking care not to carry over material from interphase.

ISOLATION OF DNA

- Add 1ml of ethanol to the aqueous solution and cap the centrifuge tube and mix the contents by inversion.
- Collect the precipitated DNA by centrifugation at maximum speed for 5min at 4ºC in a microfuge tube. Remove the supernatant using a Pasteur pipette. Centrifuge the tube briefly for 2 seconds and remove last traces of ethanol from the bottom of the tube.
- Resuspend the nuclei acid pellet in 0.4ml of T.E (pH 8.0) with RNase and incubate the solution for 5min at 35ºC.
- Add to the solution and equal volume of phenol : chloroform and extract the RNase digested solution by inversion. The organic and aqueous phase is separated by centrifugation at maximum speed for 5min at rtp in microfuge and transfer the aqueous layer to a fresh microfuge tube.
- Add 80µl of 10M ammonium acetate and 1ml of ethanol to the aqueous layer, mix the solution by gentle inversion and store the tube for 5mins at rtp.
- Collect the precipitated DNA by centrifugation for 5min in microfuge. Decant the supernatant and rinse the nucleic acid pellet with 0.5ml 70% ethanol.
- Centrifuge at maximum speed for 2min and remove the ethanol rinse using drawn out Pasteur pipette.
- Centrifuge the tube briefly (2 seconds) and remove the last traces of ethanol from the bottom of the tube.
- Allow the pellet of DNA to dry in the air for 5minutes and then dissolve the pellet in 50µl of TE (pH 8.0) and store in a fridge.

Results/Observations/Discussion/ Conclusion

EXPERIMENT 4: SOURTHERN TRANSFER OF DNA FROM AGAROSE GEL ON TO NITROCELLULOSE / NYLON MEMBRANE:

INTRODUCTION:

Southern hybridization of DNA fragments separated in electrophoretic gels is a technique which involves transfer of DNA to membrane filters for detection of specific base sequences by complimentary probes. Professor E.M Southern developed this technique in 1975 hence the name. Southern blots are used to identify and quantitate specific DNA sequences in analysis of genome organization and expression in the study of genetic diseases, genetic finger printing and PCR products analysis.

PRINCIPLE:

This technique involves fractionating DNA molecules on the basis of size in a gel and transferred on a positively charged nitrocellulose or nylon membrane by capillary or electrophoretic transfer. The DNA is immobilised onto a membrane by UV crosslinking or by baking at 80ºC. The membrane is washed, prehybridised and then hybridised with a biotin labelled probe specific to one of the DNA bands in the DNA digest provided. After hybridisation the unbound probe is removed by washing. The membrane is then with the protein block to reduce non specific interaction. The bound probe (hybridised) is detected by incubation of membrane with streptavidin HRP conjugate. The final detection is done following of substrate TMB/H_2O_2 (Tetramethyl benzidine H_2O_2 substrate) that reach with HRP to give a blue coloured DNA band on the nylon membrane.

$$H_2O_2 \xrightarrow{HRP} H_2O + [O]$$

$$TMP + [O] \rightarrow TMBO \text{ blue}$$

REAGENTS AND MATERIALS:

- DNA maker
- Biotinylated probe
- Prehybridization buffer
- Hybridisation buffer
- Wash buffer (ABCD)
- Blocking buffer
- Conjugate dilution buffer
- Substrate solution
- Electro transfer buffer
- Blocking powder
- Ethidium bromide
- Streptavidin -HRP conjugate
- Agarose

- TAE
- Filter buffer
- Nylon paper
- Distilled water
- Petri dish
- Hot air oven
- Incubator shaker (45ºC)
- UV transilluminator

PREPARATIONS OF REAGENTS

- Preparations of prehybridization buffer:
  1g of blocking powder + 10ml prehybridization buffer
- Preparation of hybridisation buffer:
  1g of blocking powder + 10ml hybridisation buffer
- Preparation of blocking buffer:
  1g of blocking powder + 10ml of blocking buffer
- Preparation of ABCD:
  Thaw and mix the buffer provided thoroughly. Dilute 15ml each of 2× buffer supplied with 15ml of distilled water to give buffers at 1× concentration 30ml each.
- Preparation of streptavidin -HRP- conjugate buffer: prepare 9ml of conjugate dilute buffer by adding 9 of tween 20 to the conjugate dilution buffer. Dilute 3 µl of streptavidin -HRP- conjugate with 9ml of conjugate dilution buffer for each experiment just before use.
- Preparation of substrate solutions: dilute 0.5 ml of substrate with 4.5 ml of distilled water to give a final volume of 5ml of substrate solution of 1× concentration.
- Preparation of electrotransfer buffer: dilute 25ml of 10× electrotransfer buffer with distilled 225 distilled water to give a final volume of 250 ml electrotransfer at 1× concentration.

PROCEDURE

: AGAROSE GEL ELECTROPHORESIS:

Prepare 1.0% agarose gel containing ethidium bromide. Load 20 µl of ready to use DNA marker supplied on the gel. Run at 50 – 100V until dye reaches 4.5 cm from the well. Cut the DNA marker lane from the agarose gel as follows: cut the gel 3mm above the first band and 2mm below the last band, ensuring the gel measures about 4 - 4.5 cm. cut the filter paper and the nylon membrane exactly to the size of the cut get ensuring there is no protrusion of the filter paper and membrane from the gel.

ELECTROBLOTTING

Assemble the electrotransfer apparatus as shown below; i.e. placing the filter paper on the cathode cassette cover followed by the cut gel and nylon membrane. Mark and place the soft side of the nylon membrane to the cut gel. Place the wet filter paper on the nylon membrane followed by the anode cassette cover. Tighten the electro cassettes with screws provided. Insert the cassette into the apparatus filled with 250 ml of 1 × electrotransfer buffer. Connect the cords to power supply according to the colour codes: red – anode, black-cathode and set voltage at 50v for 3 $^{1/2}$ hours. Turn off the power supply and remove the cassette from the apparatus, drain the buffer.

IMMOBILIZATION OF DNA ON MEMBRANE

Remove the nylon membrane gently from the cassette and place it on a thin transparent polythene sheet and then on a UV transilluminator exposing the soft side of the membrane containing transferred DNA to the UV light, with UV lamps switched on for 20minutes. This fixes DNA on the membrane. Turn off the transilluminator. Place the membrane in a plastic petri dish and incubate in an oven for 30 minutes at 70ºC. This ensures complete immobilization of DNA on to the membrane.

HYBRIDISATION

Bring the petri dish containing the membrane to room temperature after incubation. add 10 ml of prehybridization buffer to the petri dish and incubate at 45ºC in incubator shaker with mild 70-90 rpm for 45 minutes. After incubation discard prehybridization buffer taking care not to discard the membrane. Add 10ml of hybridization buffer to the petri dish containing the membrane. Keep vial of biotinylated probe for 10 minutes in a boiling water bath and immediately chill by placing it on ice for 5-10 minutes. Add this probe to the hybridization buffer in a petri dish (rinse the probe vial with 300 µl of hybridization buffer and add it to the petri dish). Incubate the petri dish at 45ºC incubator shaker with a mild shaking 70 rpm for 16 hours.

BLOCKING AND DETECTION

Decant the hybridization buffer and add 10ml of 1× wash buffer A and gently swirl the petri dish for 5min the petri dish for 5minutes at room temperature. Repeat the washes twice, discarding buffer each time. Add 10ml of 1× pre warmed (70ºC) was buffer B and gently swirl the petri dish, incubate at 70ºC for 5minutes and place in a hot air oven and gently swirl. Repeat the washes twice, discarding buffer each time. Add 10ml of 1× blocking buffer to the petri dish and incubate at room temperature for 1 hour with gentle rocking. Discard the blocking. Add 9ml of diluted streptavidin -HRP- conjugate to the petri dish and incubate at room temperature for 20minutes with gentle rocking. Discard conjugate buffer. Add 10 ml of 1× wash buffer C to the petri dish and incubate at room temperature for 5minutes with gentle rocking repeat the washes two times. Discard the buffer after each wash. Add 10ml of 1× wash buffer D to the petri dish and incubate at room temperature for 5 minutes with gentle rocking. Repeat twice and discard the buffer. Add 5ml of 1× substrate solution and gently swirl at room temperature for 45 minutes until blue colour band develops. After the colour band stop the reaction by placing the membrane into distilled water.

EXPERIMENT 5: IN VITRO SYNTHESIS OF SPECIFIC DNA FRAGMENTS USING PCR

INTRODUCTION:

The polymerase chain reaction (PCR) is a primer mediated enzymatic amplification of specifically cloned or genomic sequences. The PCR process invented more than a decade ago, has been automated for routine use in labs worldwide. The template DNA contains that target sequence which can be tens or tens of thousands of molecules of nucleotides in length. A thermal stable DNA polymerase like tag DNA polymerase catalyses the buffered reaction in which an excess of an oligonucleotide primer pair and four deoxynucleoside tri phosphates (dNTPs) are used to make millions of copies of the target sequence.

PRINCIPLE:

PCR requires repetitive series of the three fundamental steps that defines one PCR cycle: double stranded DNA template denaturation, annealing of two oligonucleotides primer to the single stranded template and enzymatic extension of primers to produce copies that can serve as templates in subsequent cycles. The target copies are double stranded and bound by annealing sites of the incorporated primers. The 3' end of the primer should complement the target exactly but the 5' end can actually be a non-complementary tail with restriction enzyme and promoter sites that will also be incorporated. As the cycle precedes both the original templates and the amplified target serve as substrates for the denaturation primer annealing and primer extension processes.

The denaturation steps occur rapidly at 94-96 ºC. Primer annealing depends on Tm (melting temperature) of the primer- temperate hybrids. The best annealing temperature is determined by optimization. Extension occurs at 72ºC for most templates. PCR can easily occur with two temperature cycles consisting of denaturation and annealing extension.

MATERIALS

- DNA template
- Sterile distilled water
- Primer pair (forward and reverse)
- 2× master mixture
- 10× PCR buffer-100mM Tris HCL (pH 9.0) 1% Triton ×100, 500mM KCL, 15mM $MgCl_2$.
- dNTPs mixture- mixture of dATP, dGTP, dCTP and dTTP each at concentration 1Mm
- Tag DNA polymerase.
- Thermal cycler
- Sterile epperndorf tubes (0.2/0.5ml)
- Sterile micro pipettes.

PROCEDURE:

1. Set up the reaction mixture by mixing the following components.

REACTION MIXTURE	VOLUME	CONCENTRATION
• 2× master mix	10µl	2×
• Forward primer	1µl	10picomole
• Reverse primer	1µl	10picomole
• DNA Template (plasmid)	3µl	100ng/volume
• Sterile distilled water	5µl	
• Final volume	20µl	

- Place the samples on a thermal cycler and program it for 30 cycles by using the following temperature values in each cycle.
- Denaturation of DNA 92-94ºC for 1minute
- Annealing or primer binding at 55ºC for 1minute
- DNA extension at 72ºC for 1-2minutes
- After the completion of the cycles perform the final extension step at 72ºC for 5minutes to ensure that all the DNA is replicated into its duplex form.
- Analyse the purity of the amplified sample by using agarose gel electrophoresis.

Results/Observations/Discussion/Conclusion

EXPERIMENT6; SDS-PAGE ANALYSIS AND MOLECULAR WEIGHT DETERMINATION OF PROTEIN.

INTRODUCTION:

Charged macromolecules may be separated based on their size and charge by electrophoresis. Using a porous polyacrylamide gel, proteins can be resolved based on sieving effect of the gel as well as their electrophoretic mobility of pH of resolving gel and the reservoir buffer is high enough (pH9), then nearly all the proteins have net negative charges and tend to move towards the anode when current is switched on.

The polyacrylamide gel is prepared by polymerising the monomer acrylamide with the help of small quantity of crosslinking reagent like N, N, and methylene bisacrylamide. Ammonium persulphate (APS) initiates the polymerising process since free radicals are generated from persulphate in the presence of promoter $N.N.N^{+}.N^{+}$ tetra methyl diamine. Gels made in the concentration range 3-30% are useful in resolving proteins of sizes up to 10^{6} daltons.

PRINCIPLE:

A form of polyacrylamide gel electrophoresis (PAGE) involves the use of a detergent sodium doclecyl sulphate (SDS) which dissociates proteins into their subunits. Being an amphilic molecule, SDS interferes with hydrophobic interactions that normally stabilize proteins. When heated most proteins bind to about 1.4g SDS per gram of protein which completely masks the intrinsic charge of proteins imparting uniformly distributed negative charge owing to the sulphate residues absorbed onto the protein surface. The outcome is that a discontinuous SDS-polyacrylamide gel migrates more slowly than the higher counterparts.

The electrophoresis mobility of biomolecules becomes inversely proportional to the $\log_{10}$ of their molecular weights. Consequently, the molecular weight of the known proteins may be determined by measuring the relative distance travelled by the protein in question, and referring to the standard curve plotted by using commercially available pure marker proteins of known molecular weight, running parallel with known protein sample in same experiment.

To accurately determine its molecular weight, a given protein must be electrophoresed in various dilutions in parallel lanes so as to avoid the development of any false band or artefact. Furthermore, its molecular weight is determined by repeating the electrophoresis in the presence of molecular weight markers running simultaneously in polyacrylamide gels of different porosities. Because SDS disrupts non covalent interactions between proteins, SDS page yields the molecular weight of individual subunits of multi-subunits proteins. Whether or non-subunits are linked by disulphide bonds may be determined by running two samples in parallel during SDS-PAGE, one treated with reducing agent B-mercapto ethanol and the other without it.

The acrylamide concentration is selected depending on the molecular weight of the protein to be resolved. Generally, the higher the molecular weight of the protein to be resolved, the lower the concentration of acrylamide in gel:

ACRYLAMIDE CONCENTRATION (%)	MOLECULAR WEIGHT (kda)
• 20	20-25
• 15	15-50
• 10	20-75
• 5	60-220

PROCEDURE:

Pouring and setting the gel

- Wipe the cleared glass plates with ethanol to free them from grease.
- Place three spacers on the three edges of intact glass plate and place the other wedged plate on it in such a way that the wedged side is away from the spacers.
- Hold the assembly in place using 1% molten agarose.
- Place the assembly vertically using a clamp and pour the resolving mixture gel in pockets created by the glass plates up to about 2 thirds of the area and allow the resolving gel to polymerise at room temperature.
- Over lay the gel with isobutanol using wash bottle.

COCKTAIL PREPARATION

SOLUTION	**RESOLVING GEL (10%)**	**STACKING GEL(5%)**
• Monomer solution	6.6ml	1.7ml
• Resolving gel	5.0ml	1.25ml
• Stacking gel		
• 10% SDS	0.2ml	0.1ml
• APS	0.1ml	0.2ml
• TEMED	40-50µl	30µl
• Total volume		
• With distilled H_2O	20ml	10ml

Preparation of discontinuous gel

- One the resolving gel sets, pour off the isobutanol layer carefully. Prepare the stacking gel mixture based on the composition and immediately pour its appropriate volume on the top of the resolving gel.

- Insert comb of appropriate size and leave it undisrupted to polymerise about (20 minutes). Avoid any bubble during the insertion of comb.

- Arranging the gel assembly for electrophoresis
- Gently remove the comb and spacer present at the bottom of the gel.
- Mount the gel plate assembly into the electrophoretic wedged plate positioned towards the inside and fill both the upper and lower reservoirs with the tank buffer so that the wells are immersed in buffer.

Loading of sample

- Before loading the sample on the SDS-PAGE gel, mix 50-100µl of the sample buffer with an equal volume of each sample such that the final concentration of the protein is about 5-10µg/µl.
- Denature the sample by heating at 100ºC for 3 minutes and load it in the gel using a syringe. Run similarly treated commercially available marker proteins parallel to be used as reference.
- Check the buffer levels in the upper and lower chambers and connect the terminal to the power pack that is upper to the cathode and lower to the anode.
- Switch on the current and run the gel initially at 10-15mAmps for 15 minutes and then 25 milli Amps (100V) till the dye front almost reaches the bottom of the gel (4-5hours) depending on the size of the gel.
- Switch off the power supply, disconnect the terminals and remove the gel assembly from the electrophoresis apparatus.
- Remove the side spacers, lift the wedged plate and slip gel gently into the stain that is kept into the trough of suitable size so as to immerse the gel in it.
- Soak the gel in it for an hour with gentle shaking.
- Transfer the gel into de-staining solution and shake gently during de-staining.
- Change the staining solution frequently till deep blue bands are clearly visible against a faded back ground.
- Determine the molecular weight of sample using the standard graph.
- Store the gel in wet condition in 7.5% acetic acid. To dry the gel, place the gel in 10% glycerol for one hour then wrap them in moistened cellophane and dry them in an oven maintained at 50ºC.

Results/Observations/Discussion/Conclusion

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