Faculty of Science

Laboratory Manual

Molecular Biology

Bachelor of Biotechnology (Hons.)

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Molecular Biology

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LINCOLN UNIVERSITY COLLEGE
FACULTY OF SCIENCE (DEPARTMENT OF BIOTECHNOLOGY)
LABORATORY SAFETY RULES

The following rules must be obeyed by all students in the science laboratory of the faculty. Wilful or repeated in advertent non-compliance may result in dismissal or suspension from the laboratories

- No entry without permission:
  - Outsiders are not allowed to enter the laboratory without permission.
  - No student is allowed to enter the laboratory unless permission has been given by a laboratory assistant or a lecturer.

- At work in the laboratory:
  - No experiment may be attempted without the knowledge and permission of a lecturer.
  - Students must wear shoes in the laboratory. Students wearing slippers or sandals are not allowed to work in the laboratory.
  - Lab coat must be worn at all times during practical work in the laboratory.
  - Do not mouth pipette chemicals.
  - Do not eat or smoke in the laboratory.
  - Do not taste any chemicals, including dilute solutions. If any acid or alkali accidentally enters your eyes or mouth, wash immediately with plenty of tap water. Inform your lecturer, and seek medical attention if necessary.
  - Paper should be used to light up the Bunsen burners.
  - Used match sticks, filter papers, and other solid waste must never be thrown into the sinks. They must be thrown into the dustbins provided. Lighted match sticks and smoldering materials must be extinguished with tap water before thrown in to the dustbins.
  - Any equipment broken or damaged must be reported to the laboratory assistant.

- Before leaving the laboratory:
  - All the equipment and benches must be cleaned at the end of each practical session.
  - Wash hands and arms with soap and water before leaving the laboratory.
  - No student is allowed to take away any chemicals, equipment or other property of the laboratory.

INTRODUCTION

1. The Scientific Method

- Making observations
- Generating hypotheses
- Making predictions
- Designing and carrying out experiments
- Constructing scientific models

2. Practical Exercises

To get the most out of the practical exercises, you need to follow carefully the instructions given. These instructions have been designed to provide you with the experience in the following skills:

- Following instructors
- Handling apparatus
- Having due regard for safely
- Making accurate observations
- Recording results in an appropriate form
- Presenting quantitative results
- Drawing conclusions

3. Following Instructions

Instructions are provided in the order in which you need to carry them out. We would advise that before carrying out the instructions, you read through the entire exercise. This will help you to remember what you have learned.

Each practical exercise in the book begins with a few lines describing its purpose in most cases the following headings are also used:

- Procedure-numbered steps that need to be carried out.
- For consideration -some questions to help you think carefully about the results you have obtained.
- Materials-a list of the apparatus, chemicals and biological materials you need.

4. Handling apparatus

Biologists need to able to use many different types of apparatus, for example, photometers (to measure water uptake by plants), respirometers (to measure oxygen uptake or carbon dioxide production), Petri dishes (for plating out bacteria and other microorganisms) and the light microscope (to magnify specimens). Many of the practical exercises are designed to help you derive the maximum benefit from a piece of apparatus.

5. Having Due Regard for Safety

Surveys have been shown that science laboratories are among the safest places to be. Nevertheless, this is no cause for complacency.

- Always move slowly and carefully in a laboratory.
- Never put your fingers in your mouth or eyes after using chemicals or touching biological specimens until you have washed your hands thoroughly with soap and warm water, and dried them.
- Make sure glass objects (e.g, thermometers, beakers) cannot roll off tables or be knocked onto the floor.
- Wear safely goggles whenever there is a risk of damage to the eyes.

<u>Situations of risk include:</u>

- Heating anything with a Bunsen burner (even heating water has its dangers')
- Handling many liquids, particularly those identified as corrosive, irritant, toxic or harmful

- Handling corrosive or irritant solids
- Some dissection work
- Allow Bunsen burners, tripods, gauzez and beakers to cool down before handling them.
- Never allow your own body fluids (especially blood and saliva) to come into contact with someone else, or theirs into contact with you.
- Keep long hair tied back and do not wear dangly earrings.
- Do not allow electrical equipment to come into contact with water.
- If you are unsure how to carry out a scientific procedure, ask.
- Make sure you understand why you are going to do something before you do it.
- Wear a lab coat when using chemicals or handling any biological specimens.
- Follow exactly agreed procedures with regard to cuts, burns, electric shocks and other accidents (e.g. with chemicals).
- Follow exactly all specific safely instructions given in this book or provided by your teacher for particular practical exercises (e.g. use of gloves, disinfection)

With practice, these procedures should become second nature to you. They will enable you to carry out practical work in safety.

6. Making Accurate Observations

In most cases the practical exercise will make it clear what you need to observe, e.g. the time taken for a certain volume of gas to be evolved or the width of a sample cells. Ensure that you know how to use any necessary equipment before starting practical. Think carefully about the precision with which you will make your observations.

7. Recording Results in an Appropriate Form

Results can be recorded in various ways. Often it is helpful to record raw data in a table. Most data will be in the form of numbers, i.e. they will be quantitative data (also known as numerical data). However, some data, e.g. flower colour, will be qualitative data.

One form in which some biological findings can be recorded is a drawing. You don't need to be professional artist to make worthwhile biological drawings. If you follow the following guidelines, a drawing can be of considerable biological value:

- Ensure that your completed drawing will cover at least a third of A4 page.
- Plan your drawing so that the various parts are is proportion and will not be drawn too small. Small marks to indicate the length and breadth of the drawing are a great help in planning and a faint outline can be rapidly drawn to show the relative positions of the parts.
- The final drawing should be made with clean, firm lines using a sharp HB pencil and, if needed, a good quality eraser (not a fluid).If important details are too small to be shown in proportion, they can be put in an enlarged drawing at the side of the main drawing.
- Avoid shading and the use of colour unless you are an excellent artist and they really help, for example when drawing soil profiles.
- When drawing structures seen with the naked eye or hand lens, use two lines to delineate such things as blood vessels and petioles. This will help you to indicate the relative widths of such structures.
- When drawing low power plan drawings from the light microscope, do not attempt to draw individual cells-just different tissues.
- When drawing plant cells at high power under the light microscope, use two lines to indicate the width of cell walls, but a single line to indicate a membrane.
- Always put a scale on each drawing.

8. Presenting Quantitative Results

Presentation of data is all about using graphs or other visual means to make it easier to see what your results tell you. The following four ways of presenting data are the most frequently used in biology: line graphs, bar charts, histograms and scatter graphs (Figure 1).

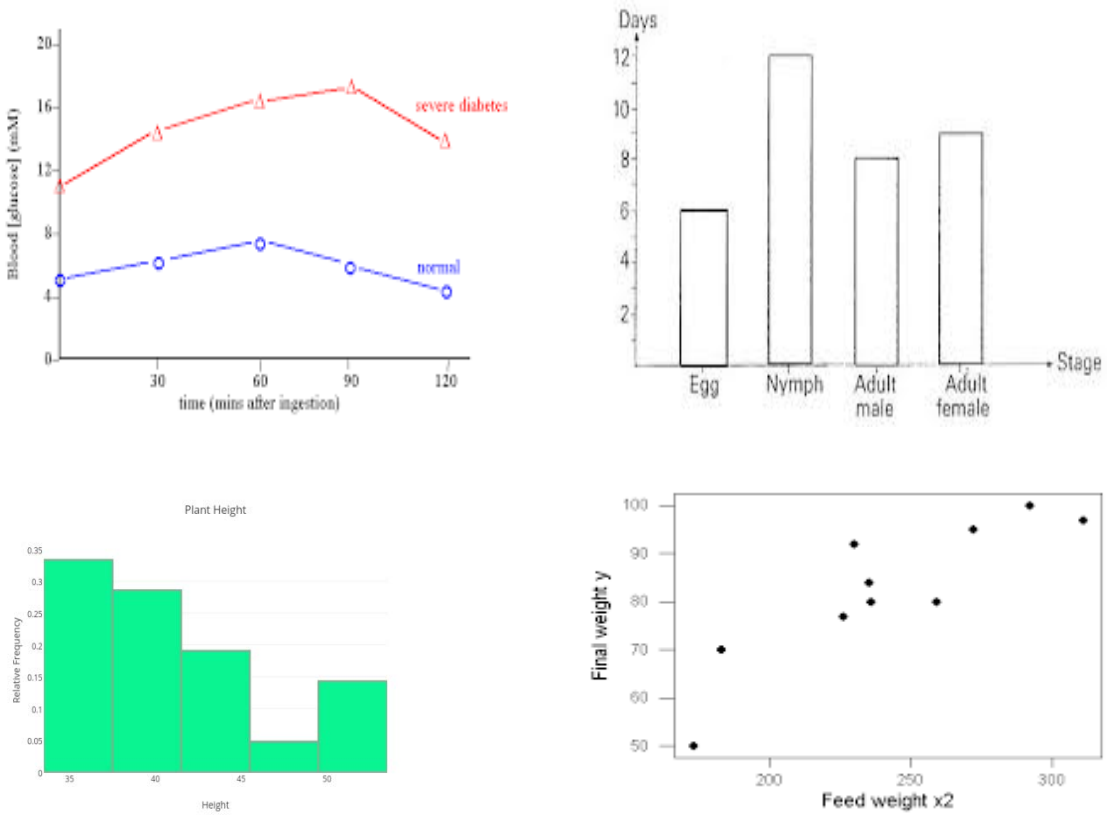


Figure 1: Line graphs, bar charts, histograms and scatter graphs

9. Drawing Conclusions

Finally, you will need to draw conclusions. If your practical exercise has involved the testing of a hypothesis, for example that the enzyme pepsin works better at low pH than in neutral or alkaline conditions, your conclusion should indicate whether the hypothesis has been refuted (i.e. shown not to be the case) or supported. Of course, even if your hypothesis has been supported, it doesn't mean that it has been confirmed with 100% certainty- in other words it isn't proved. Science proceeds more by showing that certain ideas are wrong than by showing that others are right (think about that!). Your conclusion might therefore include further ways of testing the original hypothesis, or might raise new possibilities to be investigated.

Often you will only be able to arrive at your conclusions after statistically analysing your data.

10. Writing a Scientific Lab Report

Title

- Communicate the subject investigated in the paper.

Introduction

- State the hypothesis.
- Give well-defined reasons for making the hypothesis.
- Explain the biological basis of the experiment.
- Cite sources to substantiate background information.

- Explain how the method used will produce information relevant to your hypothesis.
- State a prediction based on your hypothesis. (If the hypotheis is supported, then the results will be.)

Materials and Methods

- Use the appropriate style.
- Give enough detail so the reader could duplicate your experiment
- State the control treatment, replication and standardized variables that were used.

Results

- Summarize the data (do not include raw data).
- Present the data in an appropriate format (table or graph).
- Present tables and figures neatly so they are easily read.
- Label the axes of each graph completely.
- Give units of measurement where appropriate.
- Write a descriptive caption for each table and figure.
- Include a short paragraph pointing out important results but do not interpret the data.

Discussion

- State whether the hypothesis was supported or proven false by the results, or else state that the results were inconclusive.
- Cite specific results that support your conclusions.
- Give the reasoning for your conclusions.
- Demonstrate that you understand the biological meaning of your results.
- Compare the results, with your predictions and explain any unexpected results.
- Compare the results to other research or information available to you.
- Discuss any weaknesses in your experimental design or problems with the execution of the experiment.
- Discuss how you might extend or improve your experiment.

Conclusion

- Restate your conclusion.
- Restate important results.

Literature Cited

- Use the proper citation form in the text.
- Use proper citation form in the Literature Cited section.
- Refer in the text to any source listed in this section.

Acknowledgement

- State any appropriate acknowledgement that you think is necessary.

Practical 1

Title: Preparation of buffers and material for DNA extraction

Objective:

After completing the practical, you will be able:

1. To prepare reagents and material

Introduction

The search for a more efficient means of extracting DNA of both higher quality and yield has led to the development of a variety of protocols, however the fundamentals of DNA extraction remains the same. DNA must be purified from cellular material in a manner that prevents degradation. Because of this, even crude extraction procedures can still be adopted to prepare a sufficient amount of DNA to allow for multiple end uses.

DNA extraction from plant tissue can vary depending on the material used. Essentially any mechanical means of breaking down the cell wall and membranes to allow access to nuclear material, without its degradation is required. For this, usually an initial grinding stage with liquid nitrogen is employed to break down cell wall material and allow access to DNA while harmful cellular enzymes and chemicals remain inactivated. Once the tissue has been sufficiently ground, it can then be resuspended in a suitable buffer, such as CTAB. In order to purify DNA, insoluble particulates are removed through centrifugation while soluble proteins and other material are separated through mixing with chloroform and centrifugation. DNA must then be precipitated from the aqueous phase and washed thoroughly to remove contaminating salts. The purified DNA is then resuspended and stored in TE buffer or sterile distilled water. This method has been shown to give intact genomic DNA from plant tissue

Procedure:

- Preparation of materials, consumables, buffers and reagents

Materials and equipment need to be provided:

- Sterilized 1.5 ml centrifuge tubes
- Ethanol (96 – 100%)
- Ethanol (70%)
- Waterbath
- Pipeter
- Centrifuge
- CTAB powder
- CTAB extraction buffer
- Wash buffer
- Mortar & Pestle
- Double-distilled water
- 2-mercaptoethanol
- Chloroform
- Octanol
- Isopropanol
- Chloroform: Isoamyl alcohol (24: 1)

Preparation instructions

1. Grinding of the plant material can be done using mortar and pestle with CTAB extraction buffer. For plants such as maize and lettuce that have pronounced leaf midribs, these should be removed from the material before grinding, as it is a major source of carbohydrate contamination.
2. Preparation of CTAB extraction buffer: immediately prior to use, add 90 ul of 2-mercapthoethanol and 90 mg CTAB powder in 9 ml of CTAB extraction buffer. Preheat the solution at 65'C.
3. Chloroform: Octanol/Isoamyl (24:1). Store in dark at room temperature (15-25'C). Prepare and dispense this solution in fume hood.
4. To the CTAB wash buffer concentrate (120 ml) provided, add 360ml of ethanol (100%) prior to use.
5. Pre-hill the CTAB wash buffer and 70% ethanol prior to use.

Questions

1. What does the CTAB look like?
2. What is the function of Chloroform: Octanol/Isoamyl in DNA extraction?
3. Describe how to prepare CTAB and wash buffers?

Practical 2

Title: Total genomic DNA extraction

Objective:

After completing the practical, you will be able:

1. To extract high quality and appropriate quantity of genomic DNA

Introduction:

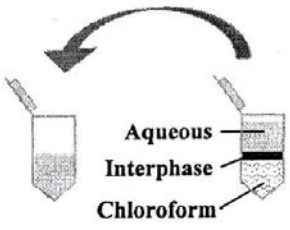
Use of molecular techniques has been widely increased in the past few years because they are the most reliable and accurate tools for the various purposes such as evaluation of genetic structure, forensic analysis, medical molecular techniques. Extraction of high quality and quantity genomic DNA is a critical pre-requisite step for any molecular techniques. In fact, the first and most important step of molecular techniques is to isolate the high quality and standard quantity of DNA.

Materials & Equipment:

- Water bath
- Centrifuge
- Mortar & Pestle
- Liquid nitrogen (optional)
- 2.0 ml centrifuge tube
- Pipette
- CTAB buffer
- Chloroform
- Isopropanol or Ethanol

Procedure:

1. Transfer 200-300mg of plant tissue into a 2.0ml sterilized centrifuge tube
2. Grind plant material using sea/quarts sands and a micro-pestle
3. Add sufficient pre-warmed CTAB buffer (Refer to experiment 1)
4. Vortex and spin
5. Incubate for 30-60 min at 65˚C with occasional inversion.
6. Centrifuge for 5 min at 13000xg
7. Transfer the aqueous phase carefully to a new tube
8. Add 1 equal volume of Chloroform:Isoamyl (24:1) and invert for 5 min
9. Centrifuge for 5 min at 13000xg
10. Transfer top aqueous phase carefully to a new tube
11. Precipitate DNA by adding 1 vol. of cold Isopropanol or ethanol
12. Centrifuge for 10min at 13000xg in 4˚C
13. Discard the isopropanol carefully
14. Add 2 volume of wash buffer (Refer to Experiment 1)
15. Centrifuge for 5min at 13000xg
16. Discard the Ethanol carefully
17. Brief air dry (not more than 5 min)
18. Dissolve in 50-100ul Elution Buffer by pipetting
19. Store in -20˚C for further analysis



Trouble shooting tips:

1. After step 8, it is very hard to collect the supernatant without disturbing the bottom layer. Collect as much as you can; recentrifuge if necessary.

2. Drying the pellet is usually a straggling step. Simply resuspend the next morning if needed or/and place on ice if not using right away.

3. You will use additional tubes per sample. It is best to label both the tubes and tops, and also write down sample names, as they can wash off in the water baths.

Questions:

1. What did the DNA look like?
2. If DNA is so small it fits in one cell, how are we able to see it with our eyes after extraction?
3. Do you see any differences in the DNA of different samples?
4. What can we do with the DNA we isolate? Why is it valuable that we are able to isolate DNA?
5. Why do we wear gloves when doing science/DNA extraction?
6. Based on this lab, why do we shampoo when we shower?

Practical 3
Title: PCR amplification

Objective:
After completing the practical, you will be able:

1. To gain hands-on experience of principles and practice of polymerase chain reaction (PCR)

Introduction:

The polymerase chain reaction (PCR) is a DNA amplification technique that has revolutionized almost all aspects of biological research. PCR was invented in 1984 by Dr. Kary Mullis at the Cetus Corporation in California. The enormous utility of the PCR method is based on its ease of use and its ability to allow the amplification of small DNA fragments. For this ground breaking technology, Mullis was awarded the Nobel Prize in Chemistry in 1993.

Before performing PCR, template DNA is extracted from various biological sources. Because PCR is very sensitive, only a few copies of the gene are required. Nevertheless, freshly isolated DNA will provide better amplification results than older DNA specimens that may have become degraded. In order to amplify the specific DNA or target sequence, two primers (short, synthetic DNA molecules) are designed to correspond to the ends of the target sequence. The primers hybridize to the DNA template, which marks this sequence to be copied by DNA polymerase. Starting from the primer, DNA polymerase builds a new strand of DNA in the 5' —> 3' direction, using the DNA template as a guide.

To perform PCR, the template DNA and a molar excess of primers are mixed with the four “free" deoxynucleotides (dATP, dCTP, dGTP, and dTTP), and a thermostable DNA polymerase. The most commonly used DNA polymerase is Taq DNA polymerase. This enzyme, originally purified from a bacterium that inhabits hot springs, is stable at very high temperatures. These components (template DNA, primers, the four deoxynucleotides, and Taq DNA polymerase) are mixed with a buffer that contains Mg^{+2}, an essential cofactor for Taq polymerase. The PCR reaction mixture is subjected to sequential heating/cooling cycles at three different temperatures in a thermal cycler.

In the first step, known as “denaturation", the mixture is heated to near boiling (94°C -96°C) to “un-zip" (or melt) the target DNA. The high temperature disrupts the hydrogen bonds between the two complementary DNA strands and causes their separation.

In the second step, known as “annealing", the reaction mixture is cooled to 45°C - 65°C, which allows the primers to base pair with the target DNA sequence.

In the third step, known as “extension", the temperature is raised to 72°C. This is the optimal temperature at which Taq polymerase can add nucleotides to the hybridized primers to synthesize the new complementary strands.

These three steps - denaturation, annealing, and extension - constitute one PCR “cycle". Each PCR cycle doubles the amount of the target DNA in less than five minutes. In order to produce enough DNA for analysis, twenty to forty cycles may be required. To simplify this process, a specialized machine, called a “thermal cycler" or a “PCR machine", was created to rapidly heat and cool the samples.

Materials and Equipment:

- PCR kit ($MgCl_2$, PCR buffer, DNA Taq polymerase)
- Thermal Cycler machine
- Pipette
- PCR tubes and tips
- ddH_2O
- dNTPmix
- Primer stocks
- DNA sample (from previous practical)

Procedure:

1. LABEL one 1.5 ml centrifuge tube with "master mix" and your group name or initials.
2. LABEL adequate number of 0.2 PCR tube for different DNA samples in addition to one for negative control.
3. FILL IN the below table with adequate amount of reagents and multiply based on the number of samples.

PCR protocol:

Reagent	**1x (10µl)**	**??X (10 µl)**
$MgCl_2$		
PCR buffer (5x) – Green Buffer		
dNTPmix		
Forward primer		
Reverse primer		
Taq polymerase		
ddH_2o		
DNA		
Total		

4. MIX the reagents by gently flicking the tube except DNA. This is the PCR master mixture. The solution should be pale green in color. NOTE: If the solution is not pale green, the PCR sample has not been correctly assembled.
5. ADD 1 µl of individual DNA samples to 0.2 ml labeled PCR tubes.
6. TRANSFER 9 µl of the PCR master mixture to the labeled 0.2 ml "PCR" tube with already added DNA.
7. PLACE the "PCR" tube on ice.
8. MIX the sample gently. Make sure the PCR mixture is completely dissolved.
9. Quickly CENTRIFUGE to collect the sample at the bottom of the tube.
10. Recommend appropriate cycling parameters for the PCR machine by filling in the below table:

Cycling parameters:

Step	Temperature	Time	Number of cycles
Initial denaturation			
Denaturation			
Annealing			
Extension			
Final extension			
Hold			

10. PLACE the PCR tubes in the PCR machine and RUN.
11. AFTER completing PCR, PLACE the tubes in -20°C for future use.

Next step: Separation of PCR Products by Electrophoresis (Refer to Practical 4)

Questions:

1. What are these things doing in my PCR reaction?

a) Primer

b) DNA Polymerase

c) Nucleotide

2. Draw out a pictorial representation of PCR. Include such steps as the separation of DNA strands, binding of primers, and elongation.

3. a. Suppose you begin a PCR reaction with 1 piece of double stranded DNA. After 28 cycles of replication, how many pieces of double stranded DNA do you now have?

b. At the end of the entire PCR reaction, are the original DNA strands bound to each other or to new DNA strands? Explain.

4. What exactly is PCR used for and why is it an effective and important technique?

Practical 4
Title: Agarose gel electrophoresis

Objectives:

After completing the practical, you will be able:

1. To familiarise with equipment and apparatus used in extraction and gel electrophoresis
2. To understand the principle of extraction and gel electrophoresis
3. To observe how dye molecules of different sizes and charges migrate through a gel during electrophoresis and will then draw conclusions about unknown dyes based on this information

Introduction

The term electrophoresis describes the migration of a charged particle under the influence of an electric field. Many important biological molecules, such as amino acids, peptides, proteins, nucleotides and nucleic acids, possess ionisable groups and, therefore, at any given pH, exist in solution as electrically charged species either as cations (+) or anions (-). Under the influence of an electric field these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge.

The equipment required for electrophoresis consists basically of two items, a power pack and an electrophoresis unit. Electrophoresis units are available for running either vertical or horizontal gel systems. Vertical slab gel units are commercially available and routinely used to separate proteins in acrylamide gels. The gel is formed between two glass plates that are clamped together but held apart by plastic spacers.

Agarose is a linear polysaccharide (average relative molecular mass about 12 000) made up of the basic repeat unit agarobiose, which comprises alternating units of galactose and 3,6-anhydrogalactose. Agarose is one of the components of agar that is a mixture of polysaccharides isolated from certain seaweeds. Agarose is usually used at concentrations of between 1% and 3%. Agarose gels are formed by suspending dry agarose in aqueous buffer, then boiling the mixture until a clear solution forms. This is poured and allowed to cool to room temperature to form a rigid gel. The gelling properties are attributed to both inter- and intramolecular hydrogen bonding within and between the long agarose chains. This cross-linked structure gives the gel good anticonvectional properties. The pore size in the gel is controlled by the initial concentration of agarose; large pore sizes are formed from low concentrations and smaller pore sizes are formed from the higher concentrations.

Electrophoresis in acrylamide gels is frequently referred to as PAGE, being an abbreviation for polyacrylamide gel electrophoresis. Cross-linked polyacrylamide gels are formed from the polymerisation of acrylamide monomer in the presence of smaller amounts of N,N'-methylene-bisacrylamide (normally referred to as 'bis'-acrylamide). Note that bisacrylamide is essentially two acrylamide molecules linked by a methylene group, and is used as a cross-linking agent. Acrylamide monomer is polymerised in a head-to-tail fashion into long chains and occasionally a bis-acrylamide molecule is built into the growing chain, thus introducing a second site for chain extension. Proceeding in this way a cross-linked matrix of well-defined structure is formed. The polymerisation of acrylamide is an example of free-radical catalysis, and is initiated by the addition of ammonium persulphate and the base N,N,N',N'-tetramethylenediamine (TEMED).

Materials & Reagents

Materials	Reagents
gel electrophoresis chamber power supply (minimum 50-V capability) roll of masking tape rack for microcentrifuge tubes 400-ml (or larger) container boiling water bath or microwave oven distilled water (380 mL)	Gel Electrophoresis Carolina Biological Kit

Procedure

1. Load dye samples into the wells (also called lanes) from left to right following the order listed below. To load the first sample (bromphenol blue) into the well, draw 20 µl of dye.

   Order of Loading
   lane 1 bromphenol blue
   lane 2 methyl orange
   lane 3 ponceau G
   lane 4 xylene cyanol
   lane 5 pyronin Y
   lane 6 unknown #1
   lane 7 unknown #2
   lane 8 unknown #3

2. Once all the dye samples have been loaded, place the lid on the electrophoresis chamber. Orient the lid with the positive end of the chamber connected to the red (positive) cord and the negative end of the chamber connected to the black (negative) cord. Then connect the electrical cords to the power supply, with the positive lead in the positive input (red to red) and the negative lead in the negative input (black to black). If using a multi-channelled power supply, make sure both electrical leads are connected to the same channel.

3. Turn on the power supply and set it to the desired voltage. Watch as the dyes slowly move into the gel and separate over time. Do not allow any of the dyes to run off the gel. Run the gel until the band in lane 3 is 0.5 cm from the end of the gel.

4. Once the desired separation of dyes has been achieved, turn off the power, disconnect the leads from the inputs, and remove the top of the electrophoresis chamber.

5. Carefully remove the casting tray and slide the gel into the plastic tray.

Questions:

1. Describe how to prepare TBE buffer.

2. What are the applications of agarose gel electrophoresis?

3. How will you prepare agarose gel?

4. What is agarose gel electrophoresis?

5. Based on the direction of migration, migration distance, and the appearance of your gel, what dye components were present in each of the unknown dye mixtures?

6. Which dye molecule travelled farthest through the gel? Which travelled the shortest distance through the gel? What properties affect migration distance?

7. What was the charge of the dye molecules that migrated toward the positive electrode and of the dye molecules that migrated toward the negative electrode? How do you know?

8. Why is electrical current necessary for separating molecules by gel electrophoresis?

9. Why the porous matrix of agarose is gels an essential component of molecule separation by gel electrophoresis?

Notes

Notes

Notes

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