Faculty of Science

Laboratory Manual

Recombinant Dna Technology

Bachelor of Biotechnology (Hons.)

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Recombinant DNA Technology

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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).

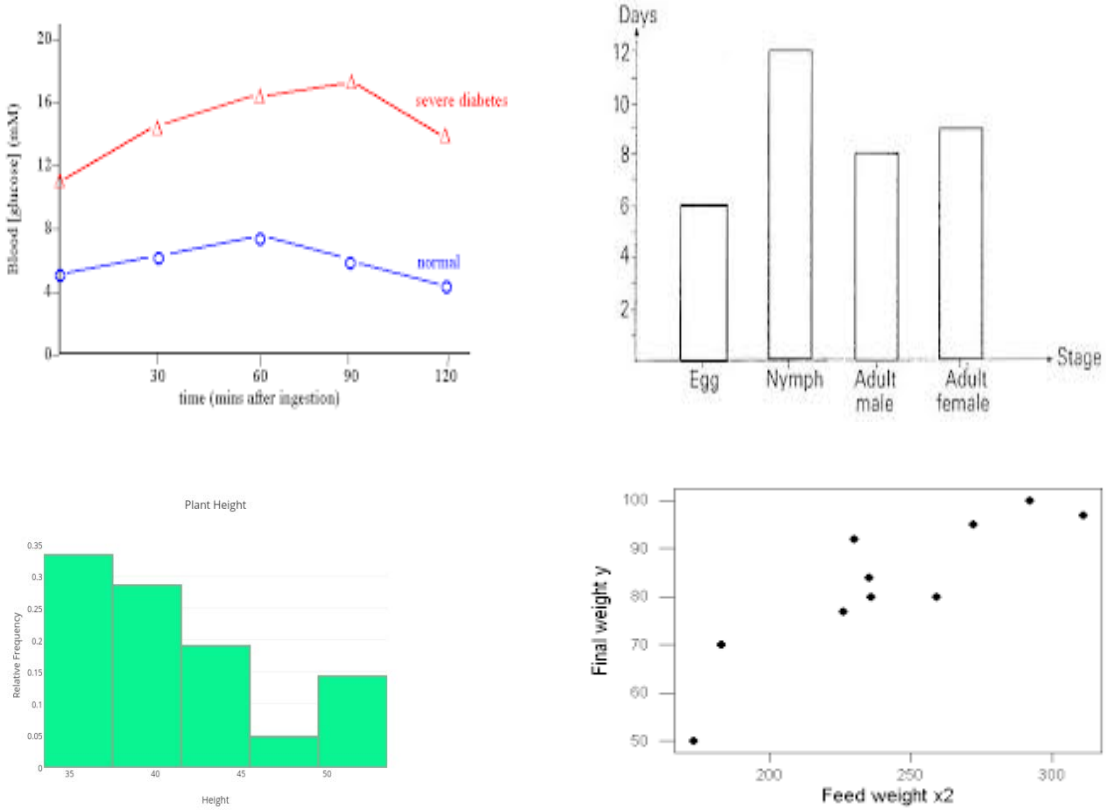


Figure 1: Line graphs, bar charts, histograms and scatter graphs

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 agarose (0.7%) gel

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

1. To prepare agarose (0.7%) gel

Introduction:

Agarose gel electrophoresis is an easy way to separate DNA fragments by their sizes and visualize them. It is a common diagnostic procedure used in molecular biology and recombinant DNA technology laboratories.

Electrophoresis:
The technique of electrophoresis is based on the fact that DNA is negatively charged at neutral pH due to its phosphate backbone. For this reason, when an electrical potential is placed on the DNA it will move toward the positive (Figure 1):

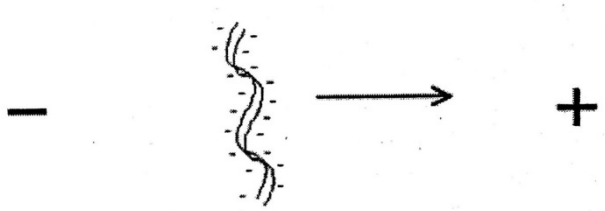


Figure 1

The rate at which the DNA will move toward the positive pole is slowed by making the DNA move through an agarose gel. The agarose forms a porous lattice in the buffer solution and the DNA must slip through the holes in the lattice in order to move toward the positive pole. This shows the molecular down. Larger molecules will be slowed down more than smaller molecules, since the smaller molecules can fit through the holes easier. As a result, a mixture of large and small fragments of DNA that has been run through an agarose gel will be separated by size. Figure 2 is a graphic representation of an agarose gel made by "running" DNA molecular weight markers, an isolated plasmid, and the same plasmid after linearization with a restriction enzyme:
These gels are visualized on a U.V. trans-illuminator by staining the DNA with a fluorescent dye (ethidium bromide). The DNA molecular weight marker is a set of DNA fragments of known molecular sizes that are used as a standard to determine the sizes of your unknown fragments.

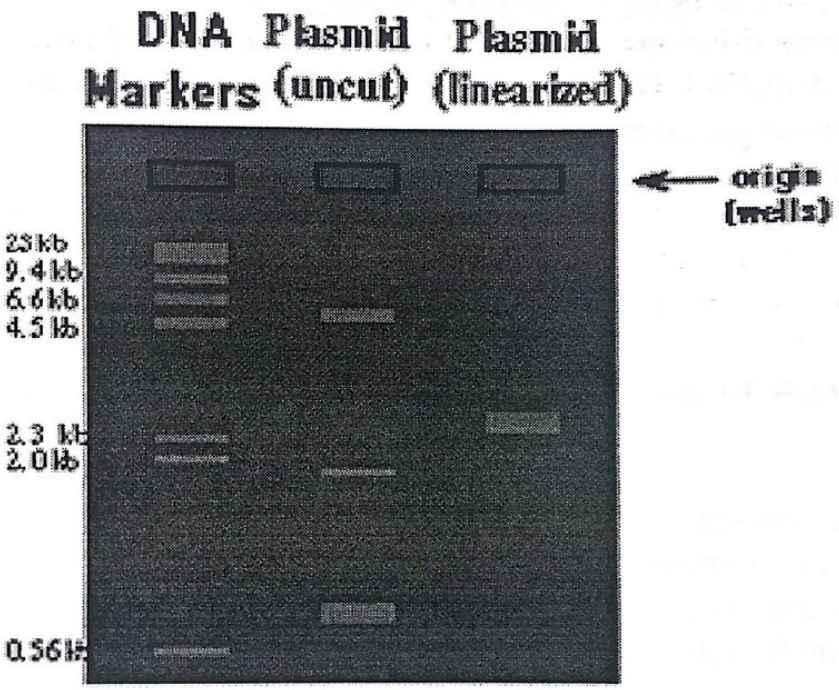


Figure 2

Materials:

- Electrophoresis caster
- Electrophoresis combs
- Gloves and Tissue
- 250-mL (or larger) conical flask
- Gel box
- Boiling water bath or microwave oven
- Tris, acetate and EDTA (TAE) or Tris, borate and EDTA (TBE)

Procedure:

- Prepare TAE* buffer (1x).
- Transfer 100ml of the buffer to a conical flask.
- Weigh 0.7 grams of agarose and add to the 100ml buffer solution.
- Keep in oven or waterbath.
- Take the solution from oven.
- Pour the solution to a gel caster.
- Place the comb.
- Incubate the cassette containing gel to solidify.
- Keep the solidified gel in gel box containing TAE buffer.

Differences encountered in real laboratory:

1. Make sure that the Agarose is fully dissolved in the buffer. If it is not dissolved well, again melt it some more time to dissolve completely.

2. Before casting the gel, the tray and comb should wipe with ethanol.

3. Labelings should be proper.

4. Before the incubation step, ensure that the water bath is set at the correct temperature that we required or not.

*Preparation of TAE buffer:

AE buffer is a solution made up of Tris base, acetic acid and EDTA (Tris-acetate-EDTA). It is historically the most common buffer used for agarose gel electrophoresis in the analyses of DNA products resulting from PCR amplification, DNA purification protocols or DNA cloning experiments.

This buffer has a low ionic strength and low buffering capacity. It is best suited to electrophoresis of large (>20 kb) pieces of DNA and will need to be replaced frequently or recirculated for longer (>4 h) gel run times. With that in mind, you may want to consider several batches of the buffer.

Given that the buffer is easy to make and the steps can be carried out quickly, making more than one batch at a time shouldn't be particularly time-consuming or difficult to pull off. Using the instructions below, it should take just 30 minutes to make the TAE buffer. Here's how:

Prepare a Stock Solution of EDTA

An EDTA (ethylenediamine tetraacetic acid) solution is prepared ahead of time. EDTA will not go completely into a solution until the pH is adjusted to about 8.0. For a 500-milliliter stock solution of 0.5 M EDTA, weigh out 93.05 grams of EDTA disodium salt (FW = 372.2). Dissolve in 400-milliliters deionized water and adjust the pH with sodium hydroxide (NaOH). Top up the solution to a final volume of 500 milliliters.

Whip Up a Stock Solution of TAE

Make a concentrated (50x) stock solution of TAE by weighing out 242 grams of Tris base (FW = 121.14) and dissolving it in approximately 750 milliliters of deionized water. Carefully add 57.1 milliliters of glacial acid and 100 milliliters of 0.5 M EDTA (pH 8.0). After that, adjust the solution to a final volume of 1 liter. This stock solution can be stored at room temperature. The pH of this buffer is not adjusted and should be about 8.5.

Prepare a Working Solution of TAE

The working solution of 1x TAE buffer is made by simply diluting the stock solution by 50x in deionized water. Final solute concentrations are 40 mM. Tris-acetate and 1 mM EDTA. The buffer is now ready for use in running an agarose gel.

Questions:

1. What is the principle of electrophoresis?
2. Why do you need to use a buffer while preparing agarose gel?
3. Circle the appropriate word below. DNA in an electrical field will move toward:

Positive electrode Negative electrode

4. How will you prepare agarose gel? Draw if needed.
5. Why do we wear gloves when doing science/gel preparation?
6. What are the applications of agarose gel electrophoresis?
7. Describe unique characteristics of agarose used in electrophoresis.

Practical 2
Title: Lambda DNA (dam- & dcm-) preparation

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

1. To instruct how to prepare Lambda DNA from heat inducible lysogenic *E. coli* strain (dam- & dcm-).

Introduction:
Lysogenic phages

In contrast to a lytic cycle, lysogenic infection is characterized by retention of the phage DNA molecule in the host bacterium, possibly for many thousands of cell divisions. With many lysogenic phages the phage DNA is inserted into the bacterial genome, in a manner similar to episomal insertion. The integrated form of the phage DNA (called the prophage) is quiescent, and a bacterium (referred to as a lysogen) that carries a prophage is usually physiologically indistinguishable from an uninfected cell. However, the prophage is eventually released from the host genome and the phage reverts to the lytic mode and lyses the cell. The infection cycle of lambda (λ), a typical lysogenic phage of this type, is shown in Figure 1.

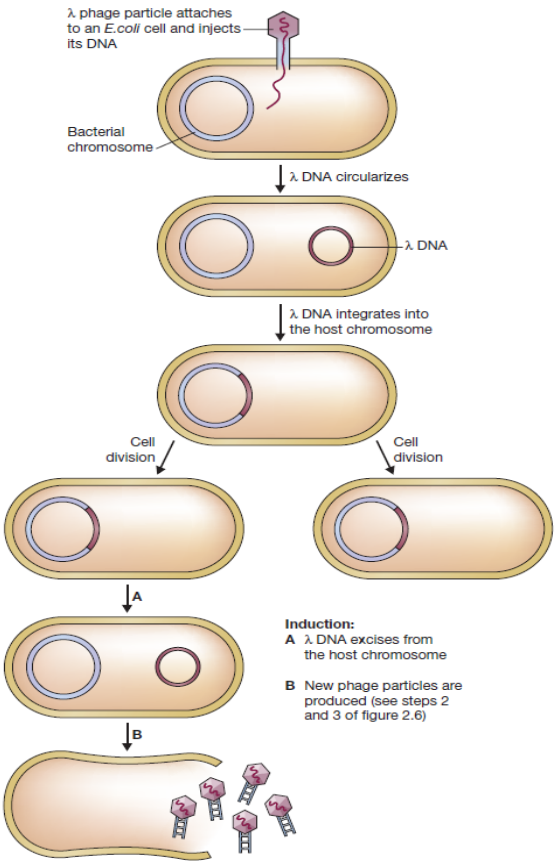


Figure 1: The lysogenic infection cycle of bacteriophage λ

Although there are many different varieties of bacteriophage, only λ and M13 have found a major role as cloning vectors.

Gene organization in the λ DNA molecule

λ is a typical example of a head-and-tail phage (Figure 2). The DNA is contained in the polyhedral head structure and the tail serves to attach the phage to the bacterial surface and to inject the DNA into the cell. The λ DNA molecule is 49 kb in size and has been intensively studied by the techniques of gene mapping, DNA sequencing and recombinant DNA technology. As a result the positions and identities of all of the genes in the λ DNA molecule are known. A feature of the λ genetic map is that genes related in terms of function are clustered together in the genome. For example, all of the genes coding for components of the capsid are grouped together in the left-hand third of the molecule, and genes controlling integration of the prophage into the host genome are clustered in the middle of the molecule. Clustering of related genes is profoundly important for controlling expression of the λ genome, as it allows genes to be switched on and off as a group rather than individually.

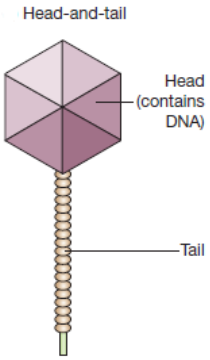


Figure 2: Head-and tail phase structure

The linear and circular forms of λ DNA

A second feature of λ that turns out to be of importance in the construction of cloning vectors is the conformation of the DNA molecule. The molecule shown in Figure 3 is linear, with two free ends, and represents the DNA present in the phage head structure. This linear molecule consists of two complementary strands of DNA, base-paired according to the Watson–Crick rules (that is, double-stranded DNA). However, at either end of the molecule is a short 12-nucleotide stretch in which the DNA is single-stranded. The two single strands are complementary, and so can base pair with one another to form a circular, completely double-stranded molecule.

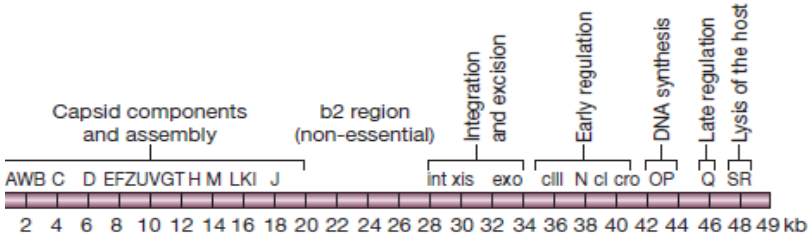


Figure 3: The λ genetic map, showing the positions of the important genes and the functions of the gene clusters

Complementary single strands are often referred to as “sticky” ends or cohesive ends, because base pairing between them can “stick” together the two ends of a DNA molecule (or the ends of two different DNA molecules). The λ cohesive ends are called the *cos* sites and they play two distinct roles during the e infection cycle. First, they allow the linear DNA molecule that is injected into the cell to be circularized, which is a necessary prerequisite for insertion into the bacterial genome.

The second role of the *cos* sites is rather different, and comes into play after the prophage has excised from the host genome. At this stage a large number of new λ DNA molecules are produced by the rolling circle mechanism of replication, in which a continuous DNA strand is “rolled off” the template molecule. The result is a catenane consisting of a series of linear λ genomes joined together at the *cos* sites. The role of the *cos* sites is now to act as recognition sequences for an endonuclease that cleaves the catenane at the cos sites, producing individual λ genomes. This endonuclease, which is the product

of gene *A* on the λ DNA molecule, creates the single stranded sticky ends, and also acts in conjunction with other proteins to package each λ genome into a phage head structure. The cleavage and packaging processes recognize just the *cos* sites and the DNA sequences to either side of them, so changing the structure of the internal regions of the λ genome, for example by inserting new genes, has effect on these events so long as the overall length of the λ genome is not altered too greatly.

Preparation of Lambda DNA from bacteriophage lambda (cI857*ind* 1 *Sam* 7) obtained from heat inducible lysogenic *E. coli* strain (dam- & dcm-)

In this experiment, we are using a ready stock of the lambda DNA that was isolated from bacteriophage lambda (cI857ind 1 Sam 7) and obtained from heat inducible lysogenic *E. coli* strain (dam- & dcm-). This product is supplied by Vivantis Technologies Sdn Bhd. The product number is NN1401 and the product supplies at the quantity of 500µg. The concentration is 0.5µg/µl. The product has been passed a quality control in which the purified DNA are assayed for contaminating exonucleases, non-specific nucleases and phosphatase.

There are a number of the Lambda DNA Purification Kit that facilitates the rapid purification of high grade lambda DNA eliminates the need for toxic phenol–chloroform extraction and time-consuming polyethylene glycol (PEG) precipitation steps used in traditional lambda miniprep procedures. In the below protocol, a Lambda DNA Purification Kit manual is shown in which uses diethylaminoethyl (DEAE) resin to remove contaminating polyanions prior to phage particle disruption. The phage is lysed with ethylenediaminetetraacetic acid (EDTA) and pronase and the lambda DNA is then selectively precipitated with the cationic detergent cetyltrimethylammonium bromide (CTAB). Following an exchange reaction with sodium chloride, the highly purified lambda DNA is precipitated with ethanol. The Lambda DNA Purification Kit can be used for either liquid or plate lysates.

Procedure:

Preparation of Plating Cultures

1. Inoculate 50 ml of LB broth containing 0.2% (v/v) maltose and 10 mM MgSO4 in a sterile flask with a single colony of XL1-Blue cells or another appropriate bacterial host strain.

Note: DO NOT add antibiotic to the overnight culture or to titering plates.

2. Grow the culture overnight with shaking at 30°C. This temperature ensures that the cells will not overgrow. Phage can adhere to nonviable cells resulting in a decreased titer.

3. Spin down the cells in a sterile conical tube for 10 minutes at 2000 rpm.

4. Carefully decant the medium off the cell pellet and gently resuspend the pellet in ~15 ml of 10 mM MgSO4 (do not vortex).

5. Dilute the cells to an OD600 of 0.5 with 10 mM MgSO4.

6. The cells may be stored for 2–3 days at 4°C.

7. Proceed to step 1 of either Preparation of Plate Lysate or Preparation of Liquid Lysate.

Preparation of Plate Lysate

1. Mix 200 µl of the prepared host cells with at least 5000 pfu of lambda phage stock (100 µl) in a Falcon® 2059 tube.

2. Incubate the tube at 37°C for 20 minutes.

3. Add 3 ml of 48°C NZY top agar and spread on a 100-mm NZY agar plate. Allow the NZY top agar to harden at room temperature for 10 minutes.

4. Incubate the plate overnight at 37°C.

5. Overlay the plate with 3 ml of SM buffer.

6. Incubate the plate at room temperature for 3 hours or at 4°C overnight.

7. Remove and transfer the SM buffer (now containing lambda phage) to a fresh Falcon 2059 tube.

8. Add 1/50 volume of chloroform and vortex the tube.

9. Incubate the tube at room temperature for 10 minutes.

10. Titer to ensure >5 × 1010 pfu/ml.

11. Proceed to Purification of Lambda DNA.

Preparation of Liquid Lysate

1. Mix 100 µl of the prepared host cells with 1 × 106 pfu of lambda phage stock (100 µl) in a Falcon 2059 tube.

2. Incubate the mixture at 37°C for 20 minutes.

3. Add 4 ml of LB broth to the tube.

4. Incubate the tube at 37°C with vigorous shaking for 5 hours until lysis occurs.

5. Add two drops of chloroform.

6. Incubate the tube at room temperature for 15 minutes with shaking.

7. Centrifuge the tube at 14,000 rpm for 10 minutes.

8. Remove and transfer the supernatant to a fresh Falcon 2059 tube.

9. Titer to ensure >1 × 1010 pfu/ml.

10. Proceed to Purification of Lambda DNA.

Purification of Lambda DNA

1. Remove any residual cell debris by centrifugation at 14,000 rpm for 10 minutes.

2. Place the supernatant in a fresh microcentrifuge tube if necessary (1 ml/tube).

3. Add 1 µl of DNase I (20 mg/ml) to the tube to a final concentration of 20 µg/ml.

Note: Add 4 µl of RNase A (2 mg/ml) to the tube to a final concentration of 8 µg/ml. When using phage prepared from plate lysate, add RNase during step 12 of this protocol.

4. Incubate the tube at room temperature for 15 minutes.

5. Microcentrifuge the tube at 14,000 rpm for 5 minutes at room temperature.

6. Transfer ~1 ml of the supernatant to a fresh tube. DO NOT CARRY OVER ANY PRECIPITATE.

7. Vigorously shake the tube containing the DEAE cellulose slurry to mix and then pipet 500 µl (~ 1/2 volume) of the DEAE slurry into the tube.

8. Incubate the tube at room temperature for 10 minutes, mixing the contents of the tube every 2 minutes by hand.

9. Microcentrifuge the tube for 1 minute at room temperature.

10. Place the supernatant in a fresh tube.

Note: Resin will inhibit the modifying enzyme activity. Spin the tube again if the resin carries over.

11. Add 40 µl of 0.5 M EDTA to the tube to a final concentration of 20 mM EDTA.

12. Add 15.4 µl of pronase stock solution (50 mg/ml) to the tube.

Note: Add RNase at this step if using phage prepared from plate lysate.

13. Incubate the tube at 37°C for 15 minutes.

14. Add 30 µl of 5% CTAB stock solution to the tube to a final concentration of 0.1% (v/v).

Note: If CTAB precipitates out before use, place the CTAB at 37–65°C to drive the CTAB back into solution.

15. Incubate the tube at 65°C for 3 minutes.

16. Add carrier glycogen to the tube to a final concentration of 20 µg/ml if phage titer is 1×10^{10} or lower to help precipitate the lambda DNA.
(Add 1 µl of glycogen supplied with the kit per ml of reaction, ~1.5 µl.)

17. Incubate the samples on ice for 5 minutes to cool.

18. Microcentrifuge the samples at 14,000 rpm for 10 minutes.

19. Remove and discard the supernatant.

20. Gently resuspend the pellet in 200 µl of 1.2 M NaCl (1/5 volume from step 2 above).DO NOT VORTEX.

Note: If the pellet does not dissolve in 10 minutes at room temperature or at 37°C, spin the sample in a microcentrifuge and transfer the supernatant, which contains the DNA, to a fresh microcentrifuge tube and continue with the next step.

21. Add 500 µl of 100% (v/v) ethanol (2.5 volumes) and invert the tube to mix.

22. Microcentrifuge at 14,000 rpm for 10 minutes at room temperature. Remove and discard the supernatant.

23. Wash the pellet with 70% (v/v) ethanol. Repeat wash once more.

24. Dry the pellet and resuspend the lyophilized pellet in TE buffer.

Note: The pellet will be spread on the side of the tube.

Expected Yields

50% of the theoretical yield

1 × 1010 pfu = 0.25 µg lambda DNA from the liquid lysate method
5 × 1010 pfu = 1 µg lambda DNA from the plate lysate method
1 × 1011 pfu = 2 µg lambda DNA from the plate lysate method

TROUBLESHOOTING

Observation	Suggestion
Precipitation of the CTAB	CTAB will precipitate when stored at 4°C or below. Warm the CTAB before use.

PREPARATION OF MEDIA AND REAGENTS

DEAE-Cellulose Suspension Buffer
100 mM NaCl
10 mM Tris-HCl (pH 7.5)
50 mM $MgSO_4$

LB Broth (per Liter)
10 g of NaCl
10 g of bacto-tryptone
5 g of bacto-yeast extract
Adjust to pH 7.0 with 5 N NaOH
Add deionized H_2O to a final volume of 1 liter
Autoclave

SM Buffer (per Liter)
5.8 g of NaCl
2.0 g of $MgSO_4 \cdot 7H_2O$
50.0 ml of 1 M Tris-HCl (pH 7.5)
5.0 ml of 2% (w/v) gelatin
Add H_2O to a final volume of 1 liter
Autoclave

TE Buffer
10 mM Tris-HCl (pH 7.5)
1 mM EDTA

NZY Broth (per Liter)
5 g of NaCl
2 g of $MgSO_4 \cdot 7H_2O$
5 g of yeast extract
10 g of NZ amine (casein hydrolysate)
Adjust the pH to 7.5 with NaOH

NZY Top Agar (per Liter)
1 liter of NZY broth
Add 0.7% (w/v) agarose

NZY Agar (per Liter)
5 g of NaCl
2 g of $MgSO_4 \cdot 7H_2O$
5 g of yeast extract
10 g of NZ amine (casein hydrolysate)
15 g of agar
Adjust the pH to 7.5 with NaOH
Autoclave
Pour into petri dishes (~80 ml/150-mm plate)

Questions:

1. Describe how the genes organized in the λ DNA molecule?
2. How Lambda DNA is used for gene cloning and recombinant DNA technology? Explain.
3. With a proper diagram show the different steps of Lambda DNA isolation.

Practical 3

Title: Restriction endonuclease digestion (cleavage) of Lambda DNA with *Bam*HI, *Eco*RI and *Hin*d III

Objective:

After completing the practical, you will be able:

1. To investigate the efficiency and outcome of cutting single-digested lambda-DNA with the restriction enzymes *Bam*HI, *Eco*RI and *Hin*d III.

Introduction:

Classification: Restriction enzymes, or restriction endonucleases, are proteins that recognize and cleave specific sequences of double stranded DNA. Most, but not all of the 3000 discovered restriction enzymes found so far, come from bacteria, where they serve as a protection system, defending bacteria from foreign DNA. Restriction enzymes are divided into three major groups based on different preferences associated with recognition/cutting-sites and environmental requirements, e.g. certain temperature, ionic strength and pH. Type I and III restriction enzymes recognise certain DNA sequences and cut some distance away, sometimes as far as 10 000 base pairs away, whereas Type II restriction enzymes cut sequences located at a close proximity to the recognition site. Most restriction enzymes recognise sequences that are relatively short, often 4-8 base pairs in length. Furthermore, Type II enzymes, which comprise the most abundant group of enzymes, often recognise palindrome sequences and cut within or adjacent to these sequences.

Applications: The discovery of restriction enzymes in the late 1960s has had an enormous impact on molecular biology research. Cutting large DNA-molecules with restriction enzymes makes it possible to purify homogenous DNA-fragments of defined lengths that can be subsequently enzymatically manipulated and analysed. Cut DNA-fragments can easily be ligated into DNA-molecules with corresponding ends, thereby making recombinant DNA-molecules. Due to the exact specificity of restriction enzymes, and the specific cleavage patterns generated when cut DNA is run on gels, restriction enzymes also enables for mapping of DNA. A common application of this is restriction fragment length polymorphism (RFLP), used for e.g. paternity testing. In RFLP, specific human genomic DNA-areas are cut by several restriction enzymes, and the fragments are subjected to electrophoresis. The generated fragment pattern is unique for a given individual, but shares certain similarities with patterns generated by related individuals. Moreover, restriction enzymes can also be used to detect specific variations/mutations in DNA caused by a single nucleotide change, so called single nucleotide polymorphisms (SNPs). The SNP may generate a new cutting site, or it may result in the loss of a cutting site. Therefore, by cutting DNA with restriction enzymes directed at the site of a possible mutation and thereafter subjecting the fragments to electrophoresis, the gain or loss of a band tells whether a SNP is present or not.

Working conditions: Finding the optimal operating condition for restriction enzymes can be a hassle. pH and salt-concentration is very important, which is why companies selling restriction enzymes frequently also provide the appropriate buffer compatible with the enzyme. Some enzymes are very sensitive to certain ions, such as potassium or sodium, whereas others work in a wide range of ionic strengths. The divalent cat ion Mg^{2+} is required by most restriction enzymes. The correct working temperature is also important, simply because the bacteria that originally house the restriction enzyme have different temperature ranges within which they operate optimally. Many bacteria have operation optima around body temperature (37°C), but some prefer extreme heat or cool temperatures.

Materials:

- Restriction endonucleases *Bam*HI, *Eco*RI and *Hin*d III
- Lambda DNA
- Sterile distilled water
- 10X reaction buffer
- Buffer 1X UB
- Viva Buffer A
- Bovine Serum Albumin (BSA)
- PCR machine
- Mini-centrifuge
- 1.5 ml tubes
- PCR tubes
- Tips
- Gloves
- Spray ethanol

Procedure:

- Prepare a restriction mixture as below:

Restriction enzyme	: 1 unit
Lambda 0.3µg/µl	: 3.33µl (1µg DNA)
10X Reaction Buffer	: 5µl
Sterile Distilled Water	: Up to 50µl

Restriction enzymes:

*Bam*HI

Concentration: 20u/µl
Supplied with : 1ml of 10X Buffer UB
0.5ml Diluent Viva Buffer A
(BSA included in all Reaction Buffer)
Storage : -20°C

Reaction Conditions:
Buffer 1X UB,

25mM Tris-acetate (pH 7.6 at 30°C), 10mM Mg-acetate, 100mM K-acetate, 7mM 2-Mercaptoethanol and 50µg/ml BSA.
Incubate at 37°C.

Dilution: Viva Buffer A

10mM Tris-HCl (pH 7.4 at 25°C), 50mM KCl, 0.1mM EDTA,
1mM DTT, 200µg/ml BSA and 50% glycerol.

Thermal Inactivation: 65°C for 20 minutes

Storage Buffer:
10mM Tris-HCl (pH 7.4), 50mM NaCl, 0.1mM EDTA, 1mM DTT, 0.15% Triton X-100, 200µg/µl BSA and 50% glycerol.

Unit Definition:
1u is defined as the amount of enzyme that is required to digest 1µg of DNA in 1 hour at 37°C in 50µl of assay buffer.

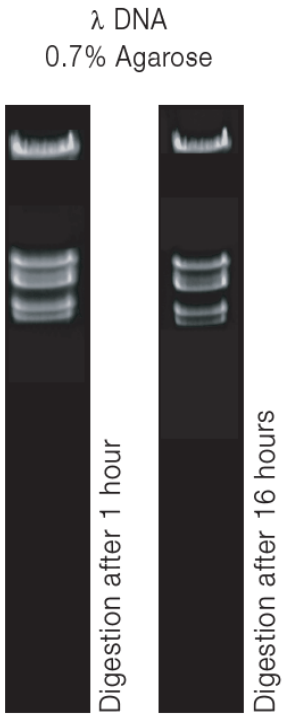
Quality Control Assays:

Ligation/ Recutting Assay:
After 20-fold overdigestion with *Bam*H I, more than 90% of the DNA fragments can be ligated and recut.

Overdigestion assay:
An unaltered banding pattern was observed after 1µg of DNA was digested with 40u of *Bam*HI for 16 hours at 37°C.

Activity in Reaction Buffer				
V1	V2	V3	V4	V5
75%	75%	50%	75%	50%

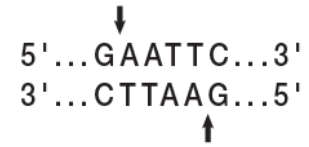
Buffer UB			
0.5X	1.0X	1.5X	2.0X
75%	100%	25%	100%



Note:

- High enzyme concentration may result in *Star Activity*.
- Total reaction volume dependent on experiment.
- The amount of enzyme to be used is very much dependent on the DNA template.
- For plasmid DNA, 5-10X more enzyme is required.

*Eco*RI



Concentration: 20u/µl
Supplied with : 1ml of 10X Buffer EcoR I
1ml of 10X Buffer UB
0.5ml Diluent Viva Buffer A
(BSA included in all Reaction Buffer)
Storage : -20°C

Reaction Conditions:
Buffer *Eco*R I,

50mM Tris-HCl (pH 7.5 at 30°C), 10mM MgCl2, 100mM NaCl, 0.02% triton X-100, and 0.1mg/ml BSA. Incubate at 37°C.

Dilution: Viva Buffer A

10mM Tris-HCl (pH 7.4 at 25°C), 50mM KCl, 0.1mM EDTA,
1mM DTT, 200µg/ml BSA and 50% glycerol.

Thermal Inactivation: 65°C for 20 minutes

Storage Buffer:
10mM Tris-HCl (pH 7.5), 200mM NaCl, 0.1mM EDTA,
7mM 2-mercaptoethanol, 200µg/ml BSA and 50% glycerol.

Unit Definition:
1u is defined as the amount of enzyme that is required to digest 1µg of DNA in 1 hour at 37°C in 50µl of assay buffer.

Quality Control Assays:

Ligation/ Recutting Assay:
After 20-fold overdigestion with *Eco*R I, more than 95% of the DNA fragments can be ligated and recut.

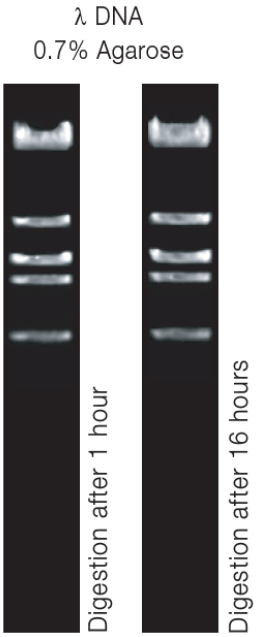
Overdigestion assay:
An unaltered banding pattern was observed after 1µg of DNA was digested with 40u of *Eco*R I or 16 hours at 37°C.

Note:

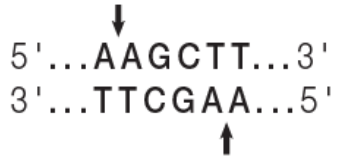
- High enzyme concentration may result in *Star Activity*
- Overdigestion in Buffer V3 and V4 will cause *Star Activity.*
- Total reaction volume dependent on experiment.
- The amount of enzyme to be used is very much dependent on the DNA template.
- For plasmid DNA, 5-10X more enzyme is required.

Activity in Reaction Buffer				
V1	V2	V3	V4	V5
50%	50%	100%	100%	50%

Buffer UB			
0.5X	1.0X	1.5X	2.0X
50%	50%	75%	75%



*Hin*d III



Concentration: 20u/µl
Supplied with : 1ml of 10X Buffer V2
1ml of 10X Buffer UB
0.5ml Diluent Viva Buffer A
(BSA included in all Reaction Buffer)
Storage : -20°C

Reaction Conditions:
Buffer V2,

10mM Tris-HCl (pH 7.5 at 30°C), 10mM MgCl2, 50mM NaCl, and 100µg/ml BSA.
Incubate at 37°C.
Dilution: Viva Buffer A

10mM Tris-HCl (pH 7.4 at 25°C), 50mM KCl, 0.1mM EDTA, 1mM DTT, 200µg/ml BSA and 50% glycerol.

Thermal Inactivation: 80°C for 20 minutes
Storage Buffer:
10mM Tris-HCl (pH 7.5), 300mM NaCl, 0.1mM EDTA, 7mM 2-mercaptoethanol, 200µg/ml BSA and 50% glycerol.

Unit Definition:
1u is defined as the amount of enzyme that is required to digest 1µg of DNA in 1 hour at 37°C in 50µl of assay buffer.

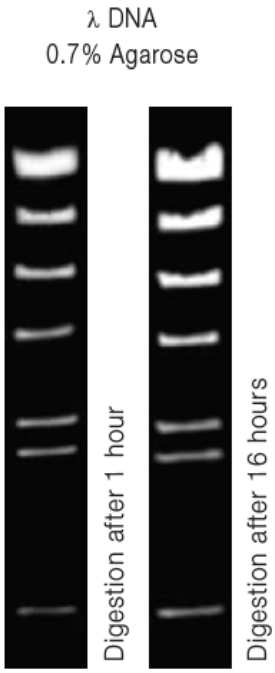
Quality Control Assays:

Ligation/ Recutting Assay:
After 20-fold overdigestion with *Hin*d III, more than 90% of the DNA fragments can be ligated and recut.

Overdigestion assay:
An unaltered banding pattern was observed after 1µg of DNA was digested with 40u of *Hin*d III for 16 hours at 37°C.

Activity in Reaction Buffer				
V1	V2	V3	V4	V5
75%	100%	75%	75%	75%

Buffer UB			
0.5X	1.0X	1.5X	2.0X
75%	75%	75%	50%



Note:

- Total reaction volume dependent on experiment.
- The amount of enzyme to be used is very much dependent on the DNA template.
- For plasmid DNA, 5-10X more enzyme is required.

Questions:

1. What are restriction enzymes?
2. Why restriction enzymes are are necessary before DNA can be electrophoresed?
3. Where do restriction enzymes come from?
4. Scientists have isolated over 100 different restriction enzymes, with names such as *Eco*RI, *Hin*dIII, *Bam*HI, *Sa*lI, *Xho*I, etc. How do restriction enzymes differ from one other?

Practical 4

Title: Agarose gel electrophoresis of digested Lambda DNA fragments

Objectives:

After completing the practical, you will be able:

1. To perform gel electrophoresis by preparation of a mixture of RE-digested DNA samples with loading buffer
2. To determine the size of the products from your RE digests

Introduction:

Gel electrophoresis is the most widely used method in molecular biology for separating macromolecules from one another on the basis of size. It is especially useful for analyzing mixtures of proteins or of nucleic acids with respect to the presence and relative abundance of molecules of different sizes, and for estimating the size of purified macromolecules; it can also be used as a step in purification of a specific protein or a specific length-class of DNA. Electrophoresis gels for nucleic acids are most commonly prepared with agarose or with polyacrylamide. In this laboratory, agarose gel electrophoresis will be used to separate DNA molecules that differ in number of nucleotides and, therefore, in length (size). With a few changes in details (gel preparation, buffers used, stains applied, etc.), the same overall approach would used be with a protein solution.

The fundamental principle of gel electrophoresis is that charged macromolecules will migrate through a gel when an electrical field is applied across that gel. The distance migrated by a DNA molecule during the time it is subjected to the electrical field is determined by three factors: (1) the size (length) of the molecule, (2) the electrical field (dimensions and voltage differential), and (3) the density of the gel matrix. When a sample containing DNA molecules of different sizes is applied as a mixture to the same position in the gel, all of the molecules are subjected to the same voltage differential, and all of the molecules are challenged to work their way through the same gel matrix. This leaves molecular size as the only factor that will determine the final relative positions of the DNA molecules present in the original mixture.

In order to perform gel electrophoresis, you will mix your RE-digested DNA samples with “loading buffer”. Loading buffer contains two important components: (1) a dye called Nucleic Acid Dye, which binds to DNA and fluoresces when exposed to short-wavelength light (~400 nm), (2) a 6X Loading Dye (with xylene cyanol gel loading dye) which allows you to view the progression of your sample as it migrates down the gel.

To determine the size of the products from your RE digests, you will load a solution of DNA of known sizes (your “markers”) onto your agarose gel along with your restriction enzyme digested samples. After the samples have been loaded into the gel, the gel will be covered with TBE/TAE buffer at pH 8. Then, the electrophoresis apparatus will be closed, electrical leads will be attached, and sufficient current will be applied to maintain a voltage differential of approximately 110 V across the gel. Electrophoresis will continue until the bromophenol blue indicator dye approaches the positive end of the gel (~ 1 hour). After the gel has been run, your instructor will disconnect the electrical leads and expose the gel to light of the appropriate wavelength to cause fluorescence of the Nucleic Acid Dye. A picture of this gel can then be taken, and the location of the fluorescent bands of the DNA markers can be measured. You can then use these measurements to estimate their length of your RE digestion products (in base pairs) by comparing the distance migrated by your sample to the distance migrated by the markers.

Materials:

- Gel electrophoresis set
- 6X Loading Dye (with xylene cyanol gel loading dye)
- Digested restriction lambda DNA products from practical 3
- Pipetter
- Tips
- Tubes
- Gloves
- TAE buffer
- DNA ladder
- Gel documentation system

Procedure 1:

1. Using a micropipetter, add 5 µl loading buffer into your RE digestion tube. Mix the contents by gently lifting the mixture into the pipet tip and then expelling the mixture back into the tube – only once.

2. Set your micropipetter to 15 µl. Place a fresh tip on the shaft, and load 15 µl from your RE digest + loading buffer mixture tube into your assigned lane on a 0.7 - 1.2% agarose gel. Each group member should write down which sample was loaded into which lane.

3. To determine the sizes of the DNA fragments generated from our RE digestions, we will compare their distance of migration with the distance of migration of DNA fragments of known sizes "DNA markers - ladders". The instructor will load 6 µl of DNA markers onto one lane of each gel.

4. After all wells are loaded, the instructor will close the apparatus, attach the electrical leads to the power supply, and switch on the current. The power supply is regulated to deliver sufficient current to sustain a voltage differential of approximately 110 V across the gel, from the negative pole to the positive pole. Watch for tiny bubbles to rise from the electrodes, indicating that current is passing through your buffer/gel system. After about one hour, the indicator dye (bromophenol blue) should be approaching the positive pole end of the gel, and the current will be switched off and the electrical leads disconnected.

5. Your instructor will demonstrate for you the removal of a gel from the electrophoresis apparatus and placement on the visualization light box. Wearing latex gloves, she will lift the gel tray from the apparatus with the tray slanting downward at one end, where it will be supported by the instructor's gloved fingers. This tray, with the gel inside, will be placed on top of a light source to illuminate the Nucleic Acid Dye. Note: When removing gels from the electrophoresis apparatus, if the tray is not slanted toward the end that is blocked by the instructor's fingers, the gel is quite likely to slip out the other end of the tray onto the bench or the floor of the laboratory. This is invariably disappointing.

Procedure 2:

Gel Electrophoresis Analysis

1. When electrophoresis is finished, inform your instructor who will help you bring your gel (in the tray) over to the gel documentation system, and set it up for picture-taking. Your instructor will take a picture of your gel as it is exposed to short-wavelength light. Each group member will receive a picture of the gel. Because of the fluorescence of the Nucleic Acid Dye, DNA bands will appear bright white and the gel will appear dark.

2. Take your picture back to your bench and find the bottom of the wells on the gel. Measure the distance from the bottom of the wells to each bright DNA band. Record these distances (in mm) in Data Tables provided on the following pages of this handout.

Use the following data tables to collect your raw data during lab. Use this information to help you in writing your lab report. Do not attach this version of the table to your lab report – to use this table in your report, reconstruct it for your report. Remember that all tables and figures in your lab report need titles and captions.

Data Table for Standard DNA (Markers) Agarose Gel

Base pairs/molecule	Distance from well to band, in mm
10,000	
8,000	
6,000	
5,000	
4,000	
3,000	
2,500	
2,000	
1,500	
1,000	
750	
500	
250	

The solution of “Markers” that you loaded into agarose gel contained DNA molecules that varied in size from 250 to 10,000 bp (base pairs). There should be 13 bands in your Marker lane (although not all may be visible).

Assignment: construct a standard curve for your gel:

*This will be an important figure in your lab report.
*Remember to write a title and caption for this figure.
To construct a standard curve:

a) Measure, in mm (estimate to 0.1 mm), the distance migrated by each of the bands in the markers lane. Measure from the bottom of the well at the beginning of the lane to the leading edge of each band. Record the distances in the Data Table for your agarose gel.
b) For each band, plot the distance on the X-axis and the molecular size on the Y-axis. Semilogarithmic graph paper will be provided so that as the molecular size of each band is plotted, the points will appear on the paper in positions that represent their logarithms; it is not necessary to look up the logarithm of each molecular size.
c) Draw a line of best fit through all the points that together clearly generate a straight line. Do not include the points for 10,000, 8,000, or 6,000bp on your graph.

Data Table for Restriction Mapping of Plasmids A and B

Template	Enzyme	How many fragments?	Distance (mm) each fragment migrated	Estimated size of Fragment size (bp)
A	*Bam*HI			
B	*Eco*RI			
C	*Hin*d III			

Use your standard curve graph to estimate the size of molecules in any other band on the same gel:

i. Measure the distance the band has migrated (in mm).

ii. Find its distance on the X-axis, follow a vertical line upward until it intersects with the line of best fit, then follow a horizontal line toward the left to its intersection with the Y-axis. The value at which this line intersects the Y-axis is the size of the DNA molecules in the band.

iii. Examine the lanes that were loaded with each of the digests, measure the distance (in mm) migrated by each of the discernible bands, and estimate the sizes of the molecules in each band (in bp) from your standard curve. Record these values in the data table above. If you wish to use this table for your lab report, do not attach this one – instead, recreate this table (or a similar type of table) for your report. Don't forget to write a title and caption for this table in your report.

iv. Keep your gel picture and attach it to your lab report, remembering to write a title and caption for this figure.

Notes

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