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Practical Note
Molecular Biology
(BCH 361)

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1. INTRODUCTION TO MOLECULAR BIOLOGY LABORATORY

"Molecular Biology" is the study of biology at the molecular level and is concerned with the understanding of the genetic material. The interactions between the nucleic acids (DNA and RNA) and synthesis of proteins, and how these processes (replication, transcription, translation) are regulated, form the basis of molecular biology. Since the late 1950s and early 1960s, Molecular Biologists have learned to characterize, isolate, and manipulate these molecular components. The techniques used for these studies are referred to as "Techniques of Molecular Biology".

The first step is to isolate DNA or RNA, for these techniques to be carried out. The DNA or RNA can be obtained either from the cells (e.g. plasmid DNA, genomic DNA, mRNA) or can be prepared [complimentary DNA (cDNA)]. Various methods are used for characterization and manipulation of the isolated DNA or RNA. These include:

i) Purification of DNA or RNA (by electroelution).

ii) Determining the purity of nucleic acids by spectrophotometer method.

iii) Determining the DNA composition by measuring its melting temperature (Tm). To calculate the GC content of the sample DNA.

iv) Digesting DNA samples with different restriction enzymes

v) Determining the size of the DNA and RNA fragments by running on agarose gel electrophoresis.

vi) Amplifying the DNA fragments of interest (by polymerase chain reaction; or by cloning)

In addition, a number of techniques are available for analyzing DNA. These include various mutation detection methods, such as amplification, denaturing gradient gel electrophoresis (DGGE), single stranded conformation polymorphism (SSCP), dot blot analysis, amplification mutation refractory system (ARMS), and reverse dot blot, chemical cleavage mismatch, Southern blotting, DNA sequencing and others. In this course some of the techniques mentioned above will be carried out.

The students should be thoroughly aware of the following lab safety methods in molecular biology and understand the steps to be adopted for conducting successful molecular biology experiment.

1.1. General Lab Safety in Molecular Biology

In molecular biology lab a number of chemicals are used that are hazardous and can cause severe burn and long term sickness requiring immediate medical attention. Hence, before conducting an experiment it is essential to know the safety precautions and risk associated with handling the chemical compounds. The following chemicals are especially noteworthy:

- **Phenol:** cause severe burns.

- **Acryl amide:** potential neurotoxin.
- **Ethidium bromide**: a strong carcinogen

In order to assure the safe handling of the chemicals, always follow the following safety precautions:

1. Wear gloves while handling hazardous chemicals
2. Never mouth pipettes any chemicals
3. Always use fresh tips or pipette for each solution and samples to avoid contamination of the samples and the solutions.
4. If any chemical is accidently spilt on the skin, immediately rinse with a lot of water and inform the instructor.
5. Always discard the waste in appropriate waste disposal as instructed by the instructor.

1.1.1 Ultra Violet Light

UV lamp will be used to visualize the DNA bands on the gel following electrophoresis. Direct exposure to UV light can cause acute eye irritation and skin allergy. Since retina cannot detect UV light, serious eye damage may be caused if exposed to UV, therefore **always wear safety goggles or eye protection when using UV lamps.**

1.1.2 Electricity

The voltage used for electrophoresis is sufficient to cause electrocution. Cover the buffer reservoir during electrophoresis and always switch off the power supply and unplug the lead before removing the gel from electrophoresis unit.

1.2. General Tips for Conducting a safe and successful experiment.

a) Always keep the work area clean of any unwanted tubes, beakers and dirty dishes.

b) All reagents should be marked clearly with reagent name and concentration.

c) All sample3s should be numbered and labeled correctly with the names and dates.

d) Make sure that after use the reagents and chemical are placed in the fridge or freezer as required.

e) In bacterial cultures make sure the reagents and dishes are autoclaved properly and label using autoclave taps.

f) Always mark the bottom of the bacterial culture dishes and not the lid, as the lids can easily be mixed up.

1.3. Preparation of Solutions

1.3.1. Calculations to prepare molar, % and X solutions

A Molar Solution is one in which 1 liter of solution contains the number of grams equal to its molecular weight e.g. to make up 100 ml of a 5M NaCl solution = 58.456 (mw of NaCl) g/mol x 5 moles/liter x 0.1 liter = 29.29 g in 100 ml of solution.

Percent Solution: w/v weight (gms) in 100ml. e.g. to make a 0.7% solution of agarose in TBE buffer, weigh 0.7 of agarose and bring up volume to 100 ml with TBE buffer. Percentage v/v is = volume (ml) in 100ml.

X Solution: Many enzyme buffers are prepared **as** concentrated solutions. e.g. 5X, 10X (five or ten times the concentrated of the working solution). These concentrated solutions are then diluted accordingly to give the final concen-tration of 1X of working buffer. e.g. to set up a restriction digestion in 25 µl of IX buffer, add 2.5 µl of a 10X buffer, the other reaction components, and water to a final volume of 25 µl.

1.3.2. Preparation of Working Solution from the Concentrated Solution

Many buffers in molecular biology require same concentration but at different times. To avoid having to make every buffer from scratch, it is useful to prepare several concentrated stock solutions and dilute as needed. e.g. to prepare 100 ml of TE buffer (10 mM Tris, 1 mM EDTA), combine 1 ml of a 1 M Tris solution and 0.2 ml of 0.5 M EDTA and 98.8 ml sterile water. The following is useful for calculating amounts of stock solution needed:

ci x vi = cf x vf , where ci = initial concentration, or conc of stock solution; vi = initial vol, or amount of stock solution needed cf = final concentration, or conc of desired solution; vf = final vol, or volume of desired solution.

1.3.3. Tips for making solutions for molecular biology

a) Careful handling and measurement of chemicals is crucial for a successful experiment in molecular biology. Following tips will help to make a solution:

b) Weigh out correctly the desired amount of chemicals. When using amount less than 0.1g use analytical balance.

c) Prepare all solution with double distilled water.

d) Autoclave all the solution for bacterial culture and where ever necessary

e) Some solutions cannot be autoclaved, for example, SDS. These should be filter sterilized through a 0.22 μm or 0.45 μm filters.

f) Media for the bacterial cultures should be autoclaved the same day prepared. Never prepare bacterial culture one or two days in advance of autoclaving.

g) Solid media for bacterial cultures can be made in advance, autoclaved and stored in a bottle. When needed the microwave can be melted and additional substance like antibiotics can be added and poured onto the culture plate.

h) Make sure the lid of the bottle of bacterial culture, is loose while placing into the microwave oven as the tight lid bottle can explode in the microwave while heating.

1.4. Glassware and Plastic Ware:

Glass and plastic ware used for molecular biology must be scrupulously clean. Dirty test tubes, bacterial contamination and traces of detergent can inhibit reactions or degrade nucleic acid.

Glassware should be rinsed with distilled water and autoclaved or baked at 150^0C for 1 hour. For experiments with RNA, glassware and solutions should be treated with diethylpyrocarbonate to inhibit RNases which can be resistant to autoclaving. Plastic ware such as pipettes and culture tubes are often supplied sterile. Tubes made of polypropylene are turbid and are resistant to many chemicals, like phenol and chloroform; polycarbonate or polystyrene tubes are clear and not resistant to many chemicals. Make sure that the tubes in use are resistant to the chemicals used in the experiment. Micropipette tips and microfuge tubes should be autoclaved before use.

2. PREPARATION OF GENOMIC DNA FROM BLOOD

2.1 Introduction

There are different protocols and several commercially available kits that can be used for the extraction of DNA from whole blood. This procedure is one routinely used both in research and clinical service provision and is cheap and robust. It can also be applied to cell pellets from dispersed tissues or cell cultures (omitting the red blood lysis step).

2.2 Theory

Successful nucleic acid isolation protocols have been published for nearly all biological materials. They involve the physical and chemical processes of tissue homogenisation (to increase the number of cells or the surface area available for lysis), cell permeabilisation, cell lysis (using hypotonic buffers), removal of nucleases, protein degradation, protein precipitation, solubilisation of nucleic acids and finally various washing steps. Cell permeabilisation may be achieved with the help of non-ionic (non DNA-binding) detergents such as SDS and Triton.

2.3 Objective

To isolate pure DNA from blood.

2.4 Materials

2.4.1 Chemicals

1 EDTA
2 NaOH
3 Tris-HCl
4 Sucrose
5 $MgCl_2$
6 Triton X
7 Sodium dodecyl sulphate**.**
8 NaCl
9 Sodium perchlorate
10 TE Buffer
11 Chloroform
12 Ethanol

2.4.2 Equipments

1. Waterbath set at 65°C.
2. Centrifuge tubes (15 mL; Falcon).
3. Microfuge (1.5 mL) tubes.

4. Tube roller/rotator.

2.4.3 Glassware

1 Glass Pasteur pipetts, heated to seal the end and curled to form a "loop" or "hook" for spooling DNA.

2 Tubes

2.4.4 Preparation of Solutions

This method uses standard chemicals that can be obtained from any major supplier, e.g.Sigma;

1. Ethylene diamine tetra acetate (EDTA) (0.5 M), pH 8.0: Add 146.1 g of anhydrous EDTA to 800 mL of distilled water. Adjust pH to 8.0 with NaOH pellets (this will require about 20 g). Make up to 1 L with distilled water. Autoclave at 15 p.s.i. for 15 min.
2. 1 M Tris-HCl, pH 7.6: Dissolve 121.1 g of Tris base in 800 mL of distilled water. Adjust pH with concentrated HCl (this requires about 60 mL). CAUTION: the addition of acid produces heat. Allow mixture to cool to room temperature before finally correcting pH. Make up to 1 L with distilled water. Autoclave at 15 p.s.i. for 15 min.
3. Reagent A: Red blood cell lysis solution: 0.01M Tris-HCl pH 7.4, 320 mM sucrose, 5 mM $MgCl_2$, 1% Triton X 100.
4. Add 10 mL of 1 M Tris, 109.54 g of sucrose, 0.47 g of $MgCl_2$, and 10 mL of Triton X-100 to 800 mL of distilled water. Adjust pH to 8.0, and make up to 1 L with distilled water. Autoclave at 10 p.s.i. for 10 min (see Note 1).
5. Reagent B: Cell lysis solution: 0.4 M Tris-HCl, 150 mM NaCl, 0.06 M EDTA, 1% sodium dodecyl sulphate (SDS), pH 8.0. Take 400 mL of 1 M Tris (pH 7.6), 120 mL of 0.5 M EDTA (pH 8.0), 8.76 g of NaCl, and adjust pH to 8.0 with NaoH . Make up to 1 L with distilled water. Autoclave for 15 min at 15. p.s.i. After autoclaving, add 10 g of SDS.

2.5 Experimental Protocol

2.5.1 Blood Collection

Draw blood in EDTA-containing Vacutainer tube by venipuncture. Store at room temperature or 4^0 C and extract within the same working day.

2.5.2 DNA Extraction

To extract DNA from cell cultures or disaggregated tissues, omit steps 1 through 3.

1. Place 3 mL of whole blood in a 15-mL falcon tube.
2. Add 12 mL of reagent A.
3. Mix on a rolling or rotating blood mixer for 4 min at room temperature.
4. Centrifuge at 3000*g* for 5 min at room temperature.
5. Discard supernatant without disturbing cell pellet. Remove remaining moisture by inverting the tube and blotting onto tissue paper.
6. Add 1 mL of reagent B and vortex briefly to resuspend the cell pellet.
7. Add 250 μL of 5 *M* sodium perchlorate and mix by inverting tube several times.
8. Place tube in water bath for 15 to 20 min at 65°C.
9. Allow to cool to room temperature.
10. Add 2 mL of ice-cold chloroform.
11. Mix on a rolling or rotating mixer for 30 to 60 min.
12. Centrifuge at 2400*g* for 2 min.
13. Transfer upper phase into a clean falcon tube using a sterile pipette.
14. Add 2 to 3 ml of ice-cold ethanol and invert gently to allow DNA to precipitate.
15. Using a freshly prepared flamed Pasteur pipette spool the DNA onto the hooked end.
16. Transfer to a 1.5-mL Eppendorf tube and allow to air dry.
17. Resuspend in 200 μL of TE buffer.

As a final step in nucleic acid isolation, the yield and purity of the extracted nucleic acid may need to be determined.

2.6 Results

2.7 Discussion

2.8 Questions

1. What do you think is the purpose of the cell lysis solution?
2. What is the purpose of ethanol?
3. Isolated DNA should be free from contaminating protein, heme and other cellular macromolecule, what precautions did you take to solve this situation?
4. Heme, the non-protein iron component of hemoglobin, is a primary contaminant of DNA from blood preparations, how can you detect this type of contaminant in the isolated DNA?
5. In this procedure, if you didn't have sodium perchlorate, what other chemical can you use instead?

2.9 References

1. Bartlett JMS and Stirling D, Methods in Molecular Biology, second edition (226):29-33

2. Helms C. Salting out Procedure for Human DNA extraction. In: The Donis-Keller Lab - Lab Manual Homepage [online]. 24 April 1990. [cited 19 Nov. 2002; 11:09 EST]. Available from: http://hdklab.wustl.edu/lab_manual/dna/dna2.html.

3. Epplen JE and Lubjuhn T. DNA profiling and DNA fingerprinting. Birhkhauser Verlag, Berlin. 1999; p.55.

3. PREPARATION OF GENOMIC DNA FROM PLANT TISSUES

3.1 Introduction

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 step with liquid nitrogen is employed to break down cell wall material and allow access to DNA while cellular enzymes and other biochemicals are 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 by 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. To check the quality of the extracted DNA, a sample is run on an agarose gel, stained with ethidium bromide, and visualised under UV light.

3.2 Theory

Method used for extraction of DNA from the plants is different from extracting DNA from animal sources as the plant contains hard cellulose cell wall. A number of protocol for isolating DNA from plant sources are available which ranges from using simple chemicals in the lab to a more sophisticated Isolation protocol by using kits. The main goal of developing all these protocol is to search, for a more efficient means of extracting DNA of both higher quality and yield. However the fundamental 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.

3.3 Objective

To isolate and purify plant genomic DNA

3.4 Materials

3.4.1 Materials

- CTAB buffer
- Microfuge tubes
- Mortar and Pestle
- Liquid Nitrogen
- Microfuge
- Absolute ethanol (ice cold)
- 70 % Ethanol (ice cold)

- 7.5 M Ammonium acetate
- 55^0 C water bath
- Chloroform: Iso Amyl alcohol (24:1)
- Water (sterile)

3.4.2 CTAB buffer 100ml

- 2.0 g CTAB (Hexadecyl trimethyl-ammonium bromide)
- 10.0 ml 1 M Tris pH 8.0
    - ml 0.5 M EDTA pH 8.0 (EDTA Di-sodium salt)
- 28.0 ml 5 M NaCl
- 40.0 ml H_2O
- 1g PVP 40 (polyvinyl pyrrolidone (vinylpyrrolidine homopolymer) Mw 40,000)
- Adjust all to pH 5.0 with HCl and make up to 100 ml with H_2O.

3.4.3 1 M Tris pH 8.0

Dissolve 121.1 g of Tris base in 800 ml of H_2O. Adjust pH to 8.0 by adding 42 ml of concentrated HCl. Allow the solution to cool to room temperature before making the final adjustments to the pH. Adjust the volume to 1 L with H2O. Sterilize using an autoclave.

3.5 Experimental Protocol

1. Grind 200 mg of plant tissue to a fine paste in approximately 500 µl of CTAB buffer.
2. Transfer CTAB/plant extract mixture to a microfuge tube.
3. Incubate the CTAB/plant extract mixture for about 15 min at 55^o C in a recirculating water bath.
4. After incubation, spin the CTAB/plant extract mixture at 12000 g for 5 min to spin down cell debris. Transfer the supernatant to clean microfuge tubes.
5. To each tube add 250 µl of chloroform: iso amyl alcohol (24:1) and mix the solution by inversion. After mixing, spin the tubes at 13000 rpm for 1 min.
6. Transfer the upper aqueous phase only (contains the DNA) to a clean microfuge tube.
7. To each tube add 50 µl of 7.5 M ammonium acetate followed by 500 µl of ice cold absolute ethanol.
8. Invert the tubes slowly several times to precipitate the DNA. Generally the DNA can be seen to precipitate out of solution. Alternatively the tubes can

be placed for 1 hr at -20 o C after the addition of ethanol to precipitate the DNA.

9. Following precipitation, the DNA can be pipetted off by slowly rotating/spinning a tip in the cold solution. The precipitated DNA sticks to the pipette and is visible as a clear thick precipitate. To wash the DNA, transfer the precipitate into a microfuge tube containing 500 µl of ice cold 70 % ethanol and slowly invert the tube.
10. Repeat (alternatively the precipitate can be isolated by spinning the tube at 13000 rpm for a minute to form a pellet. Remove the supernatant and wash the DNA pellet by adding two changes of ice cold 70 % ethanol).
11. After the wash, spin the DNA into a pellet by centrifuging at 13000 rpm for 1 min.
12. Remove all the supernatant and allow the DNA pellet to dry (approximately 15 min). Do not allow the DNA to over dry or it will be hard to re-dissolve.
13. Resuspend the DNA in sterile DNase free water (approximately 50-400 µl H2O; the amount of water needed to dissolve the DNA can vary, depending on how much is isolated). RNaseA (10 µg/ml) can be added to the water prior to dissolving the DNA to remove any RNA in the preparation (10 µl RNaseA in 10ml H2O).
14. After resuspension, the DNA is incubated at 65o C for 20 min to destroy any DNases that may be present and stored at 4o C.
15. Agarose gel electrophoresis of the DNA will show the integrity of the DNA, while spectrophotometry will give an indication of the concentration and purity of isolated DNA.

3.6 Results

3.7 Discussion

1. DNA from other sources like mitochondria and chloroplast can precipitate out with your genomic or nuclear DNA, Discuss How you can overcome this problem?
2. What are the sources of contamination of DNA in your sample?
3. What precautions you should use while isolating DNA?
4. Discuss your results and discuss how you can make this experiment more effective?

3.8 References

1. Doyle JJ, Doyle JL A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bullettin 1987; 19: 11-15.

4. PLASMID ISOLATION AND PURIFICATION

4.1 Introduction

Bacterial plasmids are closed circular molecules of double-stranded DNA that range in size from 1 to >200 kb. They are found in a variety of bacterial species, where they behave as additional genetic units, inherited and replicated independently of the bacterial chromosome. However, they rely upon enzymes and proteins provided by the host for their successful transcription and replication. Plasmids often contain genes that code for enzymes that can be advantageous to the host cell in some circumstances. The encoded enzymes may be involved in resistance to, or production of, antibiotics, resistance to toxins found in the environment e.g., complex organic compounds, or the production of toxins by the bacteria itself. Once purified, plasmid DNA can be used in a wide variety of downstream applications such as sequencing, Polymerase Chain Reaction (PCR), expression of proteins, transfection, and gene therapy.

4.2 Theory

Plasmid DNA is introduced into bacteria by the process of transformation. Transformation is inefficient and the plasmids become stably established in only a small number of bacterial populations. Selectable markers carried by the plasmid enable the transformed bacteria to be identified. The markers typically provide a specific resistance (ability to grow in the presence of) to antibiotics such as ampicillin or kanamycin. There are numerous method (and kits) available for the isolation of plasmid DNA from a transformed bacteria culture. In this experiment, alkaline lyses, together with treatment with the detergent sodium dodecyl sulphate (SDS), a method adapted from Protocol 1, Molecular-cloning [1], is used for isolating plasmids.

Sodium dodecyl sulphate is a strong anionic detergent, which lyses bacterial cell membrane, separates chromosomal DNA from proteins and releases plasmid DNA into the supernatant, at alkaline pH. The alkaline solution acts to disrupt base pairing which has no effect on the closed circular plasmid DNA. The degraded chromosomal DNA and protein, along with the components of the cell wall form large aggregated complexes that are precipitated during the plasmid isolation and removed by centrifugation.

4.3 Objective

To isolate and handling plasmid DNA.

4.4 Materials

4.4.1 Buffers/Solutionss

4.4.1.1 Alkaline Lysis Solution I

i. 50 mM glucose

ii. 25 mM Tric HCl (pH 8.0)

iii. 10 mM EDTA (pH 8.0)

(Prepare in batches ~100 ml and autoclave for 15 min at 15 psi (1.05kg^2/cm) on liquid cycle and store at 4^{0}C).

4.4.1.2 Alkaline Lysis Solution II

i) 0.2 N NaOH (freshly diluted from 10 N stock).
ii) 1% (w/v) SDS (prepare solution and store at room temperature).

4.4.1.3 Alkaline Lysis Solution III

i) 5 M potassium acetate (60 ml).
ii) Glacial acetic acid (11.5 ml)
iii) H2O 28.5 ml
iv) The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.
v) Store the solution at 4oC and transfer to an ice bucket just before use.

4.4.2 LB Broth (Luria - Bertani Medium)

Per Liter:
To 950 ml of deionized H_2O, add:

- Tryptone 10 g
- Yeast extract 5 g
- NaCl 10 g

Shake until the solutes dissolve. Adjust pH to 7.0 with 5 N NaCl (~0.2 ml). Adjust volume to 1 L with deionized H_2O. Sterilize by autoclaving for 20 minutes at 15 psi (1.05kg^2/cm) on liquid cycle.

4.4.3 Ampicillin (Amp)

4.4.4 Bacterial Colony:

4.4.5 Apparatus and Solutions

4.4.5.1 Per Group

- Tooth picks
- Gloves
- Vortexer
- Vacuum line and aspirator
- Balance

- Ice bucket and ice
- Sharpie marker
- 8 Microfuge tubes (1.5 ml) and tube rack
- 2x 10 ml test tubes with loose fitting lids
- 2 x 2ml LB broth
- 1000 µl Pipetteman and blue tips
- 200 µl Pipettman and yellow tips
- Sterile water (or TE)
- Solutions I, II and III

4.4.5.2 Solution I and III on Ice

- Set of Pipettman pipettes (automated) and tips
- Ethanol
- 250 ml flask and kim wipes
- Graduated cylinder
- Dry waste beaker
- Liquid waste beaker

4.5 Experimental Protocol

4.5.1 Preparation of Cells

Rich media with the correct antibiotic for selection is used in the bacterial culture. To ensure that the culture is adequately aerated, use a flask/tube with a volume at least 4 times greater than the volume of the culture, cap the tube loosely and incubate with vigorous agitation.

1. Prepare two test tubes containing 2 ml of LB broth with a final concentration of 100 µg/ml Amp. (The stock [Amp] = 50 mg/ml in water).
2. Inoculate each with a single colony of bacteria from the given sample.
3. Incubate overnight at 37ºC.

4.5.2 Cell Lyses and Recovery of Plasmid DNA

1. Remove 1.5 ml aliquot of the culture to a microcentrifuge tube. Repeat for the second culture into a second microcentrifuge tube. Make sure that the tubes are labeled.
2. Centrifuge at 4ºC, maximum speed for 30 seconds in a microfuge. Label the unused portions of the original culture and store at 4ºC.

3. After centrifugation, remove the medium by aspiration (as shown in Figure 1, below), leaving the bacterial pellet as dry as possible.
4. Resuspend each bacterial pellet in 100 µl of ice cold Alkaline Lysis Solution I. Vortex vigorously.
5. Add 200 µl of freshly prepared Alkaline Lysis Solution II to each bacterial suspension. Invert the tube rapidly 5 times. Do not vortex! Store the tube on ice.
6. Add 150 µl ice cold **Alkaline Lysis Solution III** to each microfuge tube. Invert the tube 3 to 5 times. Incubate tubes **on ice** for 3 to 5 minutes.
7. Centrifuge the bacterial lysate at maximum speed, 4ºC for 2 minutes. Transfer the supernatant to a fresh labeled tube.
8. Add 2 volumes of ethanol at room temperature. Vortex and allow tubes to stand at room temperature for 2 minutes
9. Centrifuge at maximum speed , 4ºC for 5 minutes. Orient the microfuge tubes so that the plastic hinges point outwards. The precipitate will collect on inside surface of the tube furthest from the center of rotation.

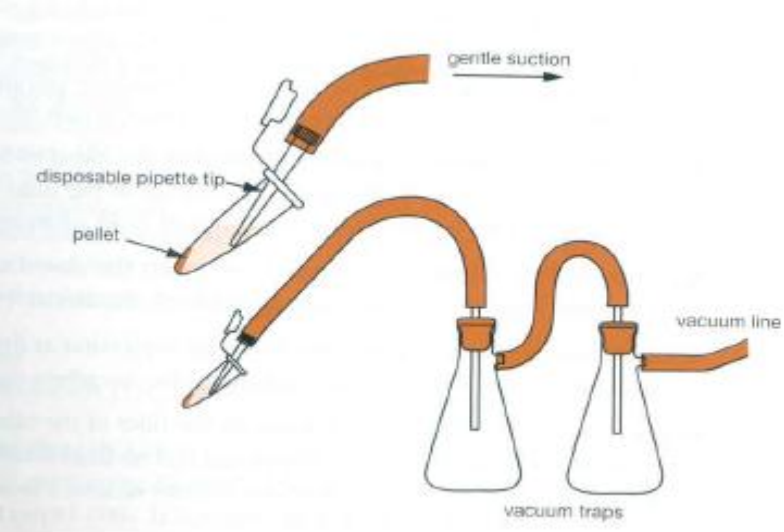

Figure 1: Aspiration of supernatant (From Sambrook,**et al 2001).**

10. Remove the supernatant by gentle aspiration. As shown in Figure 1.
11. Stand the tube in an inverted position over a paper towel to allow all fluid to drain away.
12. Add 1ml 70% ethanol, invert the closed tube several times. Centrifuge at maximum speed, 4ºC for 5 minutes.
13. Remove the supernatant by gentle aspiration.

14. Remove any beads of ethanol from the sides of the tube. Leave tube open at room temperature (upright position) until residual ethanol has evaporated (5 to 10 minutes).
15. Dissolve the pellet in 25 µl sterile water or TE and vortex the solution gently for a few seconds. The DNA can be stored at -20ºC.

4.6 Results

4.7 Discussion

In the discussion section you need to answer the following questions and discuss your results.

1. What is the importance of antibiotic gene in the plasmid? How does it help you in isolation and purification protocol for plasmid isolation?
2. Discuss the difficulties you have encountered in isolation and purification protocol.
3. Discuss the advantage and disadvantages of the particular plasmid you have taken for isolation?-
4. Discuss the importance of copy number of plasmid. How does it effect the isolation protocol?

4.8 References

1. Sambrook J, Fritsch EF & Maniatis T. Molecular Cloning. A laboratory Manual. 3rd Edition. Cold Spring Harbor Laboratory Press. New York, 2001

5. CHARACTERIZATION OF THE DNA BY: (1) THE SPECTROPHOTOMETRIC ASSAY; (2) THE MELTING TEMPERATURE (TM)

5.1 Introduction

The isolated and purified DNA can be characterized by different ways. In this experiment the purity and concentration of DNA obtained in the last experiment will be determined and the DNA will be characterized by measuring its melting temperature (Tm).

5.1.1 Determination of DNA purity and concentration by spectro-photometric assay:

A simple method for determining nucleic acid extraction efficiency and purity is via the use of UV spectrophotometer. Single stranded DNA, double stranded DNA and RNA have specific absorption coefficients of 0.027, 0.020 and 0.025 μg per ml per cm at 260 nm, respectively. Moreover, the absorption ratio of 260/280 nm is an indicator for DNA purity. RNA, protein or phenol contamination alters the ratio. Proteins have a maximum adsorption at 280 nm, and when the ratio is below 1.8 or above 2.0, a significant amount of impurities is still present within the sample. Highly purified samples of DNA have a 260/280 nm ratio of 1.8–1.9 whilst highly purified samples of RNA have a 260/280 nm ratio of 1.9–2.0. Many spectrophotometers will automatically calculate the 260/280 nm ratio and quantity of nucleic acid. Phenol/urea contamination may be assessed at 230 nm. The spectrophotometer is calibrated with a blank prior to measuring nucleic acid concentrations. This blank should comprise the solution in which the nucleic acid is resuspended in (e.g. nuclease free water, Tris EDTA buffer) only. Disposable or cleaned quartz cuvettes must be used for each new measurement. Electrophoresis of a small sample of the extract on an agarose gel along with molecular weight marker (ladder) and/or known quantities of nucleic acid, can also be used for assessing the efficiency of nucleic acid extraction.involves the. Molecular weight markers with bands comprising a known quantity of DNA are available and can be purchased.

5.1.2 Determination of melting temperature (Tm) of isolated DNA.

When DNA is heated it denatures or melts i.e. the double stranded DNA separates into its single stranded components. When the temperature is decreased the strands re-associate to form the double stranded molecule (renaturation). The **melting temperature** (T_m) is defined as the temperature at which half of the DNA strands have melted i.e half are in double-helical state and half are in the "random-coil" states. The melting temperature depends on the nucleotide composition of the DNA molecule and the length of the DNA. Higher Tm are associated with higher GC content, since GC base pairs are linked by three H-bonds while AT base pairs are linked by two H-bond. Hence GC base pairs are stronger, requiring higher temperature for melting. The Tm can be used to calculate the GC content of the DNA.

5.2 Theory

DNA melting and reassociation can be monitored by measuring the absorbance at 260 nm. Double-stranded DNA has a lower absorbance, but when it is single-stranded, the unstacking of the bases leads to an enhancement of absorbance. This is called the hyperchromic effect. Therefore, the extent to which DNA is single-stranded or double-stranded can be determined by monitoring UV absorption. Temperature for midpoint of denaturation gives the $T_{m.}$ By increasing temperature slowly and measuring absorbance at 260 nm as melting profile can be generated.

5.3 Objective

1. To determine nucleic acid extraction efficiency and purity using UV spectrophotometry.
2. To measure the AT/CG ratio and the percentage of GC content of a DNA isolated from different sources (plasmid, plant and human) using the equation.

5.4 Materials

5.4.1 Chemicals

1. DNA Template (source; plasmid, human Genomic DNA, plant Genomic DNA)
2. SSC buffer
3. UV spectrophotometer and quartz cuvettes

5.4.2 Equipments

1. Water bath
2. Spectrophotometer UV

5.4.3 Glassware

Quartz cuvette

5.4.4 20 X SSC Buffer to make 1L use:

- 175.3 g NaCl
- 88.2 g Na Citrate dihydrate
- Dil HCl

Dissolve in approximately 800 ml dH_20. Adjust to pH 7.0 with dilute HCl. Bring up to a final volume of 1L and autoclave. Store at room temperature.

5.4.5 Experimental Protocol for the Characterization of DNA by Spectrophotometer Assay.

1. Dissolve a small quantity of your extracted DNA in 3.0 ml of 0.1X SSC.
2. Turn on and blank a UV spectrophotometer at 220 nm (use 0.1X SSC as the blank). Determine the absorbance of your sample DNA at 230 nm.
3. Change the wavelength to 230 nm, reblank the spectrophotometer and measure the absorbance of the sample at 230 nm.
4. Increment the wavelength by 10 nm and repeat blanking and measuring the absorbance until readings are taken through 300 nm.
5. Compute the absorbance ratio 260 nm to 280 nm. Pure DNA (without protein or RNA) will have a 260:280 absorbance ratio of 1.85. RNA will have a 260:280 ratio of 2.0.
6. Plot the absorbance spectrum of your sample and indicate the 260:280 ratio, as well as the amount of protein contamination on the graph.

5.4.6 Experimental Protocol for Melting Point Determination

1. Dissolve your DNA preparation in SSC to give a final concentration of approximately 20 µg DNA/ml.
2. Place the dissolved DNA in a quartz cuvette along with a second cuvette containing SSC as a blank.
3. Place the cuvettes into a waterbath at 25 ° C and allow to temperature equilibrate. Remove the blank, wipe the outside dry and rapidly blank the instrument at 260 nm. Transfer the sample to the spectrophotometer (be sure to dry and work rapidly) and read the absorbance.
4. Raise the temperature of the bath to 50° C and repeat step .
5. Raise the temperature sequentially to 60° C, 65° C, 70° C, 75° C and 80° C and repeat the absorbance measurements
6. Slowly raise the temperature above 80° and make absorbance measurements every 2° until the absorbance begins to increase. At that point, increase the temperature, but continue to take readings at 1° C intervals
7. Correct all of the absorbance readings for solvent expansion relative to 25° C.
8. List the corrected values as A_t

9. Plot the value of A_t/A_{25} vs temperature and calculate the midpoint of any increased absorbance. This midpoint is the melting point (Tm) for your DNA sample.

5.5 Results

Wavelength (nm)	Absorbance
220	
230	
240	
250	
260	
270	
280	
290	
300	

Calculate the GC content of your sample using the formula
Percent of G + C = k(Tm -69.3) x 2.44.

5.6 Discussion

5.7 Questions

1. How pure is your DNA samples? Reflect on the possible sources of contaminations in your DNA samples from different sources?
2. Compare the different melting temperature of DNA obtained from different sources?
3. What does the ratio of AT/CG tells you?
4. Why does the melting temperature of the DNA sample depends on the GC content?

5.8 References

1. SantaLucia J Jr. "A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics". Proc. Natl. Acad. Sci. USA. 1998; 95 (4): 1460–5.

2. Mandel M and Marmur J. "Use of Ultravialet Absorbance-Temperature Profile for Determining the Guanine plus Cytosine Content of DNA". Methods in Enzymology 1968; 12 (2): 198–206.

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6. Primer Design

Primers are short single-stranded oligonucleotides which anneal to template DNA and serve as a "primer" for DNA synthesis. In order to achieve the geometric amplification of a DNA fragment, there must be two primers, one flanking each end of the target DNA.

The most critical step in your PCR experiment will be designing your oligonucleotide primers. Poor primers could result in little or even no PCR product. Alternatively, they could amplify many unwanted DNA fragments. Either way, it would interfere in subsequence cloning steps. Therefore it is critical that you design your primers carefully. Primer design requires extensive computer-based sequence analysis and this tutorial is designed to lead you through that analysis.

6.1 General Considerations in Primer Design

I-Specificity
II-The distance between the primers
III-Melting Temperature (T_m)
VI Primer Length
V Product Size
VI Primer Dimers
VII Hairpins
VII Primer Dimers
VIII G/C Content
IX G/C clamp

6.1.1 Specificity

Two critical issues for specificity:

1. Primers must be complementary to flanking sequences of target region
2. Primers should not be complementary to many non-target regions of genome.

Consider the following fragment of DNA. (Remember, that when both strands of DNA are shown the top strand runs 5'-3') The location of two primers is indicated by >>'s.

```
541 CATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTAC
    GTAATAGTTGTTTTATGAGGTTAACCGCTACCGGGACAGGAAAATGGTCTGTTGGTAATG

601 CTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTT
    GACAGGTGTGTTAGACGGGAAAGCTTTCTAGGGTTGCTTTTCTCTCTGGTGTACCAGGAA
    >>>>>>>>>>>>>>>>>>

661 CTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATACAAATAA
    GAACTCAAACATTGTCGACGACCCTAATGTGTACCGTACCTACTTGATATGTTTATT
                        <<<<<<<<<<<<<<<<<<<
```

The forward primer (>>>>>) which will be complementary to the lower strand and must run 5'-3' will have the sequence: 5'-CTGTCCACACAATCTGCC -3'

The reverse primer (<<<<<) which will be complementary to the upper strand and must run 3'-5' and will have the sequence: 3'-GACCCTAATGTGTACCGTAC-5'. However, we always write DNA sequences in the 5'-3' direction so the reverse primer would be written: 5'-CATGCCATGTGTAATCCCAG-3'

Ideally primers would be complementary only to the target sequence. This would ensure that the taq polymerase only copies the target region. However, when using genomic DNA as the template, there will likely be other sequences which are <u>complementary</u> or <u>nearly complementary</u> to the primers.

6.1.2 The distance between the primers

- It is rather flexible, ranging up to 10 kb. There can be, however, a considerable drop-off in synthesis efficiency with distances □ 3 kb (Jeffreys et al., 1988).

- Small distances between primers, however, lessen the ability to obtain much sequence information or to reamplify with nested internal oligonucleotides, should that be necessary.

6.1.3 Melting Temperature (T_m)

Two issues are critical for T_m.

1. The two primers should have a similar T_m.
2. The T_m should be within55-72º, around 60º is ideal.

The annealing temperature for a PCR reaction is based on the melting temperature (T_m) of the primers. The T_m is the temperature at which a population of a double stranded DNA molecule is partially denatured such that half of the molecules are in the single stranded state and half are in the double stranded state.

6.2 General points should be considered for Tm

- At temperatures above the T_m the DNA molecules will be in the single stranded form
- At temperatures below the T_m the DNA can form the double stranded form.

- If the annealing temperature is too high, the primer will not anneal to the target DNA.
- If the annealing temperature is too low the primer will mis-anneal to sequences which aren't perfectly complementary.
- In order for the primers to anneal to the target DNA the annealing temperature must be below the T_m of the primers. Typically, the annealing reaction is carried out about 5º below the T_m.
- The most important consequence of this is that the two primers designed for a PCR experiment should have very similar T_m's. Typically the T_m should be within 5º of each other. The closer the T_m's the better.
- The T_m of a molecule is dependent on its sequence, however the relationship between sequence and T_m is not simple. In general the greater the GC content of DNA the higher its T_m. The Wallace formula can be used to give a rough estimate of T_m.

$$T_m = 2(A+T) + 4(G+C).$$

6.3 Primer Length

- A primer should be 20 to 30 bases in length. . It is long enough to be specific to the target region, yet short enough to anneal efficiently.

- Must be neither too short nor too long. If primers are too short they will lack specificity. For example consider a primer only 4 nucleotides long, GATC. it will bind to thousands of sequences on the chromosomes. This could lead to amplification of unwanted sequences.

6.4 Product Size

- The choice of primers determines the size of the PCR product.
- If the two primers are complementary to nearby regions on the template DNA, then a small fragment of DNA will be amplified.
- If the two primers are complementary to regions farther apart, then a larger fragment of DNA will be amplified.
- Basic taq polymerase can easily amplify fragments up to 1000 to 2000bp. (Special polymerases can be used to amplify larger fragments.)

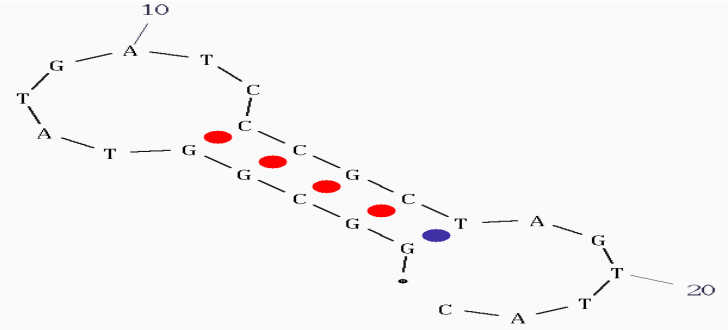
- For standard PCR, the primers should be complmentary to regions on the target DNA within 1000bp of each other.

6.5 Primer Dimers

- If the primers have self-complementary sequences the primers, which are in high concentration, will anneal with themselves.
- If they anneal with themselves they are not available to bind to the target DNA.
- There are two types of potential self-complementary sequences, those that lead to hairpins and those that lead to primer dimers.

6.6 Hairpins

- Intramolecular complementary sequences can lead to base pairing within a molecule. Consider the primer **GGC GGT ATG ATC CCG CTA GTT AC.** It can base pair internally and form the following hairpin structure.
- A primer that is base pairing with itself cannot base pair with its target DNA.
- Primers must be designed to minimize intramolecularl base pairing.
- Intramolecular base pairing is usually analyzed using computer programs.
- Avoid primers that contain more than a string of 3 intra molecular base pairs.



6.7 Primer Dimers

Primers can also participate in intermolecule base pairing. This is base pairing between two different primer molecules.

- If the base pairing is between the forward and the reverse primer it is called heterodimer formation.
- If the base pairing is between just one of the two primers it is called self-dimer formation.

```
5'       GGCGGTATGATCCCGCTAGTTAC
                |||| || || ||||
       3' CATTGATCGCCCTAGTATGGCGG
```

- The example primer used above can form several self-dimers (see the 2 boxes below). Both examples of primer dimer are problematic. The first is a highly stable structure with numerous base pairs. If the primers are base pairing with themselves they cannot base pair with the target DNA.

6.8 G/C Content

- it is important that primers be about 50% G/C and 50% A/T.
- It is also important that regions within the primer not have long runs of G/C or A/T.
- A stretch of A/T's might only weakly base pair while a stretch of G/C might promote mis-annealing.
- It is also useful to avoid a long string of a single nucleotide or even long strings of purines or pyrimidines.

6.9 G/C clamp

Stable base pairing of the 3' end of a primer and the target DNA is necessary for efficient DNA synthesis. To ensure the stability of this interaction, primers are often designed ending in either a G or a C. (GC base pairs are more stable than AT base pairs.) This terminal G or C is called a G/C clamp.

Summary

1. primers should be 17-28 bases in length;
2. base composition should be 50-60% (G+C);
3. primers should end (3') in a G or C, or CG or GC: this prevents "breathing" of ends and increases efficiency of priming;
4. Tms between 55-80^{o}C are preferred;
5. primer self-complementarity (ability to form 2^{o} structures such as hairpins or primer dimers) should be avoided;
6. it is especially important that the 3'-ends of primers should not be complementary (ie. base pair), as otherwise primer dimers will be synthesised preferentially to any other product;
7. runs of three or more Cs or Gs at the 3'-ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing), and should be avoided.

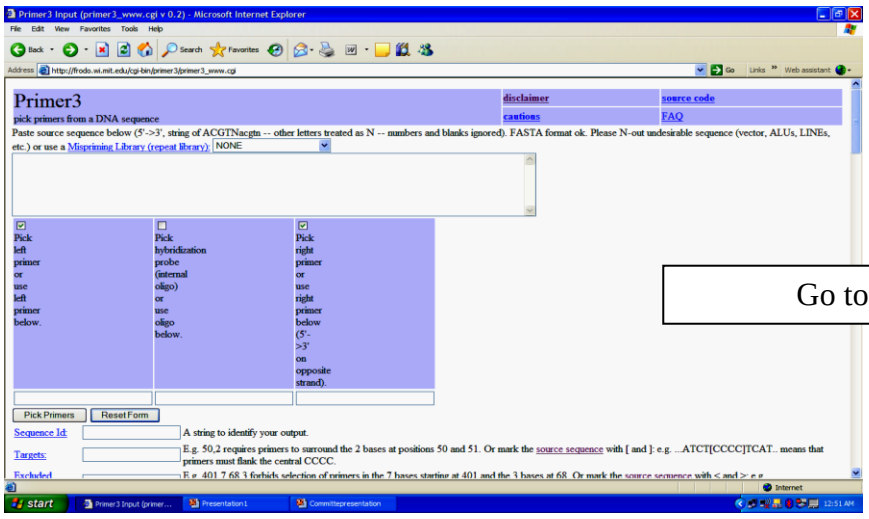
6.10 Web Based Tools for Primer Design

This semester we will be using two different internet applications for our primer design. The first is an application in Biology Workbench called Primer3. This application will analyze target regions and recommend forward and reverse primer sequences. Its analysis can be directed to specific target regions of genes and analyzes factors such as product size, primer size, tm, GC content GC clamps and dimer formation. The second application, oligocalc, <http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx> is provided by one of the companies that we order primers, IDT. It has applications for analysis of hairpins, homodimers and heterodimers. We will use it to double check the primers identified by Primer3.

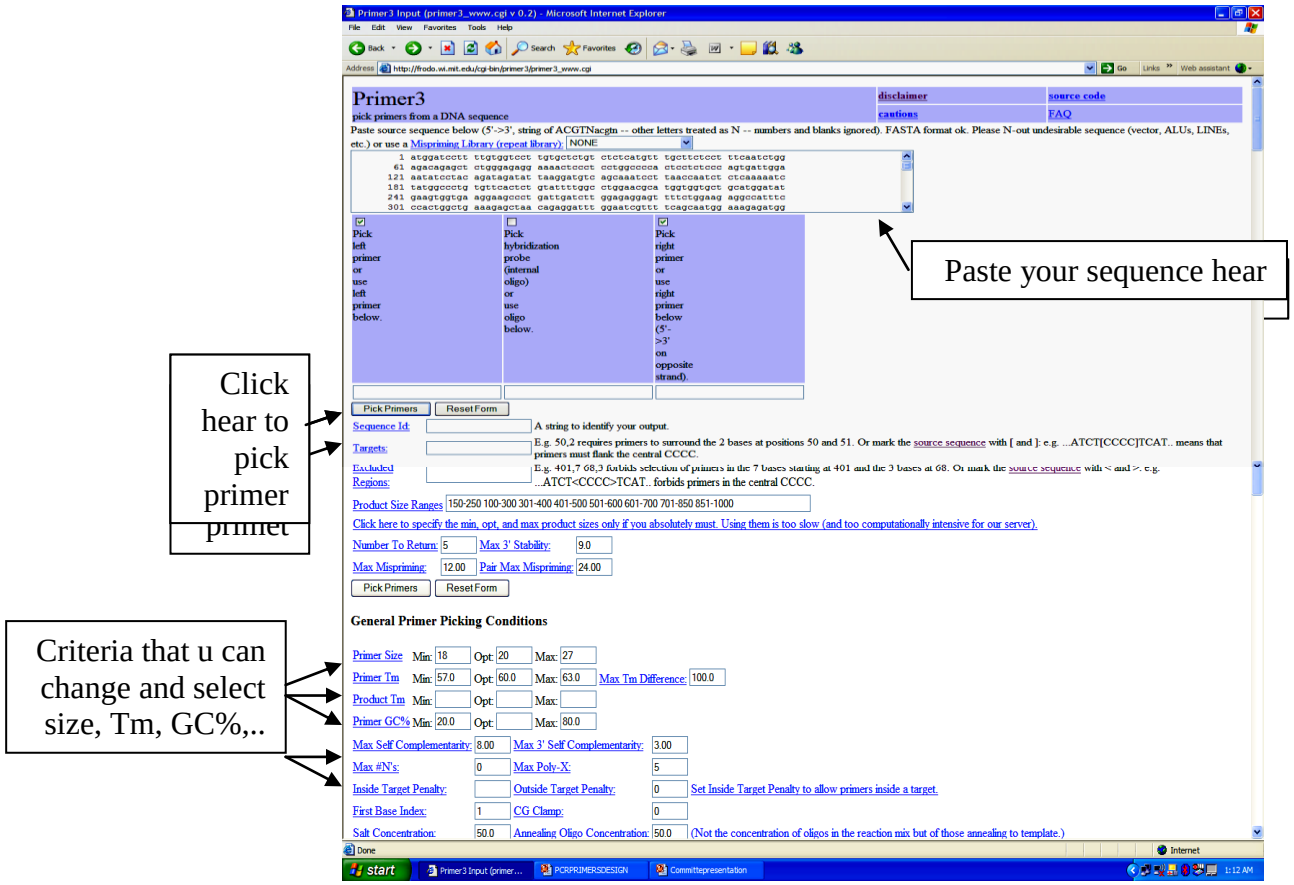
6.10.1 How to find a primer

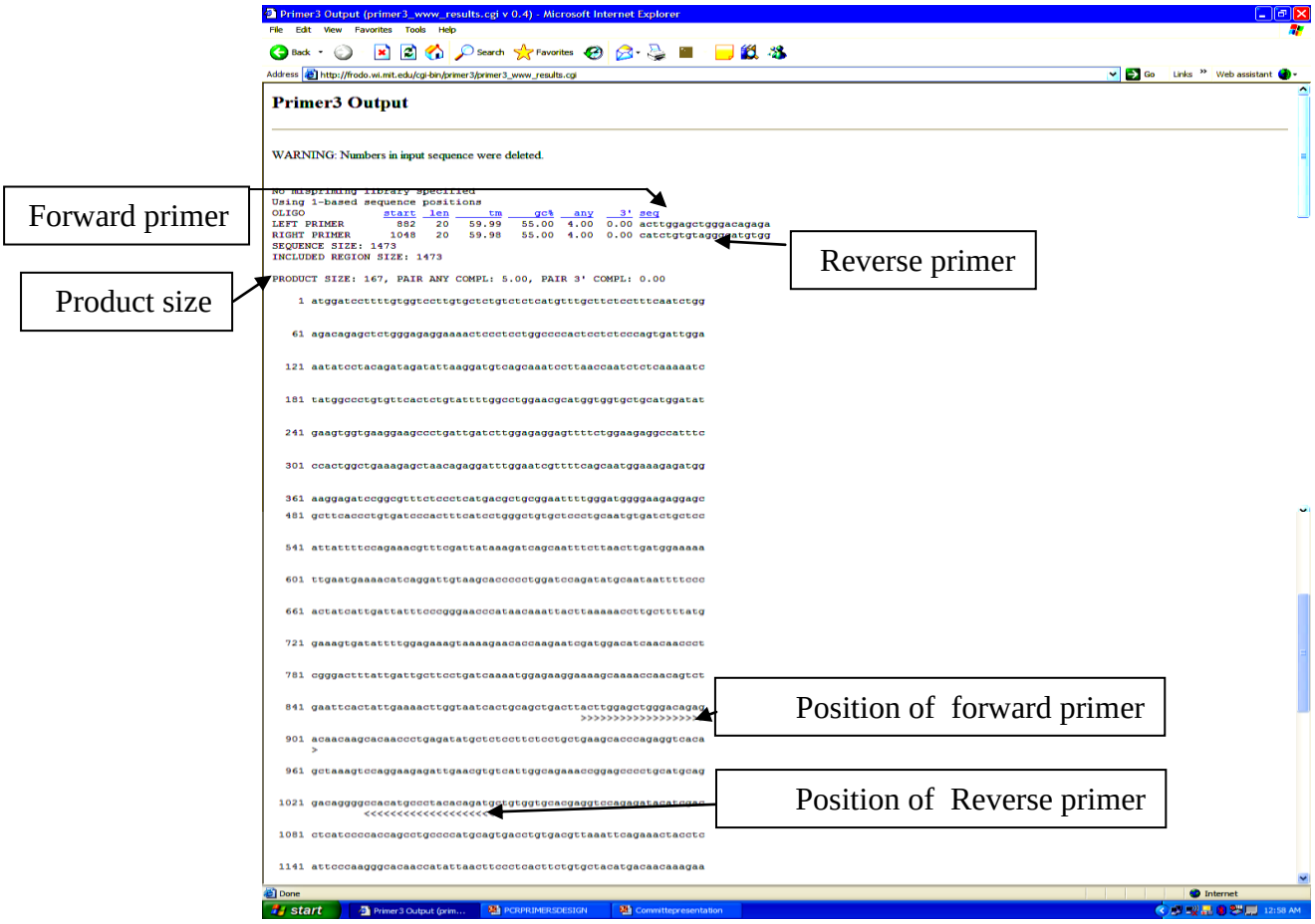
1. Access biology workbench and import the region of your gene that you plan to amplify using PCR.
2. Run primer3 program in workbench.
3. Scroll down to primer criteria on the primer 3 page and change the first two default settings.
    a. Under product size change range from 100-300 to 400-600.
    b. Change the GC clamp size from its setting of zero to a setting of one.
4. Click Submit to complete the analysis.
5. Primer3's output includes an "optimal" pair of primers. The locations of these primer sequences on the target sequence are reported. Four pairs of alternative primers are also reported.

1-



Go to primer 3 program


Primer3 Input (primer3_www.cgi v 0.2) - Microsoft Internet Explorer
Primer3
pick primers from a DNA sequence
disclaimer
source code
cautions
FAQ
Paste source sequence below (5'->3', string of ACGTNacgtn -- other letters treated as N -- numbers and blanks ignored). FASTA format ok. Please N-out undesirable sequence (vector, ALUs, LINEs, etc.) or use a Mispriming Library (repeat library): NONE
Pick left primer or use left primer below.
Pick hybridization probe (internal oligo) or use oligo below.
Pick right primer or use right primer below (5'->3' on opposite strand).
Pick Primers
Reset Form
Sequence Id:
A string to identify your output.
Targets:
E.g. 50,2 requires primers to surround the 2 bases at positions 50 and 51. Or mark the source sequence with [and]: e.g. ...ATCT[CCCC]TCAT.. means that primers must flank the central CCCC.
Excluded Regions:
...ATCT<CCCC>TCAT.. forbids primers in the central CCCC.
Product Size Ranges
150-250 100-300 301-400 401-500 501-600 601-700 701-850 851-1000
Click here to specify the min, opt, and max product sizes only if you absolutely must. Using them is too slow (and too computationally intensive for our server).
Number To Return:
Max 3' Stability:
Max Mispriming:
Pair Max Mispriming:
General Primer Picking Conditions
Primer Size
Primer Tm
Max Tm Difference:
Product Tm
Primer GC%
Max Self Complementarity:
Max 3' Self Complementarity:
Max #N's:
Max Poly-X:
Inside Target Penalty:
Outside Target Penalty:
Set Inside Target Penalty to allow primers inside a target.
First Base Index:
CG Clamp:
Salt Concentration:
Annealing Oligo Concentration:
(Not the concentration of oligos in the reaction mix but of those annealing to template.)
Paste your sequence hear
Click hear to pick primer
Criteria that u can change and select size, Tm, GC%,..



6.11 Discussion

In the discussion section you need to answer the following questions and discuss your results.

5. Did u manage to deign your own primer using manual and software methods
6. Discuss the importance of GC content Tm and length of your primer

7. Discuss the advantage and disadvantages of primer design using the software program?
8. Discuss the importance of Primer desigan and How does it can affect your PCR?

6.12 References

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7. PCR (POLYMERASE CHAIN REACTION) OPTIMIZATION (ANNEALING TEMPERATURE).

7.1 Introduction

The polymerase chain reaction (PCR) is a powerful method for fast in vitro enzymatic amplifications of specific DNA sequences. PCR amplifications can be grouped into three different categories: standard PCR, long PCR, and multiplex PCR. Standard PCR involves amplification of a single DNA sequence that is less than 5 kb in length and is useful for a variety of applications, such as cycle sequencing, cloning, mutation detection, etc. Long PCR is used for the amplification of a single sequence that is longer than 5 kb and up to 40 kb in length. Its applications include long-range sequencing; amplification of complete genes; PCR-based detection and diagnosis of medically important large-gene insertions or deletions; molecular cloning; and assembly and production of larger recombinant constructions for PCR-based mutagenesis ***(1,2)***. The third category, multiplex PCR, is used for the amplification of multiple sequences that are less than 5 kb in length. Its applications include forensic studies; pathogen identification; linkage analysis; template quantitation; genetic disease diagnosis; and population genetics ***(3–5)***. There is no single set of conditions that is optimal for all PCR. Therefore, each PCR is likely to require specific optimization for the template/primer pairs chosen. Lack of optimization often results in problems, such as no detectable PCR product or low efficiency amplification of the chosen template; the presence of nonspecific bands or smeary background; the formation of "primer-dimers" that compete with the chosen template/primer set for amplification; or mutations caused by errors in nucleotide incorporation. It is particularly important to optimize PCR that will be used for repetitive diagnostic or analytical procedures where optimal amplification is required. Optimization of a particular PCR can be time consuming and complicated because of the various parameters that are involved. These parameters include the following:

i) quality and concentration of DNA template;

ii) design and concentration of primers;

iii) concentration of magnesium ions;

iv) concentration of the four deoxynucleotides (dNTPs);

v) PCR buffer systems;

vi) selection and concentration of DNA polymerase;

vii) PCR thermal cycling conditions;

viii) addition and concentrations of PCR additives/cosolvents; and

ix) use of the "hot start" technique. Optimization of PCR may be affected by each of these parameters individually, as well as the combined interdependent effects of any of these parameters.

7.2 Theory

The purpose of a PCR is to produce a large number of copies of a DNA fragment of interest (e.g. a gene or a part of a gene). This is necessary to have enough starting template for studing DNA fragment e.g. sequencing.

7.2.1 The Amplification: The Cycling Reactions

There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler (a thermo cycler), which can heat and cool the tubes with the reaction mixture in a very short time.

7.2.2 Denaturation at around 94°C

By heating the DNA at high temperature e.g. 94^0C. the DNA is denatured. During the denaturation, the double strand DNA melts and seperates into the two single strands. All enzymatic reactions stop (for example : the extension from a previous cycle).

7.2.2.1 Annealing of the Primer to the Template, at around 54°C

The primers are in motion, known as Brownian movement. Ionic bonds are constantly formed and broken between the free bases on single stranded primer and complementary bases on the single stranded DNA template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore.

7.2.2.2 Extension at around 72°C

For the DNA polymerase the ideal working temperature is around 72^0C. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match get loose again (because of the higher temperature) and don't give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side. The DNA polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, and bases are added complementary to the template.

7.3 Objective

The objective of this procedure is:

1. to determine the parameters that may affect the specificity, fidelity, and efficiency of PCR,
2. as well as approaches that can be taken to achieve optimal PCR amplifications.

7.4 Materials

7.4.1 Chemicals and Materials

1. Template DNA (e.g., plasmid DNA, genomic DNA).
2. Forward and reverse PCR primers.
3. $MgCl_2$ (25 mM).

4. dNTPs (a mixture of 2.5 mM dATP, dCTP, dGTP, and dTTP).
5. 10× PCR buffer: 500 mM KCl, 100 mM Tris-HCl, pH 8.3, 25°C.
6. Thermal stable DNA polymerase (e.g., Taq DNA polymerase).
7. PCR additives/cosolvents (optional; e.g., betaine, glycerol, DMSO, formamide, bovine serum albumin, ammonium sulfate, polyethylene glycol, gelatin, Tween-20, Triton X-100,

7.4.2 Equipments

1. Automated pipettes
2. Microfuge
3. Thermocyclers
4. Electrophoresis tanks
5. Vortex
6. Power packs
7. –20°C freezerDist H_2O source
8. Laminar cabinet
9. Microwave
10. Work stations
11. Ice machine
12. Gel viewing system
13. Balance
14. Gel documentation system
15. pH meter

7.4.3 Setting up PCR

The common volume of a PCR is 10, 25, 50, or 100 μL. Although larger volumes are easier to pipette, they also use up a larger amount of reagents, which is less economical.

All of the reaction components can be mixed in together in a 0.5-mL PCR tube in any sequence except for the DNA polymerase, which should be added last. It is recommended to mix all the components right before PCR cycling.

For each PCR, the following components are mixed together:

1. Template DNA (1–500 ng).
2. Primers (0.05–1.0 μ*M*).
3. Mg2+ (0.5–5 m*M*).
4. dNTP (20–200 μ*M* each).
5. 1× PCR buffer: 1 m*M* Tris-HCl and 5 m*M* KCl.

6. DNA polymerase (0.5–2.5 U for each 50 μL of PCR).

As a real-life example, the following PCR was set up to amplify the *cII* gene from bacteriophage lambda DNA (total volume = 50 μL):

1. 1 μL of 1 ng/μL lambda DNA (final amount = 1 ng).
2. 1 μL of 50 μM forward PCR primer (final concentration = 1 μM).
3. 1 μL of 50 μM reverse PCR primer (final concentration = 1 μM).
4. 5 μL of 25 mM MgCl2 (final concentration = 2.5 mM).
5. 4 μL of 2.5 mM dNTPs (final concentration = 200 μM).
6. 5 μL of 10× PCR buffer (final concentration = 1×).
7. 0.25 μL of 5 U/μL Taq DNA polymerase (final amount = 1.25 U).

7.4.4 PCR Cycling

A common PCR cycling program usually starts with an initial dissociation step at 92 to 95°C for 2 to 5 min to ensure the complete separation of the DNA strands. Most PCR will reach sufficient amplification after 20 to 40 cycles of strand denaturation at 90 to 98°C for 10 s to 1 min, primer annealing at 55 to 70°C for 30 s to 1 min, and primer extension at 72 to 74°C for 1 min per kilobase of expected PCR product.

It is suggested that a final extension step of 5 to 10 min at 72°C will ensure that all amplicons are fully extended, although no solid evidence proves that this step is necessary. For example, the cycling program used to amplify the previously described lambda *cII* gene is as follows: initial denaturation for 4 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, then held at 4°C.

7.4.5 Verifying PCR Amplification

To measure the success of PCR amplification, 5 to 10 μL of the final PCR product is run on a 2% agarose gel and visualized by staining with ethidium bromide.

7.5 Results

7.6 Discussion

7.7 Questions

The critical questions are as follows which should be discussed in the Discussion section:

1. Is there a band on the gel?
2. 2) Is the band at the expected size?
3. (3) Are there any nonspecific bands beside the expected PCR band on the gel?
4. (4) Is there smear on the gel? A successful PCR amplification should display a single band with the expected size without nonspecific bands and smear.

7.8 References

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8. PREPARATION OF DNA FOR MORE ADVANCED APPLICATIONS (CUT FROM GEL AND PURIFY)

8.1 Introduction

Following digestion with appropriate restriction enzymes, the DNA of interest is subjected to electrophoresis on a preparative agarose gel. The portion of the gel containing the restriction fragment to be purified is then physically removed from the gel. This agarose slice is placed into a buffer-filled piece of dialysis tubing and again subjected to electrophoresis. The restriction fragment migrates out of the gel slice into the buffer, and the DNA is further purified and concentrated. This procedure is effective with fragment sizes ranging from 50 to 20,000 bp.

8.2 Objective

Isolation of DNA fragments from agarose gels. The isolated DNA fragments can be used for cloning, ligation reactions, PCR protocols, sequencing and so many other applications.

8.3 Theory

The DNA fragments are subjected to electrophoresis so that it came out of the agarose slice. The quality of the preparative DNA digest and the resolution of the preparative gels are critical for obtaining optimal yields of fragments from all protocols. If the preparative gel is overloaded, cross-contamination will occur as a small amount of each fragment becomes "trapped" in the other bands. This low level of contamination can be significant if the fragment is to be used for some cloning procedures, or as a probe in some hybridization. If it is necessary to obtain a large amount of highly pure fragment, the first preparation should be re-electrophoresed and repurified.

8.4 Materials

1. DNA fragment encoding sequence of interest
2. Ethidium bromide solution
3. TAE buffer pH
4. M NaCl
5. 100% and 70% ethanol
6. TE buffer, pH 8.0
7. Spectrapor 3 dialysis membrane tubing (11.5-mm diameter with MWCO of 3500; Baxter).

8.5 Experimental Protocol

1. Digest 0.1 to 25 ug DNA to completion with appropriate restriction enzyme as described before in experiment 6.
2. Load sample onto the appropriate agarose gel and subject to electrophoreseis.

3. After electrophoresis, stain the gel with ethidium bromide solution as described before and photograph the gel.
4. Using UV light for visualizing bands carefully cut out the target band with a scalpel.
5. Rinse dialysis tubing with TAE buffer.
6. Tightly tie off one end of the tubing with two knots. Slide the gel slice into the tubing.
7. Fill the tubing with TAE buffer until it is almost completely expanded. Knot or clip the top of the tubing closed.
8. Place the sealed dialysis bag in a horizontal gel electrophoresis apparatus. Fill the apparatus with 1x TAE buffer until the dialysis bag is just covered with solution.
9. Electroelute at a constant voltage of 2 V/cm between the two wires. For a 50- to 500-bp target fragment, electroelute 30 to 45 min. For a 500- to 2000-bp fragment, electroelute 2 hr. For a 2000- to 4000-bp fragment, electroelute 4 hr. For larger fragments, electroelute at 1 V/cm overnight.
10. After electroelution is complete, reverse the polarity of the electrodes and turn on voltage at 100 V for 30 sec.
11. Carefully open the top of the dialysis bag and collect TAE buffer with a Pasteur pipette. Massage gel slice out of the bag and wash the bag with a pipet of TAE buffer.
12. Add an equal volume of phenol/chloroform/isoamyl alcohol to the DNA solution to be purified in a 1.5-ml microcentrifuge tube.
13. Vortex vigorously for 10 sec and microcentrifuge 15 sec at maximum speed, room temperature.
14. Carefully remove the top (aqueous) phase containing the DNA using a 200 ml (or µl) pipette and transfer to a new tube. If a white precipitate is present at the aqueous/organic interface, repeat steps 12 to 10.
15. Add 1/10 vol. of 3 M sodium acetate, pH 5.2, to the solution of DNA. Mix by vortexing briefly or by flicking the tube several times with a finger.
16. Add 2 to 2.5 vol of ice-cold 100% ethanol. Mix by vortexing and place in crushed dry ice for 5 min or longer.
17. Microcentrifuge 5 min at maximum speed and remove the supernatant.
18. Add 1 ml room-temperature 70% ethanol. Invert the tube several times and microcentrifuge as in step 17.
19. Remove the supernatant as in step 17. Dry the pellet in a desiccator under vacuum or in a Speedvac evaporator.
20. Dissolve the dry pellet in an appropriate volume of water if it is going to be used for further enzymatic manipulations requiring specific buffers. Dissolve in TE buffer, pH 8.0, if it is going to be stored indefinitely.

8.6 Results

8.7 Discussion

8.8 Questions

1. Is agarose gel suitable for separating short DNA fragments?
2. Why do you reverse the electric current for 30 sec before the end of the run?
3. Write down the role of ethanol in precipitating nucleic acids? Can it be substituted?

8.9 References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. Current Protocols in Molecular Biology. (eds.) John Wiley & Sons, Inc. New York, 2003

9. DIGESTION OF DNA WITH RESTRICTION ENZYMES BAMHI UNDER DIFFERENT SALT CONCENTRATIONS

9.1 Introduction

Restriction endonucleases (RE) are enzymes, produced by bacterial cells that have the ability to cleave the sugar phosphate backbones in double stranded DNA at specific restriction sites. However, they do not cleave the host DNA as the restriction sites are methylated, and hence seem to function in host defense, as bacteria use them to cut DNA from foreign sources, like the viruses that infect them (i.e. bacteriophages). Each RE recognizes a specific sequence of 4-6 nucleotides in length. Table 6.1 presents examples of a few RE with their recognition sites.

Table 6.1: **Some RE and their restriction sites**

Restriction Endonuclease	**Microorganism Source**	**Restriction Site**
EcoRI	*Escherichia coli RY 13*	C-A-A-T-T-G G-T-T-A-A-C
Hind III	*Haemophilus influenzae Rd*	A-A-C-G-T-T T-T-G-C-A-A
Hpa I	*Haemophilus Para influenzae*	C-T-T-A-A-C G-A-A-T-T-C
Bam HI	*Bacillus amyloliquefaciens H.*	G-G-A-T-C-C C-C-T-A-G-G
Pst I	*Providencia stuarti I*	C-C-G-G G-G-C-C

Over 300 RE have been isolated and the nomenclature depends on the organism from which they are derived e.g. Bam HI from Bacillus amyloliquefaciens H, ECoR1 from E coli, Hind II and Hind III from Haemophilus influenzae, and XhoI from Xanthomonas holcicola. Like other enzymes each restriction enzyme functions best under optimal conditions of temperature, pH and ionic strength.

9.2 Theory

Restriction endonuclease cleavage is accomplished by incubating the genomic DNA or DNA fragments obtained following amplification using polymerase chain reaction (PCR) with the RE under appropriate experimental conditions. The RE restricts the DNA at sites where the specific sequence recognized by the RE are present, resulting in the production of different size fragments. These fragments can be separated on electrophoresis using agarose gel.

In this experiment the restriction of genomic DNA using BamHI will be investigated and the effect of different NaCl concentrations on the activity of BamHI will be studied.

9.3 Objective

To determine the optimum concentration of sodium chloride (NaCl) for the restriction enzyme digestion of DNA by BamHI.

9.4 Materials

9.4.1 Materials

1. DNA (genomic DNA or PCR product) in H_2O or TE buffer
2. Restriction endonuclease (BamHI)
3. 10X restriction endonuclease buffer
4. NaCl: 1M
5. 0.5 M EDTA, pH 8.0

9.4.2 Equipment

1. Automated pipettes
2. Weighing balance
3. pH meter
4. Water bath
5. Centrifuge

9.4.3 Glassware and Disposables

1. Autoclaved Tubes
2. Tips
3. Gloves
6. Centrifuge tubes

9.5 Experimental Protocol

1. Lable six clean microcentrifuge tubes, 1-3 in duplicates.
2. In the three microcentrifuge tubes pipet the following:

Solution	Tube 1	Tube 2	Tube 3
DNA solution	1 ul	1 ul	1 ul
10Xrestriction buffer	2 ul	2 ul	2 ul
NaCl solution	0	1 ul	2 ul
Water	17 ul	16 ul	15 ul

3. Add BamHI (3 U/µg DNA) and incubate the reaction mixture for 1 hr at 370 C in an incubator.
4. Stop the reaction by adding 0.5 µl of 0.5 M EDTA (12.5 mM final concentration), and prepare it for agarose gel electrophoresis by adding 5 µl of gel loading buffer.

9.6 Results

9.7 Discussion

Discuss the following points:

- Is the DNA digested, and if so how many fragments are produced and what are their sizes?
- Is the standard graph a straight line?
- What is the effect of NaCl exclusion and inclusion on the activity of BamHI?
- Did you fulfill your objectives?

9.8 References

1. Alberts, B.,Bray, D, Lewis, J., et al. **The Molecular Biology of the Cell.** Garland Publishing, New York.
2. Danna, AJ. Determination of fragment order through partial digests and multiple enzyme digests. Methods Enzymol. 1980; 65: 449-467.
3. Ausubel, F.M. Brent, R. Kingston, R.E. et al. (eds). Short Protocols in Molecular Biology. 1999 - John Wiley & sons, Inc. New York.

10.1. ELECTROPHORETIC SEPARATION OF DNA AND DNA FRAGMENTS IN AGAROSE GEL

10.1.1 Introduction

Agarose gel electrophoresis is the easiest and frequently used technique for separating and analyzing DNA. Gels allow separation and identification of nucleic acids based on migration under the influence of an electric charge. Migration of nucleic acid molecules in an electric field is determined by size and conformation, allowing nucleic acid fragments of different sizes to be separated. The purpose of the gel electrophoresis may also be to observe the DNA, to quantify it or to isolate a particular band.

10.1.2 Theory

Gel electrophoresis is an essential technique for analyzing DNA. When a charged molecule is placed in an electric field, it will migrate towards the electrode with the opposite charge. All nucleic acids carry a net negative charge due to the presence of phosphate in the structure. The amount of negative charge is almost the same in all DNA molecules, since all the nucleic acids carry the same amount of negative charge per unit size. Hence DNA will migrate towards the positive electrode (the red one) according to the size of the molecule. Smaller molecular weight DNA fragments will migrate faster than the larger ones. Agarose gel analysis is the most frequently used method for analyzing DNA fragments between 0.1 and 25 kb. Low agarose concentrations are used to separate large DNA fragments, while high agarose concentrations allow resolution of small DNA fragments. Talbe 9,1.1 lists the ideal concentration of agarose for different size DNA fragments.

The size of the separated DNA can be calculated by comparing the distance it migrated in the gel with standard molecular-weight markers.

Table 9,1.1 : Electrophoretic Superation of different sizes of DNA on different concentrations of Gel.

Agarose concentration (g%)	DNA fragment range (Kb)
0.3	5–60
0.5	1–30
0.7	0.8–12
1.0	0.5–10
1.2	0.4–7
1.5	0.2–3
2.0	0.1–2

10.1.3 Objectives

Separation of DNA using agarose gel electrophoresis can shed light into many applications. Electrophoretic separation of DNAfragments is carried out with the following objectives:

1. To calculate the molecular size of DNA fragment by comparing the separated band with known standard molecular weight marker.
2. To quantify DNA fragment by comparing the separated band with known quantity of DNA.
3. To obtain the purity of DNA preparation.
4. To Isolate a particular band for the purpose of purification.

10.1.4 Materials

10.1.4.1 Chemicals

1. Agarose, buffers
2. Ethidium bromide
3. Standard DNA ladder
4. DNA to be studied.

10.1.4.2 Equipment

1. Horizontal Electrophoresis apparatus
2. Power supply
3. Microwave
4. Transilluminator, and
5. photoimager machine.

10.1.4.3 Plastic wares:

1. Eppendorf tubes
2. Automatic pipettes
3. tips

10.1.4.4 Preparation of Solutions

A. Loading buffers

1. 25mg bromophenol blue
2. 4g sucrose
3. H2O to 10mL
4. Store at 4°C to avoid mould growing in the sucrose

B. Electrophoresis Running and gel buffer (TBE)

1. 10.9 g Tris base
2. 5.5 g Boric acid
3. 0.465 g EDTA

Dissolve the ingredients in 900 ml of distilled water. Adjust pH to about 8.3 using NaOH and make up to 1 L

10.1.5 Experimental Procedure

10.1.5.1 Pouring the gel

1. Prepare enough 1x electrophoresis buffer both to pour the gel and fill the electrophoresis tank. *The most commonly used buffers for agarose gel electrophoresis are TBE (Tris-borate–EDTA) and TAE (Tris-acetate–EDTA).*
2. Add an appropriate amount of agarose (depending on the concentration required) to an appropriate volume of electrophoresis buffer (depending on the type of electrophoresis apparatus being used) in a flask or bottle. T*he vessel should not be more than half full. Cover the vessel to minimize evaporation.*
3. Heat the slurry in a microwave or boiling water bath, swirling the vessel occasionally, until the agarose is dissolved. *Ensure that the lid of the flask is loose to avoid buildup of pressure. Be careful not to let the agarose solution boil over as it becomes super-heated. If the volume of liquid reduces* considerably during heating due to evaporation, make up to the original volume with distilled water. This will ensure that the agarose concentration is correct and that the gel and the electrophoresis buffer have the same buffer composition.
4. Cool the agarose to 55–60°C. Add the visualization dye, ethidium bromide (EtBr) (final concentration 0.5 ug/ml)
5. Pour the agarose solution onto the gel tray to a thickness of 3–5 mm. Insert the comb either before or immediately after pouring the gel. Leave the gel to set (30–40 min) to solidify. Ensure that there is enough space between the bottom of the comb and the glass plate (0.5–1.0 mm) to allow proper formation of the wells and avoid sample leakage. Make sure that there are no air bubbles in the gel or trapped between the wells.
6. Carefully remove the comb, from the gel. Fill the tank containing the gel with electrophoresis buffer. Add enough buffer to cover the gel with a depth of approximately 1 mm liquid above the surface of the gel. If too much buffer is used the electric current will flow through the buffer instead of the gel.

10.1.5.2 Preparation of samples:

Add 1 volume of gel loading buffer to 6 volumes DNA sample and mix. Samples should always be mixed with gel loading buffer prior to loading on a gel.

Gel loading buffer serves three main purposes:

1. To increase the density of the samples to ensure that they sink into the wells on loading.

2. To add color to the samples through use of dyes such as bromophenol blue or xylene cyanol, facilitating loading.
3. To allow tracking of the electrophoresis due to co-migration of the dyes with DNA fragments of a specific size. Do not use sample volumes close to the capacity of the wells, as samples may spill over into adjacent wells during loading. Be sure that all samples have the same buffer composition. High salt concentrations, for example in some restriction buffers, will retard the migration of the DNA fragments. Ensure that no ethanol is present in the samples, as this will cause samples to float out of the wells on loading.

10.1.5.3 Applying Samples and Running Gel Electrophoresis:

1. Apply samples in gel loading buffer to the wells of the gel. Prior to sample loading, remove air bubbles from the wells by rinsing them with electrophoresis buffer.Make sure that the entire gel is submerged in the electrophoresis buffer.To load samples, insert the pipette tip deep into the well and expel the liquid slowly. Take care not to break the agarose with the pipette tip.Once samples are loaded, do not move the gel tray/tank as this may cause samples to float out of the wells. Be sure to always include at least one lane of appropriate molecular weight markers.
2. Connect the electrodes to the Power pack, so that the DNA will migrate towards the anode (positive electrode, red electrode).
3. Turn on the power supply and run the gel at 1–10 V/cm until the dyes have migrated an appropriate distance. This will depend on the size of DNA being analyzed, the concentration of agarose in the gel, and the separation required. Avoid use of very high voltages which can cause trailing and smearing of DNA bands in the gel, particularly with high molecular weight DNA.

10.1.5.4 Visual Analysis of the gel

Ethidium bromide–DNA complexes display increased fluorescence compared to the dye in solution. This means that illumination of a stained gel under UV light (254–366 nm) allows bands of DNA to be visualized against a background of unbound dye. The gel image can be recorded by taking a Polaroid™ photograph or using a gel documentation system (a photo imager).

10.1.6 Results

10.1.7 Discussion:

1. Discuss the mobility of different sizes and shape of the DNA on the agarose gel and describe how the concentrations of the agarose gel affect the mobility of the DNA samples.
2. Why do you think selection of the right percentage of agarose gel is crucial in separation of the DNA sample?

10.1.8 Questions

1. Following electrophoresis of DNA fragments through an agarose gel, which band is closest to the positive electrode and which is the closest to the negative electrode?
2. Why should we use low agarose concentration gels to separate large DNA fragments?
3. Why do we add bromophenol blue to the loading buffer?
4. What precautions are necessary when using ethidium bromide and why?
5. Why should we add sucrose to the loading buffer?

10.1.9 References

1. A Guide to Analytical Gels: Qiagen Bench Guide *http://www1.qiagen.com/literature/render.aspx?id=23553*
2. Dale JW and von Schantz M. From Genes to Genomes, Concepts and applications of DNA technology. 2nd Ed. 2007, John Willey and Sons, INC.

10.2 Separation of PCR Product in Agarose Gel

10.2.1 Introduction

A normal way of analyzing PCR product is to separate the sample DNA in an agarose electrophoresis gel. This ascertains that only one fragment is obtained in each reaction.

10.2.2 Theory

When a DNA molecule is placed under an electric field, it will migrate towards the electrode with the opposite charge (the positive electrode, the red one) according to the size of the molecule. Smaller molecular size DNA fragments will migrate faster than the larger one. The size of PCR amplification product depends on how far is one primer from the other primer when annealed to the template DNA to be amplified.

10.2.3 Objectives of the experiment:

1. To Study the efficiency of PCR reaction.
2. To calculate the molecular size of the amplified DNA fragment by comparing the separated band with known standard molecular weight marker.

3. To Separate the amplified DNA fragment for further purification and cloning.

10.2.4 Materials Required

10.2.4.1 Chemicals

1. Agarose
2. Buffers
3. Ethidium bromide
4. Standard DNA ladder
5. PCR amplified DNA.

10.2.4.2 Equipment

1. Horizontal Electrophoresis apparatus
2. Power supply
3. Microwave
4. Transilluminator and photoimager machine.

10.2.4.3 Plastic Wares

1. Eppindorf tubes
2. Automatic pipettes.

10.2.4.4 Preparation of Solutions

A. Loading buffers

1. 25mg bromophenol blue
2. 4g sucrose
3. H2O to 10mL
4. Store at 4°C to avoid mould growing in the sucrose

B. Electrophoresis Running and gel buffer (TBE)

1. 9.29 g Tris base
2. 5.5 g Boric acid
3. 0.465 g EDTA
4. Dissolve the ingredients in 900 ml of distilled water. Adjust pH to about 8.3 using NaOH and make up to 1 L

10.2.5 Experimental Procedure

1. Weigh agarose powder, mix it with electrophoresis buffer to the desired concentration depending on the size of the PCR-amplified DNA fragment. Ex. To prepare 1% gel weight 1 g agarose and add 100 ml electrophoresis buffer (TBE). This concentration is suitable to fractionate a PCR-amplified fragment of 0.5 to 10 Kb. (Table 9.21).

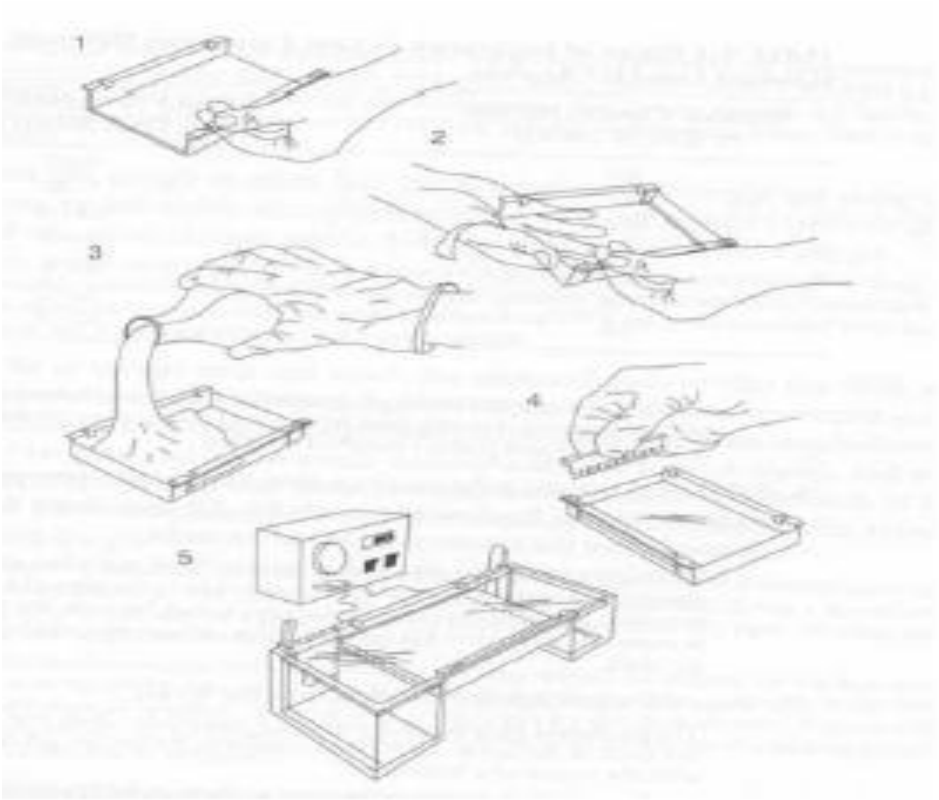

***Figure 2: Setting up Agarose Gel Electrophoresis Cell (Molecular Cloning, 3rd Edition, Sambrook and Russe*ll)**

Table 9.21 :

: **Agarose concentration (g/200 ml)**	**DNA fragment range (Kb)**
1.0	0.5–10
1.2	0.4–7
1.5	0.2–3
2.0	0.1–2

2. Heat the mixture in a microwave oven until completely melted. (It takes about 1 min).
3. Leave to cool to about 60°C and add ethidium bromide to final concentration of 0.5 mg/ml. Add 2 ml of 100 ml gel from stock containing 10 mg%.
4. Pour the solution into a casting tray containing a sample comb and allow solidifying at room temperature.
5. After the gel has solidified, remove the comb, and insert plastic tray containing the gel into the electrophoresis chamber.
6. Cover the gel with TBE buffer.
7. Mix six volumes of the DNA sample containing PCR-amplified fragment with one volume loading buffer (25 mg bromophenol blue, 4 g sucrose in10 mL H2O) and load onto the gel by pipetting into the sample wells.

8. Cover the apparatus, and connect the power supply at a voltage no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel). You can confirm that current is flowing by observing bubbles coming off the electrodes. DNA will migrate towards the positive electrode, which is usually colored red. Avoid use of very high voltages which can cause trailing and smearing of DNA bands in the gel, particularly with high-molecular-weight DNA.
9. Switch off the power when the dyes have migrated an appropriate distance (about 70% of the gel length).
7. The gel image can be recorded by exposing the gel to UV transiluminator (254–366 nm) which allow bands of DNA bound to ethidium bromide to be visualized against a background of unbound dye.
8. UV light can damage the eyes and skin. Always wear suitable eye and face protection when working with a UV light source.
9. UV light damages DNA. If DNA fragments are to be extracted from the gel, use a lower intensity UV source if possible and minimize exposure of the DNA to the UV light. Ethidium bromide is a powerful mutagen and is very toxic. Wear gloves and take appropriate safety precautions when handling.

10.2.6 Results

Calculate the size of the separated DNA fragment by comparing its size with the size of known standard.

Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the $\log_{10}$ of their molecular weight. In other words, if you plot the distance from the well that DNA fragments have migrated against the $\log_{10}$ of either their molecular weights or number of base pairs, a roughly straight line will appear.

10.2.7 Discussion

10.2.8 Questions

1. What is the function of sucrose in the loading buffer?
2. What is a mutagen?

10.2.9 References

http://www.vivo.colostate.edu/hbooks/genetics/biotech/gels/agardna.html

10.3 Separation of Plasmid and Genomic DNA in Agarose Gel

10.3.1 Introduction

Agarose gel electrophoresis is the easiest and commonest way of separating and analyzing DNA. Gels allow separation and identification of nucleic acids based on charge migration. Migration of nucleic acid molecules in an electric field is determined by size and conformation, allowing nucleic fragments of different sizes to be separated. The purpose of the gel might be to look at the DNA, to quantify it or to isolate a particular band.

10.3.2 Theory

Gel electrophoresis is a crucial technique for analyzing DNA. When a charged molecule is placed in an electric field, it will migrate towards the electrode with the opposite charge. All nucleic acids carry a net negative charge due to the presence of phosphate in its structure. The amount of negative charge is almost the same in all DNA molecules, since all the nucleic acids carry the same amount of negative charge per unit size. Hence DNA will migrate towards the positive electrode (the red one) according to the size of the molecule. Smaller molecular weight DNA will migrate faster than the larger one. Agarose gel analysis is the most commonly used method for analyzing DNA fragments between 0.1 and 25 kb. Low agarose concentrations are used to separate large DNA fragments, while high agarose concentrations allow resolution of small DNA fragments.

The size of the separated DNA can be calculated by comparing the distance it migrated in the gel with standard molecular-weight markers as described in the following table.

Agarose concentration (g%)	DNA fragment range (Kb)
0.3	5–60
0.5	1–30
0.7	0.8–12
1.0	0.5–10
1.2	0.4–7
1.5	0.2–3
2.0	0.1–2

10.3.3 Objectives

1. To calculate the molecular size of DNA fragment by comparing the separated band with known Standard Molecular weight marker.
2. Judging the quality and shape of DNA preparation.

10.3.4 Materials

10.3.4.1 Chemicals

1. Agarose
2. Buffers
3. Ethidium bromide
4. Standard DNA ladder

10.3.4.2 Equipment

1. Horizontal Electrophoresis apparatus
2. Power supply
3. Microwave
4. Transilluminator
5. Photoimager machin.

10.3.4.3 Plastic wares

1. Eppendorf tubes
2. Automatic pipettes.

10.3.4.4 Preparation of Solutions

A. Loading buffers

1. 25mg bromophenol blue
2. 4g sucrose
3. H2O to 10mL
4. Store at 4°C to avoid mould growing in the sucrose

B. Electrophoresis Running and gel buffer (TBE)

1. 10.9 g Tris base
2. 5.5 g Boric acid
3. 0.465g EDTA
4. Dissolve the ingredients in 900 ml of distilled water. pH to about 8.3 using NaOH and make up to 1 L.

10.3.5 Experimental Procedure:

1. Prepare agarose gel with adequate concentration as previously shown in experiments earlier on.
2. Mix six volumes of the genomic DNA sample with one volume loading buffer (25 mg bromophenol blue, 4 g sucrose in 10 mL H_2O) and load onto the gel by pipetting into the sample wells.
3. Cover the apparatus, and connect the power supply at a voltage no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel).

4. Switch off the power when the dyes have migrated an appropriate distance (about 70% of the gel length).
5. The gel image can be recorded by exposing the gel to UV transiluminator (254–366 nm) which allow bands of DNA bound to ethidium bromide to be visualized against a background of unbound dye.
6. Compare the migration of each DNA preparation and explain the results.

10.3.6 Results

10.3.7 Discussion

10.3.8 Questions

1. The electrophoresis apparatus creates an electrical field with positive and negative poles at the ends of the gel. DNA molecules are negatively charged. To which electrode pole of the electrophoresis field would you expect DNA to migrate? (+ or -)? Explain.
2. After DNA samples are loaded into the sample wells, they are "forced" to move through the gel matrix. What size fragments (large vs. small) would you expect to move toward the opposite end of the gel most quickly? Explain.

10.4 Effect of DNA Shape on DNA Separation on Agarose Gel

10.4.1Introduction

All nucleic acids are of very similar chemical composition and have a high charge-to-mass ratio. The migration on agarose electrophoresis gel depends primarily on their frictional coefficients, which are related to the length and conformation of the nucleic acid. There are three main conformations of plasmid dsDNA: supercoiled, circular (relaxed or nicked) and linear.

In dsDNA the two strands coil around each other once per turn of the helix (i.e. every ten base pairs). Supercoiled dsDNA is more compact and therefore has a lower frictional coefficient and hence it migrates faster during gel electrophoresis than the other forms.

10.4.2 Theory

All nucleic acids carry a net negative charge due to the presence of phosphate in its structure. The amount of negative charge is almost the same in all DNA molecules, since all the nucleic acids carry the same amount of negative charge per unit size. Hence DNA will migrate towards the positive electrode (the red one) according to the size of the molecule which is affected by the conformational 3D structure of the molecule. Smaller molecular sized DNA will migrate faster than the larger one.

10.4.3Objectives of the experiment

This experiment aims at studying the effect of DNA shape on the migration on agarose electrophoresis gel.

10.4.4 Materials

10.4.4.1 Chemicals

1. Agarose
2. Buffers
3. Ethidium bromide
4. Standard DNA ladder
5. Supercoiled circular (relaxed or nicked). And
6. linearized plasmid.

10.4.4.2 Equipment

1. Horizontal Electrophoresis apparatus
2. Power supply
3. Microwave
4. Transilluminator, and
5. Photoimager machin.

10.4.4.3 Plastic wares

1. Eppendorff tubes
2. Automatic pipettes.

10.4.4.4 Preparation of Solutions

A. Loading buffers

- 25mg bromophenol blue
- 4g sucrose
- H_2O to 10mL
- Store at 4°C to avoid mould growing in the sucrose

B. Electrophoresis Running and gel buffer (TBE)

- 10.9 g Tris base

- 5.5 g Boric acid
- 0.465 g EDTA

Dissolve the ingredients in 900 ml of distilled water. pH to about 8.3 using NaOH and make up to 1 L.

10.4.5 Experimental Procedure

1. Prepare agarose gel with adequate concentration as previously shown in Table above for the separation of DNA.
2. Mix six volumes of the DNA sample containing supercoiled, circular (relaxed or nicked) or linear with one volume loading buffer (25 mg bromophenol blue, 4 g sucrose in 10 mL H_2O) and load onto the gel by pipeting into the sample wells.
3. Cover the apparatus, and connect the power supply at a voltage no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel).
4. Switch off the power when the dyes have migrated an appropriate distance (about 70% of the gel length).
5. The gel image can be recorded by exposing the gel to UV transiluminator (254–366 nm) which allow bands of DNA bound to ethidium bromide to be visualized against a background of unbound dye.
6. Compare the migration of each DNA preparation and explain the results.

10.4.6 Results

10.4.7 Discussion

10.4.8 Questions

What would be a logical explanation as to why there is more than one band of DNA for each of the samples?

10.4.9 References:

Ninfa AJ; Ballou DP and Benore M. Fundamental Laboratory Approaches for Biochemistry and Biotechnology. 2nd Ed. John Willey and Sons, INC. 2009

11.1 RNA Extraction

11.1.1 Introduction

The difficulty in RNA isolation is that most ribonucleases are very stable and active enzymes that require no cofactors to function. The first step in all RNA isolation protocols therefore involves lysing the cell in a chemical environment that results in denaturation of ribonuclease. To avoid contamination problems, the following precautions can be taken:

1. ***Solutions***. Any water or salt solutions used in RNA preparation should be treated with the chemical diethylpyrocarbonate (DEPC). This chemical inactivates ribonucleases by covalent modification. Solutions containing Tris cannot be effectively treated with DEPC because Tris reacts with DEPC to inactivate it.
2. ***Glassware and plastic***. Autoclaving will not fully inactivate many RNases. Glassware can be baked at 300^{o}C for 4 hrs. Certain kinds of plasticware (e.g., some conical centrifuge tubes and pipettes) can be rinsed with chloroform to inactivate RNase. When done carefully, this rinse is an effective treatment. Keep in mind, however, that many plastics (e.g., gel boxes) will melt when treated with chloroform. Plastic ware straight out of the package is generally free from contamination and can be used as is.
3. Hands are a major source of contaminating RNase. **Wear gloves**.

11.1.2 Theory

Most procedures for isolating RNA from eukaryotic cells involve lysing and denaturing cells to liberate total nucleic acids. Additional steps are then required to remove DNA. This procedure allows rapid preparation of total cytoplasmic RNA by using a nonionic detergent to lyse the plasma membrane, leaving the nuclei intact. The nuclei and hence the bulk of the cellular DNA are then removed with a simple brief centrifugation. It is fast and streamlined, designed for preparing total cytoplasmic RNA from many cultures simultaneously for nuclease protection analysis.

11.1.3 Objective

1. To analysis gene expression by measuring the amount of RNA.
2. To determine the structure and amount of the RNA produced from a gene, in order to elucidate the regulatory properties of the gene.

11.1.4 Materials

11.1.4.1 Materials

A. Chemicals

1. Denaturing solution

Stock solution: Mix 293 ml water, 17.6 ml of 0.75 M sodium citrate, pH 7.0, and 26.4 ml of 10% (w/v) *N*-lauroylsarcosine (Sarkosyl). Add 250 g guanidine thiocyanate and stir at 60° to 65°C to dissolve. Store up to 3 months at room temperature.

Working solution: Add 0.35 ml 2-mercaptoethanol (2-ME) to 50 ml of stock solution. Store up to 1 month at room temperature. Final concentrations are 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, and 0.1 M 2-ME.

2. Sodium acetate, 2 M

Add 16.42 g sodium acetate (anhydrous) to 40 ml water and 35 ml glacial acetic acid. Adjust solution to pH 4 with glacial acetic acid and dilute to 100 ml final with water (solution is 2 M with respect to sodium ions). Store up to 1 year at room temperature.

3. Water-saturated phenol

Dissolve 100 g phenol crystals in water at 60° to 65°C. Aspirate the upper water phase and store up to 1 month at 4°C. Do not use buffered phenol in place of water-saturated phenol.

4. Diethylpyrocarbonate (DEPC) treatment of solutions

Add 0.2 ml DEPC to 100 ml of the solution to be treated. Shake vigorously to get the DEPC into solution. Autoclave the solution to inactivate the remaining DEPC. Many investigators keep the solutions they use for RNA work separate to ensure that "dirty" pipets do not go into them. CAUTION: Wear gloves and use a fume hood when using DEPC, as it is a suspected carcinogen.

11.1.4.2 Equipment

Sorvall SS-34 rotor (or equivalent)

11.1.4.3 Glassware

1. Glass Teflon homogenizer
2. 5-ml polypropylene centrifuge tube

11.1.5 Experimental Protocol

Use deionized, distilled water in all recipes and protocol steps.

1. Add 1 ml denaturing solution per 100 mg tissue and homogenize with a few strokes in a glass Teflon homogenizer.
2. Transfer the homogenate into a 5-ml polypropylene tube. Add 0.1 ml of 2M sodium acetate, pH 4, and mix thoroughly by inversion. Add 1 ml water-saturated phenol, mix thoroughly, and add 0.2 ml of 49:1 chloroform/isoamyl alcohol. Mix thoroughly and incubate the suspension 5 min at 0^{o}C to 4^{o}C. Make sure that caps are tightly closed when mixing.
3. Centrifuge 5 min at 10,000x g (9000 rpm in SS-34 rotor), 4^{o}C. Transfer the upper aqueous phase to a clean tube. The upper aqueous phase contains the RNA, whereas the DNA and proteins are in the interphase and lower organic phase.

4. Precipitate the RNA by adding 1 ml (1 vol.) of 100% isopropanol. Incubate the samples 10 min at −20°C. Centrifuge 10 min at 10,000x g, 4°C, and discard supernatant
5. Dissolve the RNA pellet in 0.3 ml denaturing solution and transfer into a 1.5-ml microcentrifuge tube.
6. Precipitate the RNA with 0.3 ml (1 vol) of 100% isopropanol for 10 min at −20oC. Centrifuge 10 min at 10,000x g, 4oC, and discard supernatant
7. Resuspend the RNA pellet in 75% ethanol, vortex, and incubate 10 min at room temperature to dissolve residual amounts of guanidine contaminating the pellet.
8. Centrifuge 5 min at 10,000x g, 4oC, and discard supernatant. Dry the RNA pellet for 5 min.
9. Dissolve the RNA pellet in 100 to 200 µl DEPC-treated water
10. Quantitate RNA by diluting 5 µl in 1 ml alkaline water and reading the A260 and A280.

11.1.6 Results

11.1.7 Discussion

Discuss the results and answer the following questions:

1. What is the importance of using guanidine thiocyanate?
2. Why RNA is extracted into one phase using phenol/chloroform while DNA is present in another phase?
3. Is the homogenization step of any use?
4. Identify key steps in RNA isolation?

11.1.8 References

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11.2 RNA Electrophoresis

11.2.1 Introduction

The integrity and size distribution of total RNA can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining. The respective ribosomal bands should appear as sharp bands on the stained gel. 28S ribosomal RNA bands should be present with intensity approximately twice that of the 18S rRNA band. If the ribosomal bands in a given lane are not sharp, but appear as a smear of smaller sized RNAs, it is likely that the RNA sample suffered major degradation during preparation.

11.2.2 Theory

Formaldehyde agarose gels allow separation and identification of RNA based on charge migration. Unlike DNA, RNA has a high degree of secondary structure, making it necessary to use a denaturing gel. Formaldehyde in the gel disrupts secondary RNA structure so that RNA molecules can be separated by their migration under an electric field. In an electric field, nucleic acid molecules migrate towards the anode due to negatively charged phosphates along the backbone. The migration of denatured RNA molecules is determined by their size; however, the relationship between the fragment size and rate of migration is nonlinear. Agarose gel analysis is the most commonly used method for analyzing RNA species, which generally correspond in size to the resolution range of an agarose gel. Small RNA fragments, such as tRNAs or 5S rRNAs, can be analyzed by polyacrylamide gel electrophoresis.

11.2.3 Objective

1. The objective of this experiment is to give the practice for the students How to run an RNA Agarose gel.
2. To find out the correct concentration of gel for the separation of RNA.

11.2.4 Materials

A. Chemicals

1. FA gel

- Agarose 1.0 – 1.2 g
- 10x FA Gel buffer 10 ml
- Add RNase-free water to 100 ml

2. FA Gel Buffer (10x)

Per Litre

- 20 mm mM MOPs 41.9 g
- 5 mM Sodium Acetate 6.8 g
- 0.5 M EDTA, pH 8.0 20 ml
- Adjust pH to 7.0 with NaOH

- Made in RNase free water

3. FA gel running Buffer (10x)

Per Litre

- 10x FA get buffer 100 ml
- 37% (11.2.3 M) Formaldehyde 20 ml
- RNase free water 880 ml

4. RNA Loading 5x Buffer (10x))

Per Litre

- Bromophenol blue 25 mg
- 0.5 M EDTA, pH 8.0 80 µl
- 37% (11.2.3 M) Formaldehyde 750 µl
- Glycerol 2 ml
- Formamide 3.084 ml
- 10x FA Gel Buffer 4 m

B. Equipments

Electrophoresis Apparatus (Bio-Rad or any suitable one)

11.2.5 Experimental Procedure

11.2.5.1 Pouring the gel

1. Prepare enough 10x FA gel buffer to pour the gel and to make enough FA gel running buffer to fill the electrophoresis tank.
2. Mix an appropriate amount of agarose, 10x FA gel buffer, and RNase-free water in a flask or bottle.
3. Heat the mixture in a microwave or boiling water bath, swirling the vessel occasionally until the agarose is dissolved. Ensure that the lid of the flask is loose to avoid buildup of pressure. Be careful not to let the agarose solution boil over as it becomes super-heated. If the volume of liquid reduces considerably during heating due to evaporation, make up to the original volume with RNase-free distilled water. This will ensure that the agarose concentration is correct.
4. Cool the agarose to 65–70°C in a water bath. Stir or swirl occasionally to prevent uneven cooling.
5. After cooling, add 1.8 ml of 37% (11.2.3 M) formaldehyde and 1 µl of a 10 mg/ml ethidium bromide stock solution. Formaldehyde is toxic. Use a fume hood to avoid inhalation. Wear gloves and take appropriate safety precautions when handling. Make sure that the solution has cooled sufficiently before adding formaldehyde and

ethidium bromide. Formaldehyde is volatile and may evaporate if added to a solution that is too hot. Ethidium bromide in the gel allows visualization of the RNA with UV light. Ethidium bromide is toxic and a powerful mutagen. Wear gloves and take appropriate safety precautions when handling. Use of nitrile gloves is recommended as latex gloves may not provide full protection. After use, ethidium bromide solutions should be decontaminated as described in commonly used manuals. Stock solutions of ethidium bromide (generally 10 mg/ml in water) should be stored at 2–8°C in a dark bottle or a bottle wrapped in aluminum foil.

6. Pour the agarose solution onto the gel tray in a fume hood to a thickness of 3–5 mm. Insert the comb either immediately before or immediately after pouring. Let the gel set for at least 30 min. Ensure that there is enough space between the bottom of the comb and the gel tray (0.5–1.0 mm) to allow proper well formation and avoid sample leakage. Make sure that there are no air bubbles in the gel or trapped between the wells. Air bubbles can be carefully removed with a Pasteur pipetts before the gel sets. Thicker gels can be used to increase the amount of sample volume that can be loaded. Thinner gels generally transfer better in northern blotting, but smaller sample volumes can be used. The thickness of the comb affects the sharpness of bands in the gel. A thinner comb gives sharper bands, but less sample can be loaded per well.
7. Leaving the comb in the gel, place the gel in the electrophoresis tank. Fill the tank with 1x FA gel running buffer. Add enough buffers to cover the gel with approximately 1 mm of liquid above the surface of the gel. If too much buffer is used, the electric current will flow through the buffer instead of the gel.
8. Carefully remove the comb from the gel. Prior to running, let the gel equilibrate in 1x FA gel running buffer for at least 30 min.

11.2.5.2 Running and analyzing formaldehyde agarose gels for RNA analysis RNA loading buffer

RNA loading buffer must be added to samples before oading them on a gel. The loading buffer serves three main purposes:

1. To denature the RNA sample prior to loading.
2. To increase the density of the samples to ensure that they sink into the wells on loading.
3. To add color to the samples through the use of dyes, facilitating loading and visualization on the gel while running. A key feature of the concentrated RNA loading buffer is that it allows a larger volume of RNA sample to be loaded onto the gel than conventional protocols allow.

11.2.5.3 Electrophoresis buffers

RNA gels are run at a lower pH than DNA gels since RNA has a lower pKa than DNA. Furthermore, unlike DNA, RNA is susceptible to alkali cleavage at high pH. RNA gels should therefore be run at neutral

pH. MOPS (3-[N morpholino] propanesulfonic acid) is the most commonly used buffer for RNA gels due to its high buffering capacity at pH 7.0. Formaldehyde is included in the running buffer to keep the RNA denatured. Formaldehyde is also added to the agarose gel. Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% SDS), thoroughly rinsed with RNase-free water, and then rinsed with ethanol (Plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier's instructions) and allowed to dry.

11.2.5.4 Sample preparation for Electrophoresis

1. Add 1 volume of 5x RNA loading buffer to 4 volumes of RNA sample (for example, 5 µl of loading buffer and 20 µl of RNA) and mix. Samples should always be mixed with RNA loading buffer prior to loading on a gel. Do not use sample volumes close to the capacity of the wells as samples may spill over into adjacent wells during loading. Be sure that all samples have the same buffer composition. High salt concentrations will retard the migration of RNA molecules. Ensure that no ethanol is present in the samples, for example, carried over from purification procedures. Ethanol may cause samples to float out of the wells on loading.
2. To denature RNA, incubate for 3–5 min at 65°C. Chill on ice.

11.2.5.5 Electrophoresis

1. Apply denatured samples to the wells of the gel. The gel should be submerged in electrophoresis buffer in the electrophoresis tank prior to loading. Prior to sample loading, remove air bubbles from the wells by rinsing them with electrophoresis buffer. Make sure that the entire gel is submerged in the FA gel running buffer. To load samples, insert the pipet tip deep into the well and expel the liquid slowly. Take care not to break the agarose with the pipet tip. Once samples are loaded, do not move the gel tray/tank as this may cause samples to float out of the wells. Be sure to include at least one lane of appropriate molecular weight markers.

2. Connect the electrodes of the electrophoresis apparatus so that the RNA will migrate towards the anode (usually red). The electrophoresis apparatus should always be covered to protect against electric shock. Run the gel in a fume hood to avoid exposure to formaldehyde fumes from the gel and running buffer.

3. Turn on the power supply, and run the gel at 5–7 V/cm until the bromophenol blue dye has migrated approximately 2/3 of the way through the gel. Avoid use of high voltages, which can cause trailing and smearing of RNA bands. Monitor the temperature of the buffer periodically during the run. High temperature can cause partial melting of the gel and distortion of the bands. If the buffer becomes significantly heated, reduce the voltage.

11.2.5.6 Precautions

Ethidium bromide is toxic and a powerful mutagen. Wear gloves and take appropriate safety precautions when handling. Use of nitrile gloves is recommended, as latex gloves may not provide full protection. After use, ethidium bromide solutions should be decontaminated. Stock solutions of ethidium bromide (generally 10 mg/ml in water) should be stored at 2–8°C in a dark bottle or a bottle wrapped in aluminum foil.

11.2.5.7 Visualization

Ethidium bromide in the gel allows visualization of the RNA with UV light. Ethidium bromide–RNA complexes display increased fluorescence compared to the uncomplexed dye in solution. This means that illumination of a stained gel under UV light (254–366 nm) allows bands of RNA to be visualized against a background of unbound dye. The gel image can be recorded by taking a Polaroid™ photograph or using a gel documentation system. UV light can damage the eyes and skin. Always wear suitable eye and face protection when working with a UV light source. UV light damages RNA. If RNA fragments are to be extracted from the gel, use a lower intensity UV source if possible, and minimize exposure of RNA to the UV light.

11.2.6 Results

1. Identify bands from the total RNA extract on the photograph of the agarose formaldehyde gel.
2. Estimate the molecular weight of identified RNA bands.

11.2.7 Discussion

11.2.8 Questions

1. Why do we use formaldehyde in an RNA gel?
2. What is meant by RNA denaturation?
3. Which was easier DNA or RNA electrophoresis?, Why?

11.2.9 References:

1. Qiagen Bench Guide

12. DNA FINGER PRINTING

12.1 Introduction

The genome in all humans is the same and so is the gene map. However, individual differs in one or more band pairs in the non-coding sequence at millions of sites on the DNA and makes every person a very unique individual.

Using these sequences, every person could be identified solely by the sequence of their base pairs. These patterns do not, however, give an individual "fingerprint," but they are able to determine whether two DNA samples are from the same person, related people, or non-related people. Scientists use a small number of sequences of DNA that are known to vary among individuals a great deal, and analyze those to get a certain probability of a match.

DNA fingerprinting requires the use of several biotechnological techniques, and can vary among different DNA fingerprinting protocols. One way to make a DNA fingerprint involves the use of restriction enzyme digestion, and agarose gel electrophoresis. In order to understand DNA fingerprinting as in its entirety, it is important to first understand each of these constitutive parts.

Restriction enzyme can be used to identify and cleave molecules of DNA at a specific restriction sites. Because DNA from any two individuals is unique, the digestion of every individual's DNA will result in a unique set of restriction fragments. These restriction fragments can be compared between individuals to identify restriction fragment length polymorphisms (RFLPs). These RFLPs are the basis of one type of DNA fingerprint.

12.2 Theory

Although 99.9% of human DNA sequences are the same in every person, enough of the DNA is different to distinguish one individual from another. DNA profiling uses repetitive ("repeat") sequences that are highly variable, called variable number of tandem repeats (VNTR). VNTRs loci are very similar between closely related humans, but so variable that unrelated individuals are extremely unlikely to have the same VNTRs. When genomic DNA of certain organism is subjected to restriction enzyme digestion and separated by agarose gel electrophoresis, we can see different length RFLPs characteristic for that person. If we use DNA from different individuals and cut it with the same restriction enzyme we will see different length RFLPs. DNA fingerprinting is used in medical and forensic procedures, as well as in paternity determinations to discern genetic relationships between individuals at the molecular level.

12.3 Objectives

This experiment aims to explain how restriction enzymes cleave DNA (Note: this was shown before), how electrophoresis is used to separate and visualize DNA fragments, (Note: this was shown before), and how these techniques be combined to obtain a DNA fingerprint. Students will understand the process of restriction enzyme digestion and gel electrophoresis (Note: this was shown before), and how it is used to analyze DNA. Students will understand impact of DNA technology on society.

12.4 Material

12.4.1 Chemicals

1. Agarose
2. Buffers, Ethedium bromide
3. Standard DNA ladder
4. Different DNA preparations from different organisms
5. Restriction enzyme (eg. Alu-I).

12.4.2 Equipment

1. Horizontal Electrophoresis apparatus
2. Power supply
3. Microwave
4. Transilluminator, and
5. Photoimager machin.

12.4.3 Plastic wares

1. Eppendorf tubes
2. Automatic pipettes.

12.4.4. Preparation of Solutions

A. Loading buffers

- 25mg bromophenol blue
- 4g sucrose
- H_2O to 10mL
- Store at 4°C to avoid mould growing in the sucrose

B. Electrophoresis Running and gel buffer (TBE)

- 10.9 g Tris base
- 5.5 g Boric acid
- 0.465 g EDTA
- Dissolve the ingredients in 900 ml of distilled water. pH to about 8.3 using NaOH and make up to 1 L.

12.5 Experimental Procedure

12.5.1 Digestion of DNA Samples by Alu-I Enzyme

1. Label each eppendorff tube by giving a characteristic name.
2. Pipette 10 µl of each DNA sample from the stock tubes and transfer to the corresponding tubes. Use a separate tip for each DNA sample. Make sure the sample is transferred to the bottom of the tubes.

3. Add the buffer and enzyme (10 µl of enzyme/buffer) into the bottom of each tube.
4. Cap the tubes and mix the components by gently flicking the tubes with your finger. If a microcentrifuge is available, pulse spin in the centrifuge to collect all the liquid in the bottom of the tube. Otherwise, tap the tube on a tabletop.
5. Incubate for 45 min at 37 °C.
6. After the incubation period, remove the tubes from the incubator and go on to electrophoresis experiment.

12.5.2 Separation the digested DNA on Agarose electrophoresis gel

1. Prepare agarose gel with adequate concentration as previously shown in table 1.
2. Mix six volumes of the DNA sample containing DNA with one volume loading buffer (25 mg bromophenol blue, 4 g sucrose in10 mL H_2O) and load onto the gel by pipe ting into the sample wells.
3. Cover the apparatus, and connect the power supply at a voltage no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel).
4. Switch off the power when the dyes have migrated an appropriate distance (about 70% of the gel length).
5. The gel image can be recorded by exposing the gel to UV transiluminator (254–366 nm) which allow bands of DNA bound to ethidium bromide to be visualized against a background of unbound dye.
6. Compare the migration of each DNA preparation and explain the results.

12.6 Results

12.7 Discussion

12.8 Questions

1. Based on your analysis of the gel, what is your conclusion about the DNA samples in the photograph (gel)?
2. Do any of the samples seem to be from the same source? If so, which ones? Describe the evidence that supports your conclusion.
3. How many fragments were pr
4. oduced by the restriction enzyme *Alu* I?
5. What would be a logical explanation as to why there is more than one band of DNA for each of the samples?
6. Which of the DNA samples have the same number of restriction sites for the restriction endonucleases used? Write the lane numbers.
7. Which sample has the smallest DNA fragment?

12.9 References

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4. Sambrook J, Fritsch EF and Maniatis T. Molecular Cloning: A Laboratory Manual, 2nd Edition. Cold Spring Harbor Laboratory Press. 1989.