The Neotia University

Paper: Molecular Biology Practical

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Contents

Sl No.	Name of Experiment	Page No.
1	Preparation of solutions for Molecular Biology experiments.	3-13
2	Isolation of Genomic DNA from blood / plant cell / bacteria (any one)	14-16
3	Isolation of Plasmid DNA	17-20
4	Agarose gel electrophoresis of genomic DNA / Plasmid DNA	21-23
5	Preparation of restriction enzyme digests of DNA samples	24-26
6	Spectroscopic analysis of DNA/RNA	27-28

Practical 1: Preparation of solutions for Molecular Biology experiments.

Objective: Demonstration the process of different solution preparation and disposal for molecular biology experiments.

General Laboratory Procedures, Equipment Use, and Safety Considerations

I. Safety Procedures

II. Preparation of Solutions

III. Disposal of Buffers and Chemicals

IV. Equipment

V. Working with DNA

I. Safety Procedures

A. Chemicals

A number of chemicals used in any molecular biology laboratory are hazardous. All manufacturers of hazardous materials are required by law to supply the user with pertinent information on any hazards associated with their chemicals. This information is supplied in the form of Material Safety Data Sheets or MSDS. This information contains the chemical name, CAS#, health hazard data, including first aid treatment, physical data, fire and explosion hazard data, reactivity data, spill or leak procedures, and any special precautions needed when handling this chemical. A file containing MSDS information on the hazardous substances should be kept in the lab. In addition, MSDS information can be accessed on World Wide Web. You are strongly urged to make use of this information prior to using a new chemical and certainly in the case of any accidental exposure or spill. The instructor/lab manager must be notified immediately in the case of an accident involving any potentially hazardous reagents.

The following chemicals are particularly noteworthy:

Phenol - can cause severe burns

Acrylamide - potential neurotoxin

Ethidium bromide - carcinogen

These chemicals are not harmful if used properly: always wear gloves when using potentially hazardous chemicals and never mouth-pipet them. If you accidentally splash any of these chemicals on your skin, immediately rinse the area thoroughly with water and inform the instructor. Discard the waste in appropriate containers.

B. Ultraviolet Light

Exposure to ultraviolet light can cause acute eye irritation. Since the retina cannot detect UV light, you can have serious eye damage and not realize it until 30 min to 24 hours after exposure. Therefore, always wear appropriate eye protection when using UV lamps.

C. Electricity

The voltages used for electrophoresis are sufficient to cause electrocution. Cover the buffer reservoirs during electrophoresis. Always turn off the power supply and unplug the leads before removing a gel.

D. General Housekeeping

All common areas should be kept free of clutter and all dirty dishes, electrophoresis equipment, etc. should be dealt with appropriately. Since you have only a limited amount of space to call your own, it is to your advantage to keep your own area clean. Since you will use common facilities, all solutions and everything stored in an incubator, refrigerator, etc. must be labeled. In order to limit confusion, each person should use his initials or other unique designation for labeling plates, etc. Unlabeled material found in the refrigerators, incubators, or freezers may be destroyed. Always mark the backs of the plates with your initials, the date, and relevant experimental data, e.g. strain numbers.

II. Preparation of Solutions

A. Calculation of Molar, % and "X" Solutions .

1. A molar solution is one in which 1 liter of solution contains the number of grams equal to its molecular weight. Ex. 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

2. Percent solutions. Percentage (w/v) = weight (g) in 100 ml of solution; Percentage (v/v) = volume (ml) in 100 ml of solution. Ex. To make a 0.7% solution of agarose in TBE buffer, weight 0.7 of agarose and bring up volume to 100 ml with TBE buffer.

3. "X" Solutions. Many enzyme buffers are prepared as concentrated solutions, e.g. 5X or 10X (five or ten times the concentration of the working solution) and are then diluted such that the final concentration of the buffer in the reaction is 1X. Ex. To set up a restriction digestion in 25 µ l, one would add 2.5 µ l of a 10X buffer, the other reaction components, and water to a final volume of 25 µ l.

B. Preparation of Working Solutions from Concentrated Stock Solutions .

Many buffers in molecular biology require the same components but often in varying concentrations. To avoid having to make every buffer from scratch, it is useful to prepare several concentrated stock solutions and dilute as needed. Ex. To make 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: C i x V i = C f x V f , where C i = initial concentration, or conc of stock solution; V i = initial vol, or amount of stock solution needed C f = final concentration, or conc of desired solution; V f = final vol, or volume of desired solution

C. Steps in Solution Preparation:

Refer to a laboratory reference manual for any specific instructions on preparation of the particular solution and the bottle label for any specific precautions in handling the chemical. Weigh out the desired amount of chemical(s). Use an analytical balance if the amount is less than 0.1 g. Place chemical(s) into appropriate size beaker with a stir bar. Add less than the required amount of water. Prepare all solutions with double distilled water When the chemical is dissolved, transfer to a graduated cylinder and add the required amount of distilled water to achieve the final volume. An exception is in preparing solutions containing agar or agarose. Weigh the agar or agarose directly into the final vessel. If the solution needs to be at a specific pH, check the pH meter with fresh buffer solutions and follow instructions for using a pH meter. Autoclave, if possible, at 121 deg C for 20 min. Some solutions cannot be autoclaved, for example, SDS. These should be filter sterilized through a 0.22 μ m or 0.45 μ m filter. Media for bacterial cultures must be autoclaved the same day it is prepared, preferably within an hour or two. Store at room temperature and check for contamination prior to use by holding the bottle at eye level and gently swirling it Solid media for bacterial plates can be prepared in advance, autoclaved, and stored in a bottle. When needed, the agar can be melted in a microwave, any additional components, e.g. antibiotics, can be added and the plates can then be poured.

Concentrated solutions, e.g. 1M Tris-HCl pH=8.0, 5M NaCl, can be used to make working stocks by adding autoclaved double-distilled water in a sterile vessel to the appropriate amount of the concentrated solution.

D. 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 degrees C for 1 hour. For experiments with RNA, glassware and solutions are treated with diethyl-pyrocarbonate to inhibit RNases which can be resistant to autoclaving. Plastic ware such as pipets 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 you are using are resistant to the chemicals used in your experiment. Micro pipet tips and microfuge tubes should be autoclaved before use.

III. Disposal of Buffers and Chemicals

Any uncontaminated, solidified agar or agarose should be discarded in the trash, not in the sink, and the bottles rinsed well.

Any media that becomes contaminated should be promptly autoclaved before discarding it. Petri dishes and other biological waste should be discarded in Biohazard containers which will be autoclaved prior to disposal.

Organic reagents, e.g. phenol, should be used in a fume hood and all organic waste should be disposed of in a labeled container, not in the trash or the sink.

Ethidium bromide is a mutagenic substance that should be treated before disposal and should be handled only with gloves. Ethidium bromide should be disposed of in a labeled container.

Dirty glassware should be rinsed, all traces of agar or other substance that will not come clean in a dishwasher should be removed, all labels should be removed (if possible), and the glassware should be placed in the dirty dish bin. Bottle caps, stir bars and spatulas should not be placed in the bins but should be washed with hot soapy water, rinsed well with hot water, and rinsed three times with distilled water.

IV. Equipment

A. General Comments

It is to everyone's advantage to keep the equipment in good working condition. As a rule of

thumb, don't use anything unless you have been instructed in the proper use. This is true not only for equipment in the lab but also departmental equipment. Report any malfunction immediately. Rinse out all centrifuge rotors after use and in particular if anything spills. Please do not waste supplies - use only what you need. If the supply is running low, please notify either the instructor/lab manager before the supply is completely exhausted. Occasionally, it is necessary to borrow a reagent or a piece of equipment from another lab. Except in an emergency, notify the instructor.

B. Micro pipettors

Most of the experiments you will conduct in this laboratory will depend on your ability to accurately measure volumes of solutions using micro pipettors. The accuracy of your pipetting can only be as accurate as your pipettor and several steps should be taken to insure that your pipettes are accurate and are maintained in good working order. Each pair of students will be assigned a set of pipettors and upon receipt, they should be labeled with the students' name. They should then be checked for accuracy following the instructions given by the instructor. If they need to be recalibrated, do so. We have Tarson pipettors. Since the pipettors will use different pipet tips, make sure that the pipet tip you are using is designed for your pipettor. DO NOT DROP IT ON THE FLOOR. If you suspect that something is wrong with your pipettor, first check the calibration to see if your suspicions were correct, then notify the instructor.

C. Using a pH Meter

Biological functions are very sensitive to changes in pH and hence, buffers are used to stabilize the pH. A pH meter is an instrument that measures the potential difference between a reference electrode and a glass electrode, often combined into one combination electrode. The reference electrode is often AgCl 2. An accurate pH reading depends on standardization, the degree of static charge, and the temperature of the solution.

Operation of pH Meter:

Expose hole on side of electrode by sliding the collar down. Make sure there is sufficient electrode filling solution in the electrode (it should be up to the hole). If not, fill with ROSS filling solution only (Do not use any filling solution containing silver (Ag).

Ensure that sample to be pHed is at room temperature and is stirring gently on the stir plate.

Calibrate the pH meter with the two solutions that bracket the target pH - 4 and 7 or 7 and 10 as follows:

Press the CAL key to initialize the calibration sequence. The last calibration range will be displayed (e.g. 7-4). Press YES to accept or use the scroll keys to select a different range. Press YES to accept.

The number 7 will light up on the left hand side of the screen indicating that the meter is ready to accept the pH 7 standard buffer. Rinse off electrode and place in fresh pH 7 standard buffer solution. The READY light will come on when the value has stabilized. Press YES to accept the value.

The number 4 (or 10) will light up next indicating that the meter is ready to accept the pH 4 (or 10) standard buffer solution. Rinse off electrode and place in fresh pH 4 standard buffer solution. The READY light will come on when the value has stabilized. Press YES to accept the value.

SLP will be displayed. The meter will then go MEASURE mode.

Rinse electrode and place into sample. The READY light is displayed when signal is stable.

D. Autoclave Operating Procedures

Place all material to be autoclaved in a autoclavable tray. All items should have indicator tape. Separate liquids from solids and autoclave separately. Make sure lids on all bottle are loose. Do not crowd large number of items in tray- in order for all items to reach the appropriate

temperature, one must allow sufficient air/steam circulation.

Make sure chamber pressure is at 0 before opening the door.

Place items to be autoclaved in the autoclave and close the door. Some autoclaves require that you also lock the door after it's closed.

Set time - typically 20 minutes.

Temperature should be set at 121 deg C already, but double-check and change if necessary.

Set cycle: If liquid, set "liquid cycle" or "slow exhaust". If dry, set "dry cycle" or "fast exhaust" + dry time.

Start the cycle. On some autoclaves, the cycle starts automatically at step 5. On others, turn to "sterilize".

At the end of the cycle, check that: a. the chamber pressure is at 0; b. the temp is <100 deg C

Open door.

Remove contents using gloves and immediately tighten all caps.

E. Operating Instructions for Spectrophotometer - To measure the absorbance of a solution in the short-wave range (<300 nM) use the quartz cuvettes. Disposable plastic cuvettes are available for reading in the visible range.

Turn the spectrophotometer on - the switch is on the right in the back.

Allow the instrument to calibrate. Do not open the chamber during this time. The deuterium lamp is OFF by default. To read absorbance in the UV range, turn the deuterium lamp on as follows after the machine has completed its calibration: Depress the function key until Fn5 is displayed. Press the mode key until d2on is displayed. Press enter. For best accuracy, the deuterium lamp should be warmed up for 20 minutes.

Press the function key until Fn0 is displayed. Press enter. Using the up or down arrow keys, enter in the desired wavelength.

Prepare a reference cuvette containing the same diluent as your sample.Prepare your sample.

Place the reference cuvette in cell #1 and place your samples in cells #2-6.

Press the cell key until cell #1 is in position. Press the Set Reference key to blank against the appropriate buffer. Press the cell key to advance to read the next sample.

V. Working with DNA

A. Storage

The following properties of reagents and conditions are important considerations in processing and storing DNA and RNA. Heavy metals promote phosphodiester breakage. EDTA is an excellent heavy metal chelator. Free radicals are formed from chemical breakdown and radiation and they cause phosphodiester breakage. UV light at 260 nm causes a variety of lesions, including thymine dimers and cross-link. Biological activity is rapidly lost. 320 nm irradiation can also cause cross-link, but less efficiently. Ethidium bromide causes photo oxidation of DNA with visible light and molecular oxygen. Oxidation products can cause phosphodiester breakage. If no heavy metal are present, ethanol does not damage DNA. Nucleases are found on human skin; therefore, avoid direct or indirect contact between nucleic acids and fingers. Most DNases are not very stable; however, many RNases are very stable and can adsorb to glass or plastic and remain active. 5 E C is one of the best and simplest conditions for storing DNA. -20 deg C: this temperature causes extensive single and double strand breaks. -70 E C is probable excellent for long-term storage. For long-term storage of DNA, it is best to store in high salt (>1M) in the presence of high EDTA (>10mM) at pH 8.5. Storage of DNA in buoyant CsCl with ethidium bromide in the dark at 5 E C is excellent. There is about one phosphodiester break per 200 kb of DNA per year. Storage of λ DNA in the phage is better than storing the pure DNA. [ref: Davis, R.W., D. Botstein and J.R. Roth, A Manual for Genetic Engineering: Advanced Bacterial Genetics. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y. 1980.]

B. Purification

To remove protein from nucleic acid solutions:

Treat with proteolytic enzyme, e.g., pronase, proteinase K

Purify on a silica-based column such as a Qiagen PCR Prep Column

CsCl/ethidium bromide density gradient

Phenol Extract. The simplest method for purifying DNA is to extract with phenol or phenol: chloroform and then chloroform. The phenol denatures proteins and the final extraction with chloroform removes traces of phenol

Purify on silica-based column such as Qiagen Brand columns (http://www.qiagen.com)

C. Quantitation.

Spectrophotometric. For pure solutions of DNA, the simplest method of quantitation is reading the absorbance at 260 nm where an OD of 1 in a 1 cm path length = 50 μ g/ml for double-stranded DNA, 40 μ g/ml for single-stranded DNA and RNA and 20-33 μ g/ml for oligonucleotides. An absorbance ratio of 260 nm and 280 nm gives an estimate of the purity of the solution. Pure DNA and RNA solutions have OD 260/OD 280 values of 1.8 and 2.0, respectively. This method is not useful for small quantities of DNA or RNA (<1 μ g/ml).

Ethidium bromide fluorescence. The amount of DNA is a solution is proportional to the fluorescence emitted by ethidium bromide in that solution. Dilutions of an unknown DNA in the presence of 2 μ g/ml ethidium bromide are compared to dilutions of a known amount of a standard DNA solutions spotted on an agarose gel or Saran Wrap or electrophoresed in an agarose gel.

D. Concentration

Precipitation with ethanol. DNA and RNA solutions are concentrated with ethanol as follows: The volume of DNA is measured and the monovalent cation concentration is adjusted. The final concentration should be 2-2.5M for ammonium acetate, 0.3M for sodium acetate, 0.2M for sodium chloride and 0.8M for lithium chloride. The ion used often depends on the volume

of DNA and on the subsequent manipulations; for example, sodium acetate inhibits Klenow, ammonium ions inhibit T4 polynucleotide kinase, and chloride ions inhibit RNA-dependent DNA polymerases. The addition of $MgCl_2$ to a final concentration of 10mM assists in the precipitation of small DNA fragments and oligonucleotides. Following addition of the monovalent cations, 2-2.5 volumes of ethanol are added, mixed well, and stored on ice or at -20 E C for 20 min to 1 hour. The DNA is recovered by centrifugation in a microfuge for 10 min (room temperature is okay). The supernatant is carefully decanted making certain that the DNA pellet, if visible, is not discarded (often the pellet is not visible until it is dry). To remove salts, the pellet is washed with 0.5-1.0 ml of 70% ethanol, spun again, the supernatant decanted, and the pellet dried. Ammonium acetate is very soluble in ethanol and is effectively removed by a 70% wash. Sodium acetate and sodium chloride are less effectively removed. For fast drying, the pellet can spun briefly in a Speedvac, although the method is not recommended for many DNA preparations as DNA that has been over dried is difficult to resuspend and also tends to denature small fragments of DNA. Isopropanol is also used to precipitate DNA but it tends to coprecipitate salts and is harder to evaporate since it is less volatile. However, less isopropanol is required than ethanol to precipitate DNA and it is sometimes used when volumes must be kept to a minimum, e.g., in large scale plasmid preps.

E. Restriction Enzymes

Restriction and DNA modifying enzymes are stored at -20 deg C in a non-frost free freezer, typically in 50% glycerol. The enzymes are stored in an insulated cooler which will keep the enzymes at -20 deg C for some period of time. The tubes should never be allowed to reach room temperature and gloves should be worn when handling as fingers contain nucleases. Always use a new, sterile pipet tip every time you use a restriction enzyme. Also, the volume of the enzyme should be less than 1/10 of the final volume of the reaction mixture.

Practical 2: Isolation of Genomic DNA from blood / plant cell / bacteria (any one)

Objective: To isolate the genomic DNA from E .cloacae AKS7 cells.

Principle: The isolation and purification of DNA from cells is one of the most common procedures in contemporary molecular biology and embodies a transition from cell biology to the molecular biology (from in vivo to in vitro). The isolation of DNA from bacteria is a relatively simple process. The organism to be used should be grown in a favorable medium at an optimal temperature, and should be harvested in late log to early stationary phase for maximum yield.

The genomic DNA isolation needs to separate total DNA from RNA, protein, lipid, etc. Initially the cell membranes must be disrupted in order to release the DNA in the extraction buffer. SDS (sodium dodecyl sulphate) isused to disrupt the cell membrane. Once cell is disrupted, the endogenous nucleases tend to cause extensive hydrolysis. Nucleases apparently present on human fingertips are notorious for causing spurious degradation of nucleic acids during purification. DNA can be protected from endogenous nucleases by chelating Mg2++ ions using EDTA. Mg2++ ion is considered as a necessary cofactor for action of most of the nucleases. Nucleoprotein interactions are disrupted with SDS; phenol or proteinase K. Proteinase enzyme is used to degrade the proteins in the disrupted cell soup. Phenol and chloroform are used to denature and separate proteins from DNA. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. DNA released from disrupted cells is precipitated by cold absolute ethanol or isopropanol.

Materials required

1. LB Broth

2. E. cloacae AKS7 cells
3. Reagents
4. TE buffer (pH 8.0)
5. 10% SDS
6. Proteinase K
7. Phenol-chloroform mixture
8. 5M Sodium Acetate (pH 5.2)
9. Isopropanol
10. 70% ethanol
11. Autoclaved Distilled Water
12. Eppendorf tubes 2 ml
13. Micropipette
14. Microtips
15. Microfuge

Procedure:

1. 2 ml overnight culture is taken and the cells are harvested bycentrifugation for 10 minutes
2. 875 µl of TE buffer is added to the cell pellet and the cells arere-suspended in the buffer by gentle mixing.
3. 100 µl of 10% SDS and 5 µl of Proteinase K are added to the cells.
4. The above mixture is mixed well and incubated at 37°C for an hour inan incubator.
5. 1 ml of phenol-chloroform mixture is added to the contents, mixedwell by inverting and incubated at room temperature for 5 minutes.
6. The contents are centrifuged at 10,000 rpm for 10 minutes at 4° C.
7. The highly viscous jelly like supernatant is collected using cut tipsand is transferred to a fresh tube.

8. The process is repeated once again with phenol-chloroform mixtureand the supernatant is collected in a fresh tube.
9. 100 µl of 5M sodium acetate is added to the contents and is mixedgently.
10. 2 ml of isopropanol is added and mixed gently by inversion till whitestrands of DNA precipitates out.
11. The contents are centrifuged at 5,000 rpm for 10 minutes.
12. The supernatant is removed and 1ml 70% ethanol is added.
13. The above contents are centrifuged at 5,000 rpm for 10 minutes.
14. After air drying for 5 minutes 200 µl of TE buffer or distilled water isadded.
15. 10 µl of DNA sample is taken and is diluted to 1 or 2 ml with distilledwater.
16. The concentration of DNA is determined using a spectrophotometer at260/280 nm.
17. The remaining samples are stored for further experiments.

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Results

After the careful execution of the experiment, we may expect a substantial recovery of genomic DNA from the bacteria.

Discussion

The recovery of genomic DNA would help us a lot in pursuing different molecular studies in the context of genetic engineering.

Safety precautions

- Cut tips should be used so that the DNA is not subjected tomechanical disruption.
- Depending on the source of DNA, the incubation period of ProteinaseK should be extended.
- The phenol chloroform extraction should be repeated depending onthe source of DNA.
- DNase free plastic wares and reagents should be used.

Practical 3: Isolation of Plasmid DNA

Objective: To isolate plasmid DNA from bacterial cells.

Principle: When bacteria are lysed under alkaline conditions both DNA and proteins are precipitated. After the addition of acetate-containing neutralization buffer the large and less super coiled chromosomal DNA and proteins precipitate, but the small bacterial DNA plasmids can renature and stay in solution. In prokaryotes, plasmid is double stranded, circular, and is found in the cytoplasm. The cell membranes must be disrupted in order to release the plasmid in the extraction buffer. Solution 1 contains glucose, Tris, and EDTA. Glucose provides osmotic shock leading to the disruption of cell membrane, Tris is a buffering agent used to maintain a constant pH8. Plasmid can be protected from endogenous nucleases by chelating Mg2++ions using EDTA. Mg2++ ion is considered as a necessary cofactor for most nucleases. Solution II contains NaOH and SDS and this alkaline solution issued to disrupt the cell membrane and NaOH also denatures the DNA into single strands. Solution III contains acetic acid to neutralize the pH and potassium acetate to precipitate the chromosomal DNA, proteins, along with the cellular debris. Phenol /chloroform is used to denature and separate proteins from plasmid. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. Once the plasmid DNA is released, it must be precipitated in alcohol. The plasmid DNA in the aqueous phase is precipitated with cold (0oC) ethanol or isopropanol. The precipitate is usually re-dissolved in buffer and treated with phenol or organic solvent to remove the last traces of protein, followed byre-precipitation with cold ethanol.

Materials Required:

1. Luria Broth
2. Bacterial cells containing plasmid
3. Reagents
   - TE buffer(pH 8.0)
   - Solution I
   - Solution II
   - Solution III
   - Phenol-chloroform mixture
   - Isopropanol
   - 70% ethanol
4. Autoclaved Distilled Water
5. Eppendorf tubes 2 ml
6. Micropipette
7. Microtips
8. Microfuge

Procedure:

1. Take 2 ml overnight culture and harvest cells by centrifugation for 5minutes. Discard the supernatant carefully.
2. Add 100 µl of solution I to the cell pellet and re-suspend the cells by gentle mixing.
3. Incubate the above mixture at room temperature for 5 minutes.
4. Add 200 µl of solution II to the mixture and mix by inverting the tubes for 5 minutes.
5. Incubate for 5-10 minutes at room temperature.

6. Add 500µl of ice cold solution III to the mixture and mix by inverting the tube.
7. Incubate on ice for 10 minutes.
8. Centrifuge at 10,000 rpm for 5 minutes.
9. Transfer the supernatant into fresh tube.
10. Add 400 µl of phenol-chloroform mixture to the contents, mix well by inverting and incubate them at room temperature for 5 minutes.
11. Centrifuge at 10000 rpm for 5 minutes.
12. Collect the supernatant (viscous) using cut tips and transfer to a fresh tube.
13. Add 0.8 ml of isopropanol and mix gently by inversion. Incubate for 30 min at room temperature.
14. Centrifuge the contents at 10,000 rpm for 10 minutes.
15. Discard the supernatant after centrifugation.
16. After air drying for 5 minutes, add 100 µl of TE buffer or autoclaved distilled water to the pellet to re-suspend the plasmid DNA. The contaminated salt in the DNA pellet can be removed with 70%ethanol washing.
17. Take 10 µl of plasmid sample and dilute to 1 ml with distilled water for spectrometric analysis.
18. The concentration of plasmid is determined using a spectrophotometer at 260/280 nm.
19. An aliquot of plasmid DNA is used for agarose electrophoresis for quantitative and qualitative analyses.

Result:

After the careful execution of the experiment, we may expect a substantial recovery of plasmid DNA from the bacteria.

Discussion:

The recovery of plasmid DNA would help us a lot in pursuing different molecular studies in the context of genetic engineering.

Safety precaution:

1. Cut tips should be used so that the plasmid is not subjected to mechanical disruption.
2. The phenol chloroform extraction should be repeated depending on the source of plasmid to obtain pure plasmid.
3. DNase free plastic wares and reagents should be used.

Practical 4: Agarose gel electrophoresis of genomic DNA / Plasmid DNA

Objective: Separation of Genomic and Plasmid DNA by agarose gel electrophoresis

Principle: Gel electrophoresis is the standard lab procedure for separating DNA by size (e.g., length in base pairs) for visualization and purification. Electrophoresis uses an electrical field to move the negatively charged DNA through an agarose gel matrix toward a positive electrode. Shorter DNA fragments migrate through the gel more quickly than longer ones. Thus, you can determine the approximate length of a DNA fragment by running it on an agarose gel alongside a DNA ladder (a collection of DNA fragments of known lengths).

Equipment:

1. Casting tray
2. Well combs
3. Voltage source
4. Gel box
5. UV light source
6. Microwave

Reagents

1. TAE
2. Agarose
3. Ethidum bromide (stock concentration of 10 mg/mL)

Procedure

1. Pouring a Standard 1% Agarose Gel:
2. Measure 1 g of agarose
3. Mix agarose powder with 100 mL 1xTAE in a microwavable flask Microwave for 1-3 min until the agarose is completely dissolved (but do not over boil the solution, as some of the buffer will evaporate and thus alter the final percentage of agarose in the gel. Many people prefer to microwave in pulses, swirling the flask occasionally as the solution heats up.).
4. Let agarose solution cool down to about 50 °C (about when you can comfortably keep your hand on the flask), about 5 mins.
5. (Optional) Add ethidium bromide (EtBr) to a final concentration of approximately 0.2-0.5 µg/mL (usually about 2-3 µl of lab stock solution per 100 mL gel). EtBr binds to the DNA and allows you to visualize the DNA under ultraviolet (UV) light. CAUTION: EtBr is a known mutagen. Wear a lab coat, eye protection and gloves when working with this chemical. Note: If you add EtBr to your gel, you will also want to add it to the running buffer when you run the gel. If you do not add EtBr to the gel and running buffer, you will need to soak the gel in EtBr solution and then rinse it in water before you can image the gel.
6. Pour the agarose into a gel tray with the well comb in place.
7. Place newly poured gel at 4 °C for 10-15 mins OR let sit at room temperature for 20-30 mins, until it has completely solidified.

Loading Samples and Running an Agarose Gel:

1. Add loading buffer to each of your DNA samples. Note: Loading buffer serves two purposes: 1) it provides a visible dye that helps with gel loading and allows you to gauge how far the DNA has migrated; 2) it contains a high percentage of glycerol that increases the density of your DNA sample causing it settle to the bottom of the gel well, instead of diffusing in the buffer.
2. Once solidified, place the agarose gel into the gel box (electrophoresis unit).
3. Fill gel box with 1xTAE (or TBE) until the gel is covered.
4. Carefully load a molecular weight ladder into the first lane of the gel.
5. Carefully load your samples into the additional wells of the gel.
6. Run the gel at 80-150 V until the dye line is approximately 75-80% of the way down the gel. A typical run time is about 1-1.5 hours, depending on the gel concentration and voltage.
7. Turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
8. (Optional) If you did not add EtBr to the gel and buffer, place the gel into a container filled with 100 mL of TAE running buffer and 5 µL of EtBr, place on a rocker for 20-30 mins, replace EtBr solution with water and destain for 5 mins.
9. Using any device that has UV light, visualize your DNA fragments. The fragments of DNA are usually referred to as 'bands' due to their appearance on the gel.

Analyzing Your Gel:

Using the DNA ladder in the first lane as a guide (the manufacturer's instruction will tell you the size of each band), you can infer the size of the DNA in your sample lanes.

Practical 5: Preparation of restriction enzyme digests of DNA samples

Aim: To digest the pUC18 DNA with *Bam*H1 enzyme

Principle: Restriction endonucleases are the class of enzymes that are used to cleave DNA at specific sites called Restriction sites. Every restriction enzyme has a specific restriction site at which it cuts a DNA molecule. For example, restriction sequence for *Bam*HI is GGATCC (type II restriction enzyme. The most abundantly used restriction enzymes are type II restriction enzymes which cleave at specific restriction site only. These endonucleases function adequately at pH 7.4 but different enzymes vary in their requirements for ionic strength usually provided by sodium chloride and magnesium chloride. It is also advisable to add a reducing agent such as dithiothreitol (DTT) which stabilizes the enzymes and prevents their inactivation. Any variation in the concentration of Na or Mg can lead to changes in specificity of enzyme so that it can cleave at additional or nonstandard restriction sequences. The phosphodiester bond is cleaved between specific bases, one on each DNA strand, no matter the source of the DNA. The restriction endonucleases produce either sticky orblunt ends upon cleavage. Also based on the number of sequences identified for cleavage they can be tetra cutter (4), hexacutter (6) oroctacutter (8).

Materialsrequired

- pUC18 DNA
- *Bam*H1 enzyme
- 10X buffer
- 1Kb Ladder
- Sterile water
- Agarose

- 6X loading dye
- 1.5 ml Sterile Vials
- Ethidium Bromide
- 1X TAE buffer

Procedure:

- Take 1.5 µg of PUC18 DNA (10 ul) in a fresh eppendorf.
- To this, add 11.5 µl of sterile water followed by 5 µl of 10X buffer.
- Add 1.5 µl of *BamH1* enzyme (1 unit) and incubate the mixture at37°C for 2 h.
- Prepare 0. 7% agarose gel and load the samples including 1 Kb DNAladder, undigested pUC18 DNA and *BamH1* digested PUC18 DNA.
- Run the gel at 100 V for 1 hr.
- Visualize the gel under UV illuminator.
- 10 µl of the sample and 2 µl of the dye were mixed
- Laod 10 µl of this in to the gel

Results:

The digestion of DNAgives multiple bands in contrast to undigested DNA that shows a single band.

Discussion

The digestion of DNA would help us a lot in pursuing genetic manipulations.

Safety precautions

DNase free plastic wares and reagents should be used.

Practical 6: Spectroscopic analysis of DNA/RNA

Aim: To determine the amount, concentration and purity of the given DNA sample

Principle: This experiment is purely an application of the Beer Lamberts' Law which states that the concentration of the sample is directly proportional to the absorbance of light done by the sample. It is given by following expression:

$$A = \varepsilon * c * l$$

The device UV spectrophotometer works on this principle and used to find the concentration of the sample. Concentration and quality of a sample of DNA is measured with a UV spectrophotometer.

A standard graph can be drawn using different concentrations of DNA and OD (optical density) values.

Materials required

- DNA sample
- TE buffer
- UV spectrophotometer

Procedure:

- Take the DNA sample (10 µl) in TE buffer.

- Now dilute the above sample by the factor of 100 i. e, by taking 10µlof the sample in 990µl of TE buffer.
- After doing this take the optical density value at A260 & A280 and calculate the amount of DNA recovered.

Results: Use the following formula to determine the concentration of DNA

Total DNA (µg) = (A260) (50 µg/ml/A260) X (100) X (0.1 ml) where 100 is the dilution factor and 0.1 ml is the total volume of the DNA.

Discussion: If there is a contaminant, there is some additional OD, which decreases the OD ratio between 260 and 280 nm. Clean DNA has a OD260/OD280 between 1.8 and 2.0. If the DNA is contaminated by protein, then the value of OD260/OD280 gets reduced.

Safety precautions

DNase free plastic wares and reagents should be used.