KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University Established Under Section 3 of UGC Act 1956)

Coimbatore – 641 021.

LECTURE PLAN
DEPARTMENT OF BIOTECHNOLOGY

STAFF NAME: Mr Nishu Sekar
SUBJECT NAME: Molecular Biology Practical SUB.CODE: 17BTU312
SEMESTER: III (Section- B) CLASS: II B.Sc (BT)

S.No	Lecture Duration Period	List of Practical's
1	3	Preparation of solutions for Molecular Biology experiments
2	3	Isolation of Chromosomal DNA from bacterial cells
3	3	Isolation of Plasmid DNA by alkaline lysis method
4	3	Agarose gel electrophoresis of genomic DNA and Plasmid DNA
5	3	Preparation of restriction enzyme digest of DNA samples
6	3	PCR amplification on targets DNA and DNA profiling
7	3	Demonstration of AMES test or reverse mutation for carcinogenicity

LAB MANUAL **BATCH-2017-2020**

Laboratory Safety

Safety in the Laboratory Should always is in your mind. Throughout this manual Safety recommendations are given, below are some general consideration that anyone in a laboratory should know.

General laboratory safety precaution.

- Follow all instructions carefully. Use special care when you see the word CAUTION in your laboratory instructions. Follow the safety instructions given by your teacher.
- Determine the location of Fire Extinguishers, Chemical safety showers and Eye washers, Chemical Spill Kits, and alternative exit routes for lab evacuation.
- Remember that smoking, eating, or drinking in the lab room is totally prohibited.
- Wear lab aprons when working with chemicals, hot material, or preserved specimens.
- Wear safety goggles when using dangerous chemicals, hot liquids, or burners.
- Any chemicals spilled on the hands or other parts of the skin should be washed off immediately with a plenty of running water.
- If you have an open skin wound, be sure that it is covered with a water proof bandage.
- Never work alone in the laboratory.
- Keep your work area clean & dry.
- Turn of all electrical equipment, water, and gas when it is not in use, especially at the end of the laboratory period.
- Tie back long hair.
- Report all chemicals spills or fluids to your instructor immediately for proper clean up.

Special precautions for working with heat or fire:

- Never leave a lighted Bunsen burner of hot object unattended. When an object is removed from the heat & left to cool, it should be placed where it is shielded from contact.
- Inflammable liquid bottles should not be left open, not dispensed near a naked flame, hot electric element or electric motor.
- Use test tube holders to handle hot laboratory equipments.

- When you are heating something in a container such as a test tube, always point the open end of the container away from yourself & others.
- Use only Pyrex glassware's for heating.
- Allow hot materials to cool before moving them from your lab station.
- Make sure that Bunsen burner hoses fit tightly.

Special precautions for working with chemicals

- Never taste or touch substances in the laboratory without specific instructions.
- Never smell substances in the laboratory without specific instruction
- Use materials only from containers that are properly labeled.
- Wash your hand after working with chemicals.
- Do not add water to acid. Instead, dilute the acid by adding it to water.
- Mix heat generating chemicals slowly.

Special precautions for working with electrical equipment.

- Make sure the area under & around the electrical equipment is dry.
- Never touch electrical equipment with wet hands.
- Make sure the area surrounding the electrical equipment is free of flammable materials.
- Turn off all power switches before plugging an appliance into an outlet.

Special Precaution for working with Glassware's and other laboratory equipments.

- Become familiar with the names and appearance of all the laboratory equipments you will use.
- Never use broken or chipped glassware.
- Make sure that all glassware's are clean before you using it.
- Do not pick up broken glass with your bare hands. Use a pan and a brush
- If a Mercury thermometer breaks, do not touch the mercury. Notify your teacher immediately.
- Do not aim the mirror of your microscope directly at the sun. Direct sun light can damage the eyes.
- Use care handling all sharp equipments, such as scalpels and dissecting needles.

Special precautions for working with live or preserved specimens.

- If live animals are used treat them gently. Follow instructions for their proper care.
- Always wash your hands after working with live or preserved organisms.
- Specimens for dissection should be properly mounted and supported. Do not try to cut a specimen while holding it in the air.
- Do not open Petri dishes containing live cultures unless you are directed to do so.
- Detergents (detol 5 – 10%) should be used to sterilize and clean benches, glassware and equipment.
- Safety cabinet should be used while working with microbes.
- Lab coats should be worn during the work in the lab.
- Disposable items should be collected and autoclaved.

First Aid

- Injuries: bleeding should be reduced using bandages; the wound should be cleaned with iodine alcohol mixture, and wrapped with sterile bandage.
- Acid and fire burns: body burns must be washed immediately with tap water. Eye burns must be washed using eye washer, special cream for burns can be used.
- Poisoning: if any toxic chemical is swallowed, the mouth must be sensed with water, in case of acid, milk is drunk, in case of alkaline, diluted acetic acid (vinegar) can be used.
- . Skin contamination requires washing with water and removal of contaminated clothing, if the contaminant is insoluble in water remove with soap and water.

LAB MANUAL BATCH-2017-2020

IMPORTANT MESURMENTS IN MOLECULAR BIOLOGY

SIZE

Cell biology deals with things which are relatively small. The units of measurement typically used are the micron at the light microscope level, and the nanometer at the electron microscope level. For molecular measurements, the norm is the Angstrom. These units are defined within the following table:

Measure	Symbol	Relative Length	Exponential Notation
Meter	M	1	10_0
Decimeter	dm	.1	10_{-1}
Centimeter	cm	.01	10_{-2}
Millimeter	mm	.001	10_{-3}
Micrometer or micron	µ	.000001	10_{-6}
Nanometer	nm	.000000001	10_{-9}
Angstrom	Å	.0000000001	10_{-10}

From this table it is apparent that:

10 Å = 1 nm

1000 nm = 1 mm

10 mm = 1 cm

Not apparent are that:

1 inch = 2.54 cm = 25.4 mm = 25,400 µ = 25,400,000 nm

1 inch = 2.54 cm = 2.54 X 10_1 mm = 2.54 X 10_4 µ = 2.54 X 10_7 nm

1 mm = 0.04 inches

VOLUME

Volumes are measured relative to a liter, with the most commonly used measurements, the milliliter and the microliter. The following table gives the relative volumes:

Measure	Symbol	Relative Volume	Exponential Notation
Liter	L	1	10_7
Deciliter	Dl	.1	10_{-1}
Millimeter	Ml	.001	10_{-3}
Microliter	µ l	.000001	10_{-6}

There are 1,000 µ l in 1 ml.

1 ml = 1 cm_3

1 gallon = 3.8 liters

1 quart = 0.95 liters

1 liquid ounce = 29.6 ml

WEIGHT

The most common measurements of weight at the gram, milligram and microgram.

Measure	Symbol	Relative Weight	Exponential Notation
Kilogram	Kg	1000	10^{3}
Gram	g	1	10^{0}
Milligram	mg	.0001	10^{-3}
Microgram	µ g	.0000001	10^{-6}

CONCENTRATION

Most concentrations used throughout cell biology are those of a solute disolved or suspended within a solvent, and in most cases the solvent is water. There are two general methods of identifying the concentration of a solution; as molarity or as a percent. Molarity is based on the number of moles of solute in the solvent, while percent is based on the number of parts , either grams (for a solid solute) or milliliters (for a liquid solute).

Molarity equals the number of moles of solute in 1 liter of solution. A mole is equal to the gram molecular weight (or formula weight) of the solute. Sodium Chloride (NaCl), for example has a formula weight of 58.43 (22.98 for Na and 35.43

for Cl). Thus, if 58.43 grams of NaCl are dissolved in 1 liter of water, the result would equal a 1 molar solution of NaCl. This is designated as 1 M NaCl, or as simple M NaCl.

We often deal with solutions of less volume than 1 liter, and the following should be noted:

1 M NaCl = 58.43 grams / liter = 58.43 mg/ml = 58.43 µ U g/µ U l.

A 0.002 M NaCl solution contains 0.002 moles of NaCl or 0.1168 grams (0.002 x 58.43) in one liter of solvent. Note that molar is abbreviated as M, but that there is no abbreviation for moles. The 0.002 M solution contains 0.002 moles (or 2 millimoles) or solute in one liter. A 0.002 M solution would contain 0.001 moles of solute in a half liter.The number of moles = Volume (in liters) x Molar Concentration

The number of millimoles = Volume (in ml) x Molar Concentration

Note that chemical equations are always balanced via moles. Moreover, note that for dilutions of known concentrations, one can use the simple formula:

Molarity x Volume = Molarity x Volume

If you have a 0.002 M solution of NaCl and you wish to obtain 100 ml. of a 0.001 M solution,

0.002 M x Needed Volume = 0.001 M x 100 ml

Needed Volume = 0.001 M x .1 liters / 0.002 M = .050 liters= 50 ml. Measure 50 ml of the 0.002 M solution, and dilute it to 100 ml with the solvent (usually water, or an appropriate buffer).

Molarity is appropriate for use when chemical equations are to be balanced. When we deal with physical properties of solutions, molarity is not as valuable as a similar measurement of concentration, molality. For colligative properties of solutions (freezing point depression, boiling point elevation, osmotic pressure, density, viscosity), there is a better correlation between the property and molality.

Molality (designated with a lower case m) is equal to the number of moles of solute in 1000 gm of solvent. At first this may not appear any different from molarity, since a ml of water equals 1.0 gm. Indeed, for dilute solutions in water, there is little or no practical difference between a molar solution and a molal solution. In concentrated solutions, with temperature fluctuations and with changes in solvent, there is appreciable difference.

For example:

A 2 m (2 molal) solution of sucrose contains 684.4 gm of sucrose (twice the molecular weight or two moles of sucrose) dissolved in 1000 gm (approximately 1 liter) of water. The weight of this solution is 684.4 gm + 1000 gm or 1684.4 gm.

This solution (2 m sucrose) has a density of 1.18 gm/ml or 1180 gm/liter.

Since there are 1684.4 gms, division by the density (1180 gm/liter) would indicate that there are 1.43 liters of solution. That is, 684.4 gm of sucrose dissolved in 1000 gm of water would yield 1.43 liters of solution. This solution would contain 2 moles of sucrose, however and would have a molarity = 2 moles/1.43 liters or 1.40 M. So, a 2 m sucrose solution equals a 1.4 M sucrose solution.

PERCENT SOLUTIONS

In the example above of 2 m sucrose, there were 684.4 gm of sucrose in the final solution which weighed 1684.4 gm (684.4 gm sucrose + 1000 gm water). The percent of sucrose on the basis of weight is therefore 684.4/1684.4 x 100, or 40.6%.

There are three means of expressing concentration in the form of a percent figure:

1. Percent by weight (w/w); gm solute / 100 gm solvent

2. Percent weight by volume (w/v); gm solute /100 ml solvent
3. Percent by volume (v/v); ml solute / 100 ml solution

For dilute solutions, these differences are not significant, but at higher concentrations, they are. Chemists (when they use Percent designations) usually use w/w. Biochemists and physiologists more often use w/v. Both use v/v if the solute is a liquid. It is important to distinguish among these alternatives.

Using ethanol as an example, consider a 20% solution of ethanol in water, mixed according to the three designations of w/w, w/v and v/v.

1. w/w would contain 20 g of absolute ethanol mixed with 80 gm of water to yield a 20% (w/w) solution.
2. w/v would contain 20 g of absolute ethanol mixed with water to form a final volume of 100 ml.
3. v/v would contain 20 ml of absolute ethanol diluted to 100 ml with water.

The three solutions are not the same. First, the density of alcohol is not equal to that of water, and thus conversion of g to ml is not equivalent. A 20% (w/w) solution of ethanol, for example, has a density of 0.97 g/ml and 20 gm of ethanol plus 80 gm of water would have a volume of 103 ml. The % (w/v) for this solution would be 20 gm ethanol / 103 ml, or 19.4% (w/v). Similarly, absolute ethanol has a density of 0.79 gm/ml and thus 20 ml of ethanol would weigh 15.8 gm. A 20% (v/v) solution would contain 15.8 gm of ethanol in 100 ml and be a 15.8% (w/v) solution.

So, for ethanol:

20% (w/w) = 19.4 % (w/v)
20% (w/v) = 20.0 % (w/v)
20% (v/v) = 15.8 % (w/v)

In cell biology, the most common use of Percent solution is as (w/v). In practice, these are simple solutions to mix. For a 20% (w/v) sucrose solution, for example, simply weigh 20 gm of sucrose and dissolve to 100 ml with water.

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Ex.No : 1

Date:

PREPARATION OF REAGENS AND BUFFERS FOR MOLECUALR BIOLOGY EXPERIMENTS

10 M Ammonium Acetate

To prepare a 10 M solution in 100 ml, dissolve 77 g of ammonium acetate in 70 ml of H2O at room temperature. To prepare a 5 M solution in 100 ml, dissolve 38.5 g in 70 ml of H2O. Adjust the volume to 100 ml with H2O. Sterilize the solution by passing it through a 0.22µm filter. Store the solution in tightly sealed bottles at 4 ° C or at room temperature. Ammonium acetate decomposes in hot H2O and solutions containing it should not be autoclaved.

Ampicillin

Prepare a stock of 100 mg/ml in water. Sterilize by filtration. Store at -20°C but avoid repeated freeze/thaw cycles. Use at a final concentration of 100 µg/ml.

EDTA stock

To prepare 1 liter, 0.5M EDTA pH 8.0: Add 186.1 g of disodium EDTA-2H 2O to 800 ml of H 2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (approx. 20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approx. 8.0 by the addition of NaOH. For tetrasodium EDTA, use 226.1 g of EDTA and adjust pH with HCl.

6x gel loading buffer

0.25% Bromophenol blue

0.25%Xylene cyanol FF

15% Ficoll Type 4000

120 mM EDTA

LB Medium

To make 1 liter, use 10 g tryptone, 5 g yeast extract, 10 g NaCl. Adjust pH to 7.0. Sterilize by autoclaving. LB Agar Dispense 15 g per liter of agar directly into final vessel. Prepare LB medium as above and add to agar. NOTE: Agar will not go into solution until it is

autoclaved (or boiled). If adding antibiotics, autoclave medium first and allow to cool until warm to the touch, then add the antibiotic. Dispense about 30 ml per plate. Allow plates to dry either at 37°C overnight or 20 minutes in a laminar flow hood (lids removed). Store in original Petri plate bags, inverted, at 4°C for up to 2 weeks.

NaCl

To prepare 1 liter of a 5 M solution: Dissolve 292 g of NaCl in 800 ml of H 2O. Adjust the volume to 1 liter with H 2O. Dispense into aliquots and sterilize by autoclaving. Store the NaCl solution at room temperature.

NaOH

The preparation of 10 N NaOH involves a highly exothermic reaction, which can cause breakage of glass containers. Prepare this solution with extreme care in plastic beakers. To 800 ml of H2O, slowly add 400g of NaOH pellets, stirring continuously. As an added precaution, place the beaker on ice. When the pellets have dissolved completely, adjust the volume to 1 liter with H2O. Store the solution in a plastic container at room temperature. Sterilization is not necessary.

20X SB (electrophoresis buffer)

(Buffer diluted to 1X should be 10 mM Sodium hydroxide and pH 8.5) for 1 liter, weigh out 8 g NaOH and ~40 g boric acid - add water, dissolve and add additional boric acid until pH = 8.0; bring final volume to 1 liter.

SDS stock 10% or 20% (w/v) SDS.

Also called sodium lauryl (or dodecyl) sulfate. To prepare a 20% (w/v) solution, dissolve 200 g of electrophoresis-grade SDS in 900 ml of H2O. Heat to 68 ° C and stir with a magnetic stirrer to assist dissolution. If necessary, adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 liter with H 2O. Store at room temperature. Sterilization is not necessary. Do not autoclave.

20x SSC 0.3M

Na(3) citrate 3M NaCl

SOB Medium: per liter:

Bacto-tryptone 20 g Yeast extract 5 g NaCl 0.584 g KCl 0.186 g Mix components and adjust pH to 7.0 with NaOH and autoclave. 2 M Mg ++ stock: MgCl 2-6H2O 20.33 g MgSO 4 -7H2O 24.65 g Distilled water to 100 ml. Autoclave or filter sterilize. 2 M Glucose Glucose 36.04 g Distilled water to 100 ml. Filter sterilize.

For SOB Medium + magnesium: Add 1 ml of 2 M Mg ++ stock to 99 ml SOB Medium. For SOCMedium: Add 1 ml of 2 M Mg ++ stock and 1 ml of 2 M Glucose to 98 ml of SOB Medium.

3M Sodium Acetate - pH 5.2

To prepare a 3 M solution: Dissolve 408.3 g of sodium acetate-3H2O in 800 ml of H 2O. Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume to 1 liter with H2O. Dispense into aliquots and sterilize by autoclaving.

50x TAE

Prepare a 50x stock solution in 1 liter of H2O: 242 g of Tris base 57.1 ml of glacial acetic acid 100 ml of 0.5 M EDTA (pH 8.0) The 1x working solution is 40 mM Tris-acetate/1 mM EDTA.

5X (or 10X) TBE

Prepare a 5x stock solution in 1 liter of H2O: 54 g of Tris base 27.5 g of boric acid 20 ml of 0.5 M EDTA (pH 8.0) The pH of the concentrated stock buffer should be approx. 8.3.. Some investigators prefer to use more concentrated stock solutions of TBE (10x as opposed to 5x). However, 5x stock solution is more stable because the solutes do not precipitate during storage. Passing the 5x or 10x buffer stocks through a 0.22μm filter can prevent or delay formation of precipitates.

TE buffer:

10 mM Tris-Cl (pH, usually 7.6 or 8.0)

1 mM EDTA (pH 8.0)

Use concentrated stock solutions to prepare. If sterile water and sterile stocks are used, there is no need to autoclave. Otherwise, sterilize solutions by autoclaving for 20 minutes. Store the buffer at room temperature.

1 M Tris-Cl – used at various pHs Using Tris base :

To make 1 liter, dissolve 121 g Tris Base in 800 ml of water. Adjust pH to the desired value by adding approximately the following: pH = 7.4 about 70 ml of concentrated HCl pH = 7.6 about 60 ml of concentrated HCl pH = 8.0 about 42 ml of concentrated HCl Make sure solution is at room temperature before making final pH adjustments. Bring final volume to 1 liter. Sterilize by autoclaving.

Using Trizma tables: an alternate procedure for preparing Tris solutions is to combine the proper amount of Tris Base and Tris Hydrochloride to achieve the desired value using Sigma's Tris tables.

X-gal 5-bromo-4-chloro-3-indolyl-b-D-galactoside (same recipe for X-phosphate)

Make a 2% (w/v) stock solution by dissolving X-gal in dimethylformamide at a concentration of 20 mg/ml solution. Use a glass or polypropylene tube. Wrap the tube containing the solution in aluminum foil to prevent damage by light and store at -20 ° C. It is not necessary to sterilize X-gal solutions.

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EX.NO:2
Date:

ISOLATION OF TOTAL GENOMIC DNA FROM MICROBES (*E.coli*)

Aim: To isolate total genomic DNA from the bacterial cells and visualizing the same DNA by gel electrophoresis.

Principle:

Genomic DNA preparation differs from the plasmid DNA preparation. Genomic DNA is extracted from bacterial cells by immediate and complete lysis whereas plasmid DNA is isolated by slow-cell lysis to form a sphaeroplast.

The procedure of genomic DNA extraction can be divided into 4 stages:

1. A culture of bacterial cell is grown and harvested.
2. The cells are broken open to release their contents.
3. The cells extracted are treated to remove all components except the DNA.
4. The resulting DNA is then purified.

Materials required:

1. TE buffer (pH 8.0)
2. 10% (w/v) sodium dodecyl sulfate (SDS)
3. 20 mg/mL proteinase K
4. Phenol/chloroform (50:50)
5. Isopropanol
6. 70% ethanol
7. 3M sodium acetate pH 5.2

Procedure:

1) *E. coli* culture was grown overnight in Luria broth.
2) The overnight culture was transfered into 1.5 mL to a microcentrifuge tube and centrifuged at 10000 rpm for 5 min. The supernatant was decanted and repeated with another 1.5 mL of cells.

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3) The bacterial pellet was resuspended in 467 µL TE buffer by repeated pipetting. 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K was added, mixed, and incubated for 1 hr at 37°C.
4) Equal volume of buffer saturated phenol: chloroform: isoamyl alcohol (25:24:1) or chloroform: Isoamyl alcohol (24:1) was added and mixed well by inverting the tube until the phases are completely mixed. (Caution: Phenol causes severe burns. Wear gloves, goggles, and a lab coat, and keep tubes capped tightly)
5) Centrifuged at 12000 rpm for 5 min.
6) The upper aqueous layer phase was transferred to a new 1.5 ml eppendorf tube and equal volume of chloroform:Isoamyl alcohol (24:1) was added, Again mixed well by inverting and transfered to a new tube and centrifuged at 12000 rpm for 5 minutes.
7) The upper aqueous phase was again transferred to a new 1.5 ml eppendorf tube.
8) To the aqueous phase, 1/10 volume of 3M sodium acetate (pH 5.2) was added.
9) Then 0.6 volume of isopropanol was added and mixed gently until the DNA gets precipitates.
10) Centrifuged at 12000 rpm for 10 min and the supernatant was discarded.
11) The DNA pellet was washed by adding 200 µL of 70% ethanol (Centrifuge at 10000 rpm for 3 min)
12) Finally the DNA pellet was suspended in a 100–200 µL TE buffer. Complete resuspension may take several days.
13) After DNA has dissolved, the purity of the DNA was checked by electrophoresis and spectrophotometric analysis.
14) 0.8 % agarose gel was prepared using 1X TAE buffer.
15) Isolated DNA samples were loaded into the wells, recording which samples are loaded into which wells as lane 1, 2, etc. The power supply was started after sample loading, with the voltage set to 50 V.
16) Ran the gel until the second dye from the well has reached 3/4 th of the gel.
17) After the run was completed, the gel was observed under UV transilluminater.
18) Then DNA was stored at 4°C short term, –20°C or –80°C long term

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Results and Observations:

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EX.NO:3

Date:

ISOLATION OF PLASMID DNA BY ALKALINE LYSIS METHOD

Aim: To isolate plasmid DNA from the bacterial cells by alkaline lysis miniprep method and visualizing the same DNA by gel electrophoresis.

Principle:

Plasmids are extrachromosomal, double-stranded, closed-circular DNA present in many microorganisms. Plasmid DNA needs to be extracted (from bacterial hosts, mostly *E.coli*) almost routinely in cloning experiments. Many methods have been described for successful extraction of plasmid DNA; however, the alkaline miniprep method is most useful for quick extraction of plasmids, mostly for analytical use. In alkaline lysis miniprep method, the bacterial cells are lysed followed by SDS, NaOH treatment. The high pH of NaOH denatures the bacterial DNA but not the covalently closed-circular plasmid DNA. Neutralization of the high pH by sodium or potassium acetate makes the bacterial DNA to precipitate. The plasmid DNA is then purified by organic solvent.

Materials required:

1. Luria Broth - containing Trypton, 1%; Yeast extract, 0.5% NaCl, 1%
2. Antibiotics - Ampicillin, 50 mg/mL
3. Solution I - containing

50 mM glucose

25 mM Tris-CI (pH 8.0)

10 mM EDT A (pH 8.0)

Solution I can be prepared in batches of approximately 100 mL autoclaved for 15 minutes at 10 lb/sq and stored at 4°C). add RNase A (100mg/ml) to get 100 ug/ml of solution I.

4. Solution II -containing

0.2 N NaOH (freshly diluted from 10 N stock)

1% SDS

5. Solution III - containing

5 M potassium acetate 60 mL

Glacial acetic acid 11.5 mL

Distilled Water 28.5 mL

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

6. 50 % PEG 6000 solution

7. 5M NaCl

8. TE buffer: 10 mM Tris, 1 mM EDTA, pH 8.0

9. 70% and 100% ethanol.

Procedure:

Harvesting and Lysis of Bacteria

Harvesting - A single bacterial colony was inoculated into 2 mL of LB medium containing the appropriate antibiotic in a loosely capped 15-mL tube.

1) the culture was incubated for overnight at 37°C with vigorous shaking.
2) 1.5 mL of the culture was transferred into a centrifuge tube.
3) Centrifuged at 12000 g of 5 min using microcentrifuge.
4) the medium was removed by decantation, leaving the bacterial pellet as dry as possible.

Lysis by Alkali

1) The bacterial pellet was resuspended in 300 µL of ice-cold Solution I by vigorous vortexing.
2) 300 µL of freshly prepared Solution II was added.
3) The tube was closed tightly and the contents were mixed by inverting the tube rapidly for 5 minutes. (Make sure that the entire surface of the tube comes in contact with Solution II. Do not vortex). Then 300 µL of ice-cold Solution III was added.
4) the content was invert mixed and incubated on ice for 3–5 minutes.
5) After incubation, centrifuged at 14000 rpm for 10 minutes in a microcentrifuge. Then the supernatant was transferred to a fresh tube.
6) To the supernatnant, 166 µl of 50 % PEG6000 and 118 µl of 5M NaCl were added and mixed thoroughly by vortexing. Then centrifuged at 14000 rpm for 10 minutes in a microcentrifuge,

7) The supernatant was removed by gentle aspiration. The tube was kept in an inverted position on a paper towel to allow all of the fluid to drain away. Any drops of fluid adhering to the walls of the tube was also removed.
8) The pellet of DNA was rinsed with 200 µL of 70% ethanol.
9) Centrifuged at 12000 rpm for 10 minutes in a microcentrifuge.
10) The supernatant was removed as described in previous step, and the pellet of DNA was allowed to dry in the air for 1 minute.
11) The DNA pellet was resuspended in 50 µL of TE (pH 8.0). Vortexed briefly.
12) The isolated plasmid DNA was analyzed in 1.2 % agarose gel electrophoresis.

Results and Observations:

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EX. NO: 4

Date:

AGAROSE GEL ELECTROPHORESIS OF GENOMIC AND PLASMID DNA

Aim: To separate and analyze the isolated DNA sample from the plant/animal/bacterial source.

Principle:

Electrophoresis is the migration of charged molecules in response to an electric field. Their rate of migration depends on the strength of the electric field; on the net charge, size and shape of the molecules, and also on the ionic strength, viscosity, and temperature of the medium in which the molecules are moving.

Electrophoresis through agarose is the standard method used to separate, identify, and purify DNA fragments. The technique is simple, rapid to perform, and capable of resolving fragments of DNA. Furthermore, the location of DNA within the gel can be determined directly by staining with low concentrations of the fluorescent intercalating dye ethidium bromide; bands containing as little as 1–10 mg of DNA can be detected by direct examination of the gel in ultraviolet light. If necessary, these bands of DNA can be recovered from the gel and used for a variety of cloning purposes. Movement of the DNA in the gel depends on its molecular weight, conformation, and concentration of the agarose, voltage applied, and strength of the electrophoresis buffer.

Materials required:

1) Submarine gel apparatus, including glass plate, comb, gel tray and surround.
2) Agarose.
3) Stock solutions –

(i) 50 X TAE buffer (0.04 M tris-acetate, 0.001 M EDTA, pH 8.0)

For 1 litre – 242 g Tris base

57.1 mL glacial acetic acid

100 mL 0.5 M EDTA (pH 8.0)

(ii) Ethidium bromide: 10 mg/mL

(iii) 6 X gel-loading buffer – containing 0.25 % bromophenol blue and
40 % sucrose in water.

4) Agarose solution in 0.5 X TBE or 1 X TAE buffer (generally 0.7%–1%).
5) 1X TAE or 0.5 X TBE (same buffer as in agarose).
6) UV- transilluminator

Protocol:

Preparation of Agarose Gels

1. The ends of gel tray was sealed with tape.
2. The comb was placed in the gel tray about 1 inch from one end of the tray and the comb was positioned vertically, so that the teeth are about 1–2 mm above the surface of the tray.
3. 1X TAE was prepared by diluting the appropriate amount of 50X TAE buffer with distilled water.
4. 1 gm of agarose was added to the prepared 100 mL of 1X TAE in a 250-mL conical flask and boiled to dissolve agarose.
5. When the agarose gel solution temperature was around 60°C, 10 µl of Ethidium bromide stock solution was added to get a final concentration of 0.5 µg/mL and mixed thoroughly.
6. Then the cooled agarose solution was poured in the gel tank without air bubbles and was 0.5–0.9 cm thick.The set was kept undisturbed until the agarose solidifies.
7. Once the gel was solidified, 1X TAE buffer poured slowly into the gel until the buffer level stands at 0.5 to 0.8 cm above the gel surface.
8. The wells were formed by gently lifting the comb.

Electrophoresis

1. To run, the comb and the sealed tapes were removed gently, the agarose gel along with tray was placed in an electrophoresis chamber, and covered (just until wells are submerged) with 1 X TAE electrophoresis buffer (the same buffer used to prepare the agarose).

2. To prepare samples for electrophoresis, 1 µL of 6X gel loading dye was added for every 5 µL of DNA solution, and mixed well. 5–12 µL of DNA per well was loaded (for minigel).
3. The electrophoresis was ran at 50–150 volts, until dye markers have migrated an appropriate distance, depending on the size of the DNA to be visualized.
4. After electrophoresis, the agarose gel was placed onto the UV trans-illuminator platform and visualized the DNA bands under shortwave UV light.

Note: If the gel was not stained with ethidium during the run, stain the gel in 0.5 µg/mL ethidium bromide until the DNA has taken up the dye and is visible under shortwave UV light.

Result and observations:

LAB MANUAL **BATCH-2017-2020**

EX.NO:5
Date:

PREPARATION OF RESTRICTION DIGESTION OF DNA SAMPLES

Aim: To perform restriction digestion of isolated plant genomic DNA with *EcoR* I and *Hind* III enzymes and to analyze the restriction pattern by agarose gel electrophoresis.

Principle:

Type II restriction enzymes hydrolyse the backbone of DNA between deoxy ribose and phosphate groups within the recognition sites and generate two different types of ends. This leaves a phosphate group on the 5' ends and a hydroxyl group on the 3' ends of both strands. The 5' or 3' overhangs generated by enzymes that cut asymmetrically are called sticky or cohesive ends, because they will readily stick or anneal with their complementary sequences by base pairing (Example: *EcoR* I). Some enzymes cut at precisely opposite sites in two strands of DNA and generate blunt ends without overhangs called blunt ends (Example – *Hae* III).

Materials required:

1. 10X restriction enzyme buffer (see manufacturer's recommendation)
2. DNA
3. restriction enzymes – *EcoR* I and *Hind* III
4. sterile water
5. phenol:chloroform (1:1)

Procedure

1. The following contents were added to a microfuge tube:

2 µL of appropriate 10X restriction enzyme buffer, 0.1 to 5 mg DNA, and sterile water to a final volume of 19 µL (Note: These volumes are for analytical digests only. Larger volumes may be necessary for preparative digests or for chromosomal DNA digests.)

2. 1 to 2 µL (3 to 20 units) enzyme was added, mixed gently and centrifuged for a few seconds in the microfuge.

3. Incubated at the appropriate temperature (usually 37°C) for 1 to 2 hours.

4. Meanwhile, 1 % agarose gel was prepared for electrophoresis.

5. After an restrict digestion incubation, 3 µL of 6x gel loading buffer was added to the samples.

6. The digested samples, 10 µL of control DNA, 10 µL of DNA size marker were loaded, (note down the order of loading).

7. Electophoresis was performed at 50 – 100 V for 1-2 hours.

8. After electrophoresis, the gel was observed under UV transilluminator.

(Note) If the DNA is to be used for another manipulation, heat-inactivate the enzyme (if it is heat-labile) at 70°C for 15 min, phenol/chloroform extract, and ethanol precipitate, or purify on DNA purification column.

EcoR I restriction site:

Hind III restriction site

Results and Observations:

LAB MANUAL BATCH-2017-2020

Ex.No: 6

Date :

DEMONSTRATION OF AMES TEST OR REVERSE MUTATION FOR CARCINOGENICITY

Bacterial Reverse Mutation Assay

Introduction

The bacteria reversed mutation assay (Ames Test) is used to evaluate the mutagenic properties of test articles. The test uses amino acid-dependent strains of *E.coli*. In the absence of an external histidine source, the cells cannot grow to form colonies. Colony growth is resumed if a reversion of the mutation occurs, allowing the production of histidine to be resumed. Spontaneous reversions occur with each of the strains; mutagenic compounds cause an increase in the number of revertant colonies relative to the background level.

Principal of test method

Bacterial culture medium is inoculated with the appropriate Salmonella or E. coli strain and incubated overnight. A dose rangefinder for the test chemical is carried out using strain TA100 only over a wide dose of range. Bacterial culture, test chemical and S9 mix are incubated for one hour. Thereafter the incubation solution is mixed with soft agar and added to minimal agar plates. The plates are incubated for 48 – 72 hours. After this time the numbers of revertant colonies are counted.

Tester Strains

Characteristics of Tester Strains

E.coli strains are histidine-, the used E. coli strain tryptophan dependent. Revertants are identified as colonies that grow in low levels of histidine or tryptophan. Frameshift and base-pair substitution defects are represented to identify of both types. Additional genetic markers serve to make the strains more sensitive to certain types of mutagens.

The DNA repair mutation (uvrA/B) eliminates excision repair, a repair pathway for DNA damage from UV light and certain mutagens. The presence of the uvrA/B mutation makes the

strains more sensitive to the test articles that induce damage in this manner. The uvrA/B mutation is part of a deletion mutation extending into a gene for biotin synthesis; therefore, the biotin requirement is a result of the deletion of this region. The uvrA/B mutation is indicated by sensitivity to UV light.

The rfa mutation changes the properties of the bacterial cell wall and results in the partial loss of the lipopolysaccharide (LPS) barrier increasing permeability of cells to certain types of chemicals. The rfa mutation is indicated by sensitivity to crystal violet.

The R factor plasmid (pKM101) makes the strains more responsive to a variety of mutagens. The plasmid carries an ampicillin resistance gene; therefore ampicillin resistance indicates that the strains retain the plasmid. The pAQ1 plasmid carries a tetracycline resistance gene, therefore tetracycline resistance indicates that strain TA102 retains the plasmid.

Exposure concentrations

The maximum test item concentration for non cytotoxic substances is 5 mg/plate or 5 µl/plate, but depends on solubility.

Controls

Strains are tested with the solvent or vehicle as the negative control and with known mutagens to demonstrate that the assay is working efficiently (positive control) and also to demonstrate that the metabolic activation system is operating.

Evaluation/Analysis

Besides cytotoxicity, precipitation and viability the number of revertant colonies per plate is determined. The mutant frequency is expressed as the quotient of the number of revertant colonies over the number of colonies in the negative control.

Interpretation of Results

A mutagenic potential of a test item is assumed if the mutant frequency is 2.0 or higher. A dose effect relationship could underline this conclusion. A possible mutagenic potential is assumed if the quotient ranges between 1.7 to 1.9 in combination with a dose effect relationship.

No mutagenic potential is assumed if all quotients range between 1.0 (or lower) to 1.6. A nonexistent dose effect relationship could underline this conclusion.