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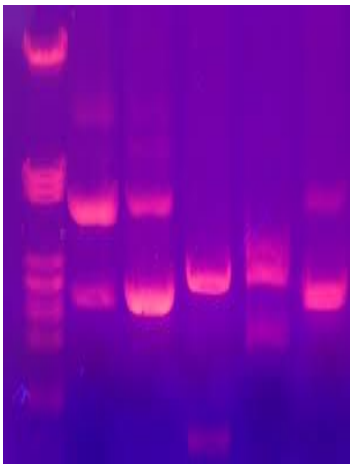
ज्ञानम् आत्म प्रदीपाय

Fundamentals of Biochemistry & Biotechnology
Practical Manual
Course No, CC-AGP102

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Practical 1: Preparation of Solutions

https://www.youtube.com/watch?v=A2YyIo8vSCA

Requirements:

Equipment: Weighing balance, magnetic stirrer, spatula

Reagent: Provided salt, distilled water

Glass Goods: Beaker, conical flask, measuring cylinder, glass rod

Additional: Tissue paper, duster, brush, marker pen

Preparing Chemical Solutions

Lab experiments and different types of research often require preparation of chemical solution. Preparation of these chemical solutions by weight (w/v) and by volume (v/v) are given.

Solute - The substance which dissolves in a solution

Solvent - The substance which dissolves another to form a solution. For example, in a sugar and water solution, water is the solvent; sugar is the solute.

Solution - A mixture of two or more pure substances. In a solution one pure substance is dissolved in another pure substance homogenously. For example, in a sugar and water solution, the solution has the same concentration throughout, ie. it is homogenous.

Mole - A fundamental unit of mass (like a "dozen" to a baker) used by chemists. This term refers to a large number of elementary particles (atoms, molecules, ions, electrons, etc) of any substance. 1 mole is 6.02×10^{23} molecules of that substance. (Avogadro's number).M

Introduction to preparation of solutions.

Many experiments involving chemicals call for their use in solution form. That is, two or more substances are mixed together in known quantities. This may involve weighing a precise amount of dry material or measuring a precise amount of liquid. Preparing solutions accurately will improve an experiment's safety and chances for success.

Solution 1: Using percentage by weight (w/v)

Formula: For weight percent (w/v) is: [Mass of solute (g) / Volume of solution (ml)] x 100

Solution 2: Using percentage by volume (v/v)

Formula: The formula for volume percent (v/v) is: [Volume of solute (ml) / Volume of solution (ml)] x 100

Solution 3: Molar Solutions

Molar solutions are the most useful in chemical reaction calculations because they directly relate the moles of solute to the volume of solution.

Formula: The formula for molarity (M) is: moles of solute / 1 liter of solution or gram-molecular masses of solute / 1 liter of solution.

Problem to be solved (On spot given by the Course Instructor):

Problem 1:

Problem 2:

Practical 2: pH and Buffer

https://www.youtube.com/watch?v=rIvEvwViJGk

Requirements:

Equipment: Weighing balance, magnetic stirrer, pH meter, Hot plate

Reagent: Different salt, distilled water, litmus paper, indicator solution, diluted HCl, 0.1% NAOH solution, pH paper

Glass Goods: Beaker, dropper, conical flask, measuring cylinder, glass rod, forceps

Stationary: Blotting paper, tissue paper, duster, marker pen

Definition: A pH value is a number, usually between 0 and 14, that represents the acidity or basicity of a solution. The "pH" is always written with a lowercase "p" and an uppercase "H", which stands for "power of hydrogen." pH values are related to hydrogen ion (H+) concentrations.

The mathematical relationship between pH and H+ is described by the equation

$$pH = -\log(H+)$$

Measuring the pH by pH indicators:

There are substances which have the property of changing their color when they come in contact with an acidic or basic environment. These substances are called **pH indicators**. Usually, they are used as dissolved substances, as for instance phenolphthalein and bromothymol blue. Often, to measure the pH, special papers which have been soaked with indicators are used. These papers change color when they are immersed in acidic or basic liquids. This is the case of the well-known litmus paper. More recently, it has become possible to measure the pH with electrical instruments like the **pH meter.**

Indicator Paper based pH measurement

1.

2.

3.

4.

5.

pH meter based measurement

1.

2.

3.

4.

5.

Table: Known Solutions given in laboratory

Substance	Colour in litmus paper	pH-meter reading	Colour in indicator Solution

Precautions

- Use only the standard colour pH chart supplied with the pH paper for assessing the pH value.
- Keep the pH strips away from chemical fumes.
- Either use fresh fine dropper or glass rod for each different sample, or wash the dropper or glass rod well with water every time.
- To correctly view the colour produced on the pH paper, keep the pH paper on a white tile while performing the experiment.

Table: Unknown Solutions given in laboratory

Substance	Colour in litmus paper	pH-meter reading	Colour in indicator Solution

Assignment: Prepare your own indicator at home:

Material	Colour	Acidic Colour	Basic Colour

Practical 3: Titrimetric Analysis

https://www.youtube.com/watch?v=sFpFCPTDv2w

Requirements:

Equipment: Weighing balance, magnetic stirrer, water bath, Spirit lamp, pH meter, Brix mete

Reagent: Fruit juice, distilled water, 0.1% NAOH solution, Phenolphthalein, Indicator stripes

Glass Goods: Burette, dropper, beaker, conical flask, measuring cylinder, glass rod, forceps

Stationary: Blotting paper, tissue paper, duster, marker pen

Test Unknown Sample 1:

Sample Name:

Sample Preparation:

Procedure: Titrate fruit juice against the 0.1% NaOH and record the reading.

Observation:

Burette Reading	$(cm)^3$	Rough	1	2	Final
	Initial				
	Final				
	Volume used				
Mean Titre					

Calculation:

Molarity of Unknown Solution	
M_1V_1 X M_2V_2	=

Precaution:

Inference:

Practical 4: Qualitative Test of Carbohydrate & Protein

https://www.youtube.com/watch?v=Ewe7i1D9lSQ

Requirements:

Equipment: Weighing balance, magnetic stirrer, water bath, Spirit lamp

Reagent: Known/unknown sugar, Available test reagent, distilled water, diluted HCl, 0.1% NAOH solution

Glass Goods: Beaker, dropper, conical flask, measuring cylinder, glass rod, forceps

Stationary: Blotting paper, tissue paper, duster

Carbohydrate:

Objective: To characterize carbohydrates present in an unknown solution on the basis of various chemical assays.

Test Unknown Sample 1:
Sample Name:

Requirement:

Procedure:

Observation:

Precaution:

Inference:

Test Unknown Sample 2:

Sample Name:

Requirement:

Procedure:

Observation:

Precaution:

Inference:

Protein: The presence of different amino acids produce different test result in samples

Objective: To characterize protein present in an unknown solution on the basis of various chemical assays.

Test known Sample 1:

Sample Name:

Requirement:

Procedure:

Observation:

Precaution:

Inference:

Practical 5: Quantitative Tests of Carbohydrates

https://www.youtube.com/watch?v=PCIm6oEKWWA

Requirements:

Equipment: Weighing balance, magnetic stirrer, water bath, Spirit lamp, Spectrophotometer

Reagent: Available test reagent, distilled water, diluted HCl, 0.1% NAOH solution, Benedicts Reagent

Glass Goods: test tube, test tube holder, Beaker, dropper, conical flask, measuring cylinder, glass rod, forceps

Stationary: Blotting paper, tissue paper, duster

Theory:

During qualitative analysis of sugars we have already learnt that glucose reduces copper sulphate in Benedict's reagent under alkaline conditions and a red precipitate is formed. This qualitative method has been exploited for its use in quantitative analysis.

Procedure:

Pipette out in a conical flask 25 ml of the Benedict's quantitative reagent. Add about 5 to 10 gm. of Na_2CO_3 and a few porcelain chips to the flask to prevent bumping. Heat the contents of conical flask to boiling and then run in the glucose solution from a burette at first rapidly and then slowly until the blue colour becomes fade.

Allow it to boil for 2-3 minutes more and add glucose solution drop by drop till the solution becomes colourless. Note down the volume of the glucose solution used and calculate the percentage of glucose in solution as described below. Sometimes the solution in the flask becomes too much concentrated due to evaporation of water. To avoid it more water may be added.

Calculation:

Observation	**Burette Reading ml**	**Equivalent concentration of Carbohydrate**	**Average**
1			
2			
3			

Test Unknown Carbohydrate Sample 1:

Sample Name:

Requirement:

Procedure:

Observation:

Precaution:

Inference:

Practical 6A: Quantitative Test of Protein

https://youtu.be/eVmFrf4T93A

Requirements for Protein:

Equipment: Weighing balance, magnetic stirrer, water bath, Spirit lamp, Spectrophotometer

Reagent: Test Protein, Fehling A, B, C reagent, distilled water, diluted HCl, 0.1% NAOH solution

Glass Goods: test tube, test tube holder, Beaker, dropper, conical flask, measuring cylinder, glass rod, forceps

Stationary: Blotting paper, tissue paper, duster

Principle:

The blue colour developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured in the Lowry's method.

Protein Solution (Stock Standard): Weigh accurately 50 mg of bovine serum albumin (Fraction V) and dissolve in distilled water and make up to 50 ml in a standard flask.

Working Standard: Dilute 10 ml of the stock solution to 50 ml with distilled water in a standard flask. One ml of this solution contains 200 µg protein.

Test Procedure:

Extraction of Protein from Sample:

Extraction is usually carried out with buffers used for the enzyme assay. Weigh 500 mg of the sample and grind well with a pestle and mortar in 5-10 ml of the buffer. Centrifuge and use the supernatant for protein estimation.

Estimation of Protein:

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes.
2. Pipette out 0.1 ml and 0.2 ml of the sample extract in two other test tubes.
3. The volume to 1 ml in all the test tubes was made. A tube with 1 ml of water serves as the blank.
4. 5 ml of reagent C to each tube including the blank is added and mixed well and allow to stand for 10 min.
5. Then 0.5 ml of reagent D is added and mix well and incubate at room temp, in the dark for 30 min. Blue colour is developed.

6. The readings at 660 nm. is taken.

7. A standard graph is drawn and calculate the amount of protein in the sample.

Calculation:

Express the amount of protein as mg/g or 100 g sample.

Calculate:

Observation	**Burette Reading ml**	**Equivalent concentration of Carbbohydrate**	**Average**
1			
2			
3			
4			
5			
6			
7			
8			

Practical 6B: Quantitative Test of Fat (Optional)

https://www.youtube.com/watch?v=fQ1hSNGnXYY

Requirements:

Equipment: Weighing balance, magnetic stirrer, water bath, Spirit lamp

Reagent: Available test reagent, distilled water, diluted HCl, 0.1% NAOH solution

Glass Goods: test tube, test tube holder, Beaker, dropper, conical flask, measuring cylinder, glass rod, forceps

Stationary: Blotting paper, tissue paper, duster

Principle

The free fatty acid in oil is estimated by titrating it against KOH in the presence of phenolphthalein indicator. The acid number is defined as the mg KOH required to neutralize the free fatty acids present in 1g of sample. However, the free fatty acid content is expressed as oleic acid equivalents.

Materials

» 1% phenolphthalein in 95% ethanol.
» 0.1N potassium hydroxide
» *Neutral Solvent*: Mix 25mL ether, 25mL 95% alcohol and 1mL of 1% phenolphthalein solution and neutralize with N/10 alkali.

Procedure

1. Dissolve 1-10g of oil or melted fat in 50mL of the standard solvent in a 250mL conical lask.
2. Add a few drops of phenolphthalein.
3. Titrate the contents against 0.1N potassium hydroxide.
4. Shake constantly until a pink color which persists for fifteen seconds is obtained.

Calculation

$$\text{Acid value (mg KOH/g)} = \frac{\text{Titrate value x Normality of KOH x 56.1}}{\text{Weight of sample (g)}}$$

The free fatty acid is calculated as oleic acid using the equation
1mL N/10 KOH = 0.028g oleic acid

Note

To find out the exact strength of KOH, prepare 0.1N oxalic acid solution (630mg in 100mL water) and titrate against KOH with phenolphthalein as indicator. Calculate the strength of KOH by the formula $V_1N_1 = V_2N_2$.

Test Unknown Protein Sample 1:

Sample Name:

Requirement:

Procedure:

Observation:

Precaution

Inference

Practical 7: Paper Chromatography

https://www.youtube.com/watch?v=yosecfE98Ok

Requirements:

Equipment: Chromatographic chamber with lid

Reagent: Isopropyl alcohol, distilled water, what man filter paper strip (20 x 2 cm)

Glass Goods: fine capillary tube, conical flask

Stationary: pencil, scale, blotting paper, tissue paper, duster

Principle:

It is based on the fact that paper chromatography separates compounds on the basis of their different rates of migration of filter paper (cellulose). The rates of migration depend upon the solvent which is flowing up or down the paper and on the relative adsorption which holds the molecules more or less tightly to the paper. If the solvent flows towards upper side on the paper it is called ascending chromatogram, and if it flows towards lower side then it is known as descending chromatogram.

Method:

Calculation:

Rf value can be measured by the following formula:

$$\text{Rf} = \frac{\text{Distance travelled by a given spot}}{\text{Distance travelled by solvent front}}$$

Distance travelled by a given spot is measured from the center of the spot.

Calculation:

1. Carefully observe that the amino acid spot should not be touched by the phenol

2. The paper should not touch the wall of the test tubes).

Unknown Sample:

Experiment to extract free amino acids from germinating seeds and to separate them by paper chromatography method. Also determine the Rf value of each amino acid:

Calculation:

Sample	Distance travelled by sample	Distance travelled by solvent	Rf

Inference:

Practical 8: TLC for separation of Amino acid/ monosaccharide

https://www.youtube.com/watch?v=rMGQavOMAmc

Requirements:

Equipment: TLC Plate, UV lamp

Reagent: methanol, ethyl acetate, ethanol, distilled water

Glass goods: fine capillary tube, conical flask

Stationary: Gloves, blotting paper, tissue paper, duster

Principle: Thin layer chromatographic (TLC) technique readily provides qualitative information and with careful attention to details, it is possible to obtain quantitative data. Thin layer chromatography is a technique used to separate and identify compounds of interest. A TLC plate is made up of a thin layer of silica adhered to glass or aluminum for support. The silica gel acts as the stationary phase and the solvent mixture acts as the mobile phase. In the ideal solvent system the compounds of interest are soluble to different degrees. Separation results from the partition equilibrium of the components in the mixture.

Procedure: In the simplest form of the technique, a narrow zone or spot of the sample mixture to be separated is applied near one end of the TLC plate and allowed to dry. The strip or plate is then placed with this end dipping in to the solvent mixture, taking care that the sample spot/zone is not immersed in the solvent. As the solvent moves towards the other end of the strip, the test mixture separates into various components. This is called as the development of TLC plates.

Calculation: The plate is removed after an optimal development time and dried and the spots/zones are detected using a suitable location reagent. An important characteristic used in thin layer chromatography is Rf value.

$$\mathrm{Rf} = \frac{\text{Distance travelled by a given spot}}{\text{Distance travelled by solvent front}}$$

Precaution: The separation depends on several factors;

(a) Solubility: the more soluble a compound is in a solvent, the faster it will move up the plate.

(b) Attractions between the compound and the silica, the more the compound interacts with silica, the lesser it moves,

(c) Size of the compound, the larger the compound the slower it moves up the plate.

Unknown Sample:

Requirements:

Sample Preparation:

Calculation:

Sample	Distance travelled by sample	Distance travelled by solvent	Rf

Inference:

Practical 9: Sterilization Techniques

https://www.youtube.com/watch?v=zDmP14twN8g

Equipment: Laminar Air Flow cabinet, Hot Air oven, Spirit lamp, magnetic stirrer, heater, micro oven

Reagent: Ethanol, Sodium hypochlorite, Soap, double distilled water, bavistin

Glass goods: Beaker, conical flask, Culture tube, Culture bottle, forceps, scalpel

Stationary: Gloves, blotting paper, tissue paper, duster, mask, head cover

Principle: Sterilization is the basic requirement for successful invitro plant generation. The explants as well as the reagent, growth chamber, media, instruments should be free of contaminants. Different sterilization includes

Wet-Heat (Autoclaving)

Dry Heat (Flaming, baking)

Filtration

Solvents

Radiation

Flame Sterilization/ Incineration:

Disinfectants:

Solve the questions given by course instructor:

Problem 1.

Problem 2.

Problem 3

Problem 4:

Problem 5:

Practical: 10 Composition of various Tissue Culture Media

https://www.youtube.com/watch?v=ltbdM3boWmU

Equipment: Autoclave, Laminar Air Flow cabinet, Hot Air oven, pH meter, Spirit lamp, magnetic stirrer, heater, micro oven

Reagent: MS salt, agar powder, hormone**,** ethanol, Sodium hypochlorite, Soap, double distilled water

Glass goods: Beaker, conical flask, Culture tube, Culture bottle, forceps, scalpel

Stationary: Gloves, blotting paper, tissue paper, duster, mask, head cover

Principle: Growth and morphogenesis of plant tissues in vitro are largely governed by the composition of the culture media. Although the basic requirements for culturing plant tissue is same but in practice there are some specific nutritional requirements for promoting optimal growth from different kinds of explants in case of different plant species. MS Media, Whites Media and Gamborg Media are example.

Composition of MS Medium:

Ingredient	Murashige & Skoog	White	Gamborg

Methods:

1. A packet of MS medium (in powdered form) is used for preparation of 1 litter medium.
2. 800 ml of distilled water is filled in a beaker.
3. MS powdered medium is slowly added into the beaker.
4. 30 g of sucrose is added.
5. PH is set at 5.8.
5. 8 g agar technical is added to the beaker.
6. Hormone is added.
7. The media is made up to 1 litre by using volumetric flask by adding 200 ml of distilled water.
9. The media is autoclaved.
10. The melting media is dispensed into sterile tubes. Each tube is labelled.
11. Each of the media listed below is prepared:

1. MS basal (without hormone)
2. 1.0 mg/1 BAP + 0.5 mg/1 NAA
3. 3.0 mg/1 BAP
4. ½MS + 3.0 mg/1 kinetin + 0.5mg/1 IAA
5. The condition of prepared medium is observed and used for culture a week later.

Precautions:

1. pH should be properly adjusted
2. Glass-goods should be clean and grease free
3. Thorough mixing of ingredients are required
4. .Autoclaved media should be kept in inoculation room

Table: Gap filling for different Media and Hormone required for Culture

Type of Culture	Media Strength	Hormone
Embryo	1 MS	BAP, NAA
Root	½ MS	?, NAA
Cell	?	?
Shoot Tip	MS	?
Callus	MS	?
Leaf	?	?

Observation:

Inference

Solve the questions:

Problem 1.

Problem 2.

Problem 3

Problem 4:

Practical 11: Preparation of Stock Solution for MS nutrient medium

Equipment: Laminar Air Flow cabinet, Hot Air oven, pH meter, Spirit lamp, magnetic stirrer, heater, micro oven

Reagent: MS salt, agar, Hormone**,** Ethanol, Sodium hypochlorite, Soap, double distilled water

Glass goods: Beaker, conical flask, Culture tube, Culture bottle, forceps, scalpel

Stationary: Gloves, blotting paper, tissue paper, duster, mask, head cover

Preparation of stock solutions: Since it is a time - consuming and tedious process to weigh the necessary products each time a medium is prepared, concentrated solutions of the desired composition of a medium are used which one dilutes adequately. These concentrated solutions are called stock solutions. Simple stock solutions comprise only one constituent at a time. Complex stock solutions comprise several chemicals. Stock solutions of macro and micronutrients, vitamins and growth regulators are prepared in distilled or high purity demineralized water. Chemicals should be of the highest grade.

i. **Macronutrient stock solution(s):** Usually, the stock solution of macronutrients is prepared as 10x. Dissolve all the macronutrients one by one except ($CaCl_2$ for macronutrient stock solution. The stock solution of $CaCl_2$ should be prepared separately. Another way is to dissolve the different macronutrients one after the other and $CaCl_2$ is dissolved separately and later added to the rest of the stock solution in order to avoid precipitation.

ii. **Micronutrient stock solution:** A stock solution of all the micronutrients with 100x is generally prepared. Since copper and cobalt are required in very small quantities, it is preferable to first make a separate stock solution of these two salts (100x) and then an appropriate volume can be pipetted and put into the main micronutrient stock solution. These nutrient solutions can be dispensed in plastic bags with zipper seals and stored frozen (e.g. 10x macronutrient solution is dispensed into a bag containing 100ml of solution to prepare 1 litre medium).

iii. **Iron-EDTA:** Iron EDTA should be added fresh. If stock solution (100x) is prepared, then it should be stored after autoclaving in an amber bottle or a bottle covered with an aluminium foil.

iv. **Vitamins and growth regulators stock solutions:** These are simple stock solutions. All the growth regulators are not soluble in water. Solubility of different growth regulators is given in Table 4.3. The compound should be dissolved in a few ml of solvent and then water is slowly added to make the requisite volume. Concentrations of compounds can be taken as mg/l or in molarity.

STORAGE OF MEDIA

After cooling, the media containers are stored preferably at 4-10^{o}C but that is not absolutely necessary. Medium should be used after 3-4 days of preparation, so that it medium is not properly sterilized, contamination will start to appear.

Table 4 Preparation of stock solutions of Murashige and Skoog (MS) medium

Constituent	Concentration in MS medium (mg/l)	Concentration in the stock solution (mg/l)	Volume to be taken/litre of medium
Macronutrients (10x) Stock solution I			
NH_4NO_3	1650	16500	100 ml
KNO_3	1900	19000	
$MgSO_4 . 7H_2O$	370	3700	
KH_2PO_4	170	1700	
Macronutrient (10x) Stock solution II			
$CaCl_2\ 2H_2O$	440	4400	100 ml
Micronutrients (100x) Stock solution III			
H_3BO_3	6.2	620	10 ml
$MnSO_4 . 4H_2O$	22.3	2230	
$ZnSO_4 . 7H_2O$	8.6	860	
Kl	0.83	83	
$Na_2MoO_4.2H_2O$	0.25	25	
$CuSO_45H_2O$	0.025	2.5	
$CoCl_2 . 6H_2O$	0.025	2.5	
Iron source			
Fe EDTA Na salt	40	Added fresh	
Vitamins			
Nicotinic acid	0.5	50 mg/100 ml	1 ml
Thiamine HCl	0.1	50 mg/100 ml	0.2 ml
Pyridoxine HCl	0.5	50 mg/100 ml	1 ml
Myo-inositol	100	Added fresh	
Others			
Glycine	2.0	50 mg/100 ml	4 ml
Sucrose	30,000	Added fresh	
Agar	8000	Added fresh	
pH 5.8			

Nutrient-medium chart for preparation of culture medium

Constituents	**Stock solution (conc.)**	**Quantity required for 1 L**	**Quantity for required volume of medium under preparation (e.g. 500ml)**	**Remarks**
Macro stock solution I	10x	100ml	50 ml	
Macro stock solution II ($CaCl_2$)	10x	100 ml	50 ml	
Micro stock solution III	100x	10 ml	5 ml	
Iron-EDTA Na salt	Added fresh	40 mg	20 mg	
Vitamins				
Nicotinic acid	50 mg/100 ml	0.5 mg/l = 1 ml	0.5 ml	
Thiamine HCl	50 mg/100 ml	0.1 mg/1 = 0.2ml	0.1 ml	
Pyridoxine HCl	50 mg/100 ml	0.5 mg/l = 1ml	0.5 ml	
Myo-inositol	Added fresh	100 mg	50 mg	
Others				
Glycine	50 mg/100 ml	2 mg/l = 4ml	2.0 ml	
Growth regulators				
Sucrose	Added fresh	30 g	15 g	
Agar	Added fresh	8 g	4 g	
pH				

Solve the questions given by course instructor:

Problem 1.

Problem 2.

Problem 3

Problem 4:

Practical 12: Callus induction from various explant

https://www.youtube.com/watch?v=Bfwrlajl07Y

Equipment: Laminar Air Flow cabinet, Hot Air oven, pH meter, Spirit lamp, magnetic stirrer, heater, micro oven
Reagent: MS salt, agar, Hormone**,** Ethanol, Sodium hypochlorite, Soap, double distilled water

Glass goods: Beaker, conical flask, Culture tube, Culture bottle, forceps, scalpel

Stationary: Gloves, blotting paper, tissue paper, duster, mask, head cover

Principle: Callus is an actively-dividing non-organized mass of undifferentiated and differentiated cells often developing either from injury (Wounding) or in tissue culture in the presence of growth regulators. Explants from both mature and immature organs can be induced to form callus. However, explants with mitotically active cells (young, juvenile cells) are generally good for callus initiation. Callus is produced on explants *in vitro* from peripheral layers as a result of wounding and in response to growth regulators, either endogenous or exogenously supplied in the medium. The season of the year, donor conditions of the plant, the age and physiological state of the parent plant contribute to the success of organogenesis in cell cultures.

Aim: To induce callus from the explants of ***Phaseolus mungo*** **(Green Gram)**

Reagents and other requirements

Growth regulators – 2, 4-D

Plant material –

Media

Seed Germination: MS Medium

Callus Induction: MS + 2, 4-D (2mg/lL)

I. Seed Germination

II. Callus Induction

Result:

Solve the questions:

Problem 1.

Problem 2.

Problem 3

Problem 4:

Practical 13: Micro propagation, Hardening & Acclimatization

https://www.youtube.com/watch?v=TORRxwbz7aY

Equipment: Laminar Air Flow cabinet, Hot Air oven, Spirit lamp, magnetic stirrer, heater, micro oven

Reagent: MS salt, agar, Hormone**,** Ethanol, Sodium hypochlorite, Soap, double distilled water

Glass goods: Beaker, conical flask, Culture tube, Culture bottle, forceps, scalpel

Stationary: Gloves, blotting paper, tissue paper, duster, mask, head cover

Hardening: The plantlets (size: ~3-4 cm long with 3-4 roots) were raised from different explants sources of all the three species. The fully differentiated plantlets with 3-4 roots from the axenic culture condition were selected for the present investigation. The plantlets were maintained in a highly reduced level of MS (Murashige and Skoog, 1962) salt solution (1/10th strength) devoid of any plant growth regulators and sucrose or any other organic **carbon source**s.

Acclimatization: The hardened plants from all the three species were transplanted to Community Potting Mix (CMP) (charcoal pieces: brick chips: moss at 1:1:1 ratio for epiphytes and charcoal pieces: brick chips: moss: forest litter at 1:1:1:1 ratio for terrestrial orchids) along with the content of the culture vials. The potted plants were maintained in the poly house as well as directly in the natural condition. Initially the transplanted plants were watered at regular interval. The plantlets were transplanted at different seasons of the year both in the natural condition as well as in the poly house. A part of the hardened plants were maintained in the culture vials for three year and are watered at regular interval.

Solve the questions:

Problem 1.

Problem 2.

Problem 3

Problem 4:

Solve the questions:

Problem 5:

Problem 6:

Problem 7:

Problem 8:

Practical 14: DNA Isolation from plants

https://www.youtube.com/watch?v=-rxLZRZ0DMQ

Equipment: Laminar Air Flow cabinet, Refrigerated Centrifuge, double distillation set, weighing machine, pH meter, water bath, vortex, Spirit lamp, magnetic stirrer, heater, micro oven, shaker-cum-incubator, micro-pipette, micro-tip box,

Reagent: MS salt, Ethanol, Sodium hypochlorite, Soap, double distilled water, Tris, EDTA, SDS, beta-mercaptoethanol, PVP, isopropanol, ethanol, NH4-acetate, Na-acetate

Glass goods: Beaker, conical flask, blade, eppendorf tube, micro tip

Stationary: Gloves, blotting paper, tissue paper, duster, mask, head cover

Solution and solvent

- Extraction buffer: 1% SDS, 0.5 M NaCl (no EDTA, Tris–HCl or pH adjustment needed); (ii) isopropanol; (iii) 70% (v/v) ethanol.

Procedure:

1. Take half of a young dry leaf and cut it into small pieces then grind it using a porcelain mortar and pestle in 400 µl of the extraction buffer. Add more buffer until it reaches a final volume of 1200 µl in order to have enough homogenate to place into microfuge tube. Harvest the homogenate into 1.7 ml microfuge tubes.

2. Spin (13,500 rpm, 4 min, RT) using a microcentrifuge.

3. Transfer the supernatant into a new microfuge tube and add an equal volume of isopropanol (500 µl in our study) and mix gently by inversion. Place the mixture on ice for 5 min.

4. Spin (13,500 rpm, 4 min, RT) using a microcentrifuge.

5. Discard the supernatant and wash the DNA pellet with 500 µl 70% (v/v) ethanol.

6. Spin (13,500 rpm, 2 min, RT) using a table microcentrifuge.

7. Discard the ethanol. Blot away the excess ethanol from the pellet by inverting/placing it on a clean paper-towel. Let the pellet air-dry.

8. Dissolve the DNA in 50 µl ddH_2O and store it at 4 °C for immediate use or − 20 °C for long-term storage. If the lab is equipped with a − 80 °C freezer, recommend storing at − 80 °C.

Specimen Name:

Different DNA Extraction Method:

Method	Extraction buffer	Solvent

Sample 1:

Scientific Name:

Method:

Precaution:

Inference

Practical 15: Genetic Fingerprinting (Designing PCR Primer)

(https://www.youtube.com/watch?v=mcOwlFVEino0

Requirements: Laptop/Desktop, calculator

What is a primer: A primer is a short, single-stranded DNA sequence used in the polymerase chain reaction (PCR) technique. In the PCR method, a pair of primers is used to hybridize with the sample DNA and define the region of the DNA that will be amplified. Primers are also referred to as oligonucleotides

Size of Primer: The size of the primer is very important as well. Short primers are mainly used for amplifying a small, simple fragment of DNA. On the other hand, a long primer is used to amplify a eukaryotic genomic DNA sample. However, a primer should not be too long (> 30-mer primers) or too short. Short primers produce inaccurate, nonspecific DNA amplification product, and long primers result in a slower hybridizing rate.

Amplicon Size: On average, the DNA fragment that needs to be amplified should be within 1-10 kB in size

Structure of the primer: should be relatively simple and contain no internal secondary structure to avoid internal folding. One also needs to avoid primer-primer annealing which creates primer dimers and disrupts the amplification process. When designing, if unsure about what nucleotide to put at a certain position within the primer, one can include more than one nucleotide at that position termed a mixed site. One can also use a nucleotide-based molecular insert (inosine) instead of a regular nucleotide for broader pairing capabilities.

Primer Property:

- Length of 18-24 bases
- 50-60% G/C content
- Start and end with 1-2 G/C pairs
- Melting temperature ™ of 50-60°C
- Primer pairs should have a Tm within 5°C of each other
- Primer pairs should not have complementary regions

Melting Temperature (Tm Value) Calculation: Primers should be end with a G or C or CG or GC base, deceasing breathing of ends, increases efficiency.
Tm Calculation = 2(A+T) + 4(G+C) ◦C

Annealing Temperature Calculation (Ta): The annealing temperature (Ta) of PCR reaction is also dependent on the Tm of primers. According to Rychlik formula
Ta = 0.3X Tm of Primer – 0.7X Tm of Product – 14.9

Avoiding primer-dimers formation: Another issue with the 3’ primer ends is the possibility of homologies within the primer pair, leading to the dreaded primer-dimer effect. Primer-dimer is when the PCR product obtained is the result of amplification of the primers themselves. This sets up a competitive annealing situation between the template and the primer-dimer product during amplification, negatively affecting results downstream. The risk is greatly increased when multiplexing, and multiple primers are included in the PCR reaction.

Problem 1: Calculate the Tm Value of each Primer

Primers	Sequences	References
DPKup	5′-GTTATCCCAGAAGGCTTTGCAGGCTTCA-3′	21
DPKlow	5′-GCCGACTGAGCCCTGGGAGGTAGGTA-3′	21
2D6dupl-F	5′-CCTGGGAAGGCCCCATGGAAG-3′	22
2D6dupl-R	5′-CAGTTACGGCAGTGGTCAGCT-3′	22
5′2D6dup	5′-GCCACCATGGTGTCTTTGCTTTCCTGG-3′	23
3′2D6dup	5′-GGTTTCTTGGCCCGCTGTCCCCACTC-3′	23
5′2D6*5	5′-CACCAGGCACCTGTACTCCTC-3′	24
3′2D6*5	5′-CAGGCATGAGCTAAGGCACCCAGAC-3′	24
5′2D6*3	5′-GGTCAGTGGTAAGGACAGGCAGGCCC-3′	25
3′2D6*3	5′-TCTCGGGGGGGCTGGGCTGGGTCCCAGGTTGGCC-3′	25
5′2D6*4	5′-TGCGCAACTTGGGCCTGGGCAAGAAGTCGCTGGACCCG-3′	25
3′2D6*4/*6	5′-CTCGGGAGCTCGCCTTGCAGAGACTC-3′	25
5′2D6*6	5′-TGCGCAACTTGGGCCTGGGCAAGAAGTCGCTGGACTAG-3′	25
5′2D6*10	5′-CCTGATGCACCGGCGCCAACGCTGGGCTGCACAGTAC-3′	25
3′2D6*10	5′-CAAACCTGCTTCCCCTTCTCAGCC-3′	25
5′2D6*17	5′-GGCGAGGACACCGCCGACCGCCCGCCTGTGCCCAGTA-3′	25
3′2D6*1×N	5′-CCTGTCTCAAATGCGGCCAGGCGGTGGGGTA-3′	25

Calculated Tm:

Tm of Forward Primer	Tm of Reverse Primer	Tm of Primer Pair	Ta of the Primer Pair

Problem 2: Calculate the GC value of the Primers from above Table

Calculated GC %:

Primer Name	Total Base Count	No, of G base	No. of C base	Total GC	% GC

Problem 3: Calculate the Tm Value of the Primer Pair mentioned by the Instructor

Gene	Primer sequence	RT-PCR product length (bp)	Reference
$GABA_AR\ \alpha1$	CCA AGT CTC CTT CTG GCT CAA CA GGG AGG GAA TTT CTG GCA CTG AT	111	Tan et al., 2011
$GABA_AR\ \alpha2$	TTA CAG TCC AAG CCG AAT GTC CC ACT TCT GAG GTT GTG TAA GCG TAG C	103	Tan et al., 2011
$GABA_AR\ \alpha3$	CAA GAA CCT GGG GAC TTT GTG AA AGC CGA TCC AAG ATT CTA GTG AA	119	Tan et al., 2011
$GABA_AR\ \alpha4$	GAG ACT GGT GGA TTT TCC TAT GG GGT CCA GGT GTA GAT CAT CTC ACT	94	Tan et al., 2011
$GABA_AR\ \alpha5$	CCC TCC TTG TCT TCT GTA TTT CC TGA TGT TGT CAT TGG TCT CGT CT	99	Tan et al., 2011
$GABA_AR\ \alpha6$	TAC AAA GGA AGA TGG GCT ATT ACG ATG GGC AAA GTC AGA GAG	439	Glassmeier et al., 1998
$GABA_AR\ \beta1$	GGG GCT TCT CTC TTT TCC CGT GA GGT GTC TGG TAC CCA GAG TTG GT	334	Gustincich et al., 1999
$GABA_AR\ \beta2$	CAA CTC TGG GTG CCT GAC ACC TA TCC TAA TGC AAC CCG TGC AGC AG	495	Gustincich et al., 1999
$GABA_AR\ \beta3$	GGT TTG CTG CGC TCA GAG CGT AA TAC AGC ACT GTC CCA TCA GGG T	390	Gustincich et al., 1999
$GABA_AR\ \gamma1$	CAG TTT GCA TTT GTA GGG TTA CG AGA CAC CCA GGA AAG AAC CAC TG	165	Gustincich et al., 1999
$GABA_AR\ \gamma2$	GGT GGA GTA TGG CAC CCT GCA TT AGG CGG TAG GGA AGA AGA TCC GA	322	Gustincich et al., 1999
$GABA_AR\ \gamma3$	TGC TCG GTC CAG GAG GGT AGA CTG ATC AGC TGC CTC AAC TGA ATT TTT	592	Gustincich et al., 1999
$GABA_AR\ \delta$	GAC TAC GTG GGC TCC AAC CTG GA ACT GTG GAG GTG ATG CGG ATG CT	398	Gustincich et al., 1999
$GABA_AR\ \varepsilon$	CAA TGC GAA GAA CAC TTG GAA GC CTG GCA GCA GCA GCT TCT ATC TT	225	Gustincich et al., 1999
β-actin	AGG CCA ACC GTG AAA AGA TG ACC AGA GGC ATA CAG GGA CAA	101	Gustincich et al., 1999

Calculated Tm:

Tm of Forward Primer	Tm of Reverse Primer	Tm of Primer Pair	Ta of the Primer Pair

Practical 16: Demonstration of Gel Electrophoresis

https://www.jove.com/v/3923/agarose-gel-electrophoresis-for-the-separation-of-dna-fragments

Equipment: Horizontal gel electrophoresis tank with all other unit, Power pack for electric supply, Laminar Air Flow cabinet, weighing machine, Micropipette, pH meter, water bath, vortex, Spirit lamp, magnetic stirrer, heater, micro oven, shaker-cum-incubator

Reagent: Agarose, ethydium bromide, loading dye, TAE buffer, ethanol, BFB, Ladder DNA

Glass goods: Beaker, conical flask, blade, eppendorf tube, micro tip

Stationary: Gloves, blotting paper, tissue paper, duster, mask, head cover

What is Gel Electrophoreses Used For?

The purpose of gel electrophoresis is to **visualize, identify and distinguish molecules** that have been processed by a previous method such as PCR, enzymatic digestion or an experimental condition. Often, mixtures of nucleic acids or proteins that are collected from a previous experiment/method are run through gel electrophoresis to determine the identity or differentiate between molecules.

Gel Electrophoresis Steps: The broad steps involved in a common DNA gel electrophoresis protocol (Each spet will be elaborated in class:

1. Preparing the samples for running

2. An agarose TAE gel solution is prepared

3. Casting the gel

4. Setting up the electrophoresis chamber

5. Loading the gel

6. Electrophoresis

7. Stopping electrophoresis and visualizing the DNA

Precaution:

Inference:

Solve the Questions:

1. The gel in gel electrophoresis is analogous to what apparatus?

- A. Power Supply
- B. Sieve
- C. Current Generator
- D. A Ladder

The gel serves to separate large and small molecules with its porous meshwork, through which smaller molecules can travel faster than larger molecules.

2. What does gel electrophoresis accomplish?

- A. Adds electric charge to molecules
- B. Switches the electric charge of molecules
- C. Adds electric charge to gels
- D. Separates molecules by their size

The current added to the system pulls charged molecules across the gel. DNA and RNA have a slightly negative charge, so they are pulled by the current. According to their size, they will travel faster or slower. Given enough time, gel electrophoresis can separate a number of molecules of different sizes.

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Additional Sheet

Additional Sheet

Additional Sheet

Additional Sheet