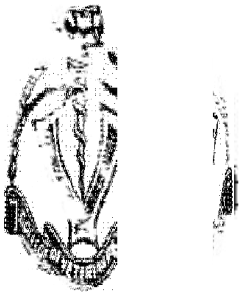
LABORATORY MANUAL ON TECHNIQUES IN BIOCHEMISTRY



COURSE NO. VBC-602 (TECHNIQUES IN BIOCHEMISTRY)

Course Teacher: Dr. Shantanu T

DEPARTMENT OF VETERINARY BIOCHEMISTRY
College of Veterinary Science, Assam Agricultural University
Khanapara, Guwahati – 781022

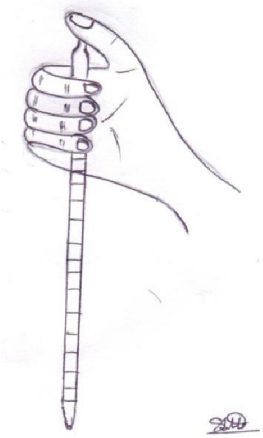
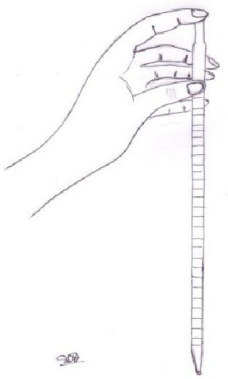
Name of the student: ……..
(Write name in capital letters) ……..

Registration No.: ……..

SAFETY RULES IN THE BIOCHEMISTRY LABORATORY

The following is a list of general safety rules to be followed by all the students!

1. Eating, drinking, and/or smoking in the laboratory is strictly forbidden.
2. Laboratory chemicals are not to be tasted since many are toxic. When instructed to smell reagents, do so with great caution, and NEVER put your nose over the bottle! Avoid looking into the mouth of any reaction vessel in which a reaction is in progress. Never point a test tube that contains a heated liquid at anyone.
3. Proper attire must be worn at all times such as apron. THE OPEN-TOED OR SANDALS ARE STRICTLY PROHIBITED.
4. No one will perform any unauthorized experiments, nor will anyone work in the lab alone, or outside of regularly scheduled hours.
5. Report ALL injuries, allergies and/or medical problems to the lab instructor.
6. Firepolish all glass tubing and stirring rods; protect your hands with a towel and lubricate the glass tubing or thermometer when inserting into stoppers.
7. When pouring something out of a reagent bottle, always READ THE LABEL TWICE to be certain that you are using the correct material; always hold the bottle by placing your hand over the label.
8. Label every chemical container to avoid mix-ups.
9. If you spill something, clean it up (GET HELP WITH HAZARDOUS MATERIALS)! Wash your hands immediately after skin contact with any chemical reagent. Also wash them after lab. If liquids drip down the side of the bottle, while pouring, wash the bottle off.
10. NEVER return excess chemicals to the reagent bottle.
11. Dispose of the excess chemicals in the proper waste container, as indicated by the lab instructor.
12. The **MOBILE PHONES** are strictly prohibited in the lab.
13. DO NOT DRAW THE STRONG ACIDS USING MOUTH PIPETTING.

	
Incorrect way of holding pipette	Correct way of holding pipette

POWER PREFIXES

Prefix	Symbol	Factor
yotta	Y	10^{24}
zetta	Z	10^{21}
exa	E	10^{18}
peta	P	10^{15}
tera	T	10^{12}
giga	G	10^{9}
mega	M	10^{6}
kilo	k	10^{3}
hecto	h	10^{2}
deka	da	10^{1}
deci	d	10^{-1}
centi	c	10^{-2}
milli	m	10^{-3}
micro	μ	10^{-6}
nano	n	10^{-9}
pico	p	10^{-12}
femto	f	10^{-15}
atto	a	10^{-18}
zepto	z	10^{-21}
yocto	y	10^{-24}

IMPORTANT CONVERSIONS:

1 cm^3 = 1 ml	1 deci liter (dl)= 100 ml	$C^o = \frac{(F^o - 32) \times 5}{9}$	{ C^o=degree Celsius F^o= degree Fahrenheit}
1 mole= 6.02×10^{23} (Avagadro's Number)			

METHODS FOR EXPRESSION OF CONCENTRATION

A solution is a soluble solid substance dissolved in a liquid. The former is called the solute and the latter the solvent. There are different methods of expressing concentration of solution -

1. In percent (%):

    a) % w/v or w/v ratio - The concentration of a solution is expressed by the weight of a solute in the total volume of the solvent. It is the most commonly used method of expressing the concentration of solutions. It can be defined gram of solute in 100ml of solution. It can be expressed in different way also e.g. %, g/dl, g/100ml

    b) % v/v or v/v ratio

2. Molar or molal: The concentration of a solution is expressed by volume of solute in the total volume of the solvent. It is commonly used for expressing the concentration of liquid solute such as ethanol. It can be defined as ml of solute in total 100 ml of solution. Eg. 100 ml of 70% ethanol indicates 70 ml of ethanol added to 30 ml of water. To prepare 'V' ml of X % of a solute→

$$\text{ml of liquid solute to be taken} = \frac{X}{100} \times V$$

According to system international units (SI units) solute concentrations are expressed in mole or millimole per litre.

One mole of a substance is defined as the atomic or molecular weight of that substance. A gram symbol or a formula weight of any substance contains 6.02 x 10^{23} (Avogrado number of particles). When one mol of substance dissolved in 1 kg of water the concentration is defined as 1 molal (1m) solution. When 1 mol of substance dissolved in 1 litre of a final solution the concentration is defined as 1 molar (1M or 1 mol/ L) solution.

Molar solution is a part of metric system, hence M = g MW/l, mM = mg MW/L, μM = μg MW/L etc.

Problem:

3) Normality: used to express the concentration of acid, base, oxidizing and reducing solution. Equivalent weight in grams in 1000 ml gives one normal. A normal solution of an acid contains the weight of acid per litre of solution, which donates formula weight of H^+ (6.02 x 10^{23} H^+).

A normal oxidizing solution contains sufficient oxidizing agent liter to increase the positive valence or decrease the negative valence of a gram-formulae weight of reducing agent by one unit and vice-versa.

Problem:
Relationship between molarity and normality: -

Molarity and normality is equal when valence is one. But one molar substance is two normal when the valency of the substance is 2 and so on.

Problem:

Relationship between millimole, milliosmol and milliequivalent: -

The term millimole is used to express the concentration of solute in solution. One millimole means atomic weight or molecular weight of that substance in 1000 ml solution.

The osmole proportion of a solute in solutions is related to the number of particles in solution and not to its weight or its charge. One osmole is equal to molecular weight in grams, which gives 6.02 x 10^{23} particles in 1000 ml of solution. In case of non-electrolyte millimole and milliosmol is same but in case of electrolyte, it depends on number of dissociable ions.

The term milliequivalent is used to express the concentration of ions (electrolytes) in biological fluids. Equivalent weight in grams in 1000 ml gives 1Equivalent weight. For univalent ions, 1 mole equals to 1 equivalent (Eq). For multivalent ions, 1 Equivalent weight is equal to the molecular weight in grams (i.e. 1 mole) divided by charge of particle

IMPORTANT FORMULAE

1. Preparation of a solution of concentration of '**M**' Molar of 'V' volume:

$$\textbf{Grams of solute to be dissolved in V ml of water} = \frac{\textbf{Molecular Weight} \times (\textbf{M}) \times \textbf{V}}{\textbf{1000}}$$

2. Preparation of a solution of concentration of '**N**' Normal of 'V' volume:

$$\text{Grams } \textbf{of solute to be dissolved in V ml of water} = \frac{\textbf{Molecular Weight} \times (\textbf{N}) \times \textbf{V}}{\textbf{Valency} \times \textbf{1000}}$$

3. Preparation of Solution of concentration of 'Os' Osmolar of 'V' volume:

$$\text{Grams } \textbf{of solute to be dissolved in V ml of water} = \frac{\textbf{Molecular Weight} \times (\textbf{N}) \times \textbf{V}}{\textbf{n} \times \textbf{1000}}$$

n = number of dissociated particles of a ionizable compound

STANDARD SOLUTION

By a standard solution we mean a solution of known strength i.e., a solution that contains a known weight of the solute present in a known volume of it.

Two commonly used methods preparing standard solution are –

A) Gravimetric analysis: This analysis enables us to ascertain quantitatively the composition of a given substance by direct weighing. Substance which are obtained in very pure state a normal (N) seminormal (N/2 or 0.5 N) centinormal (0.01N) and two normal (2N) solution of it is used as the standing point in volumetric analysis e.g. Na_2Ca_3 oxalic acid.

B) Volumetric analysis: This analysis enables us to ascertain quantitatively the composition of a given substance by means of a solution of known strength. The operation of determining the strength of an unknown solution by allowing it to react with the standard solution is titration.

It is always convenient and advisable to prepare standard solution by volumetric analysis. First a strong standard solution is prepared and then it is diluted to the desired concentration. For this purpose, a primary standard or reference standard and indicator are essential.

Indicators are substances, which indicate the end point of titration by the change of their colour. Acid-base indicators, are also called neutralization indicators, are weak organic acids or bases. The characteristics of good indicators are:

(a) A sharp contrast of colour in acid and alkaline solution
(b) The colour change must occur within a short and definite range of pH and
(c) The colour change should be at the end point of the reaction being studied.

Table 1. Uses and suitability of indicators

Indicators	**Colour in neutral solution**	**Colour with acid**	**Colour with alkali**	**Uses**
Methyl orange	Orange	Pink or red	Yellow	Strong acid /weak base
Phenolphthalein	Colourless	Colourless	Pink	Weak acid/strong base
Litmus solution	Purple or violet	Red	Blue	Weak acid/strong base

N.B. Any type of indicator can be used to titrate strong acid / strong base. Titration is not done in weak acid / weak base. Mixed indicator is a mixture of methyl orange, methyl red, Bromophenol blue, bromothymol blue and phenolphthalein. It is also a used as a universal indicator to cover wide range acid-alkali.

OBJECTIVE: PREPARATION OF 250 ML 0.1 N OXALIC ACID SOLUTION

METHOD: GRAVIMETRIC ANALYSIS

DATE:

Procedure:

1. Exactly _____g oxalic acid can be weighed in a balance calculation is shown in page
2. Transformed it to 250 ml volumetric flask
3. Diluted to the mark with distilled water mixed.

EXPERIMENT NO:

OBJECTIVE: PREPARATION OF NORMAL SOLUTION OF ACID (APPROXIMATE)

METHOD: GRAVIMETRIC ANALYSIS

DATE:

Principle:

Many acids are liquid at the ordinary temperature, hygroscopic and not pure, it is not possible to weigh the exact amount of acid. An approximate strength of acid can be prepared by following formula:

$$\mathbf{V1} = \frac{(\mathbf{Equivalent\ Weight\ of\ Acid}) \times (\mathbf{N1}) \times (\mathbf{V2})}{\mathbf{10} \times (\mathbf{Specific\ Gravity}) \times (\mathbf{Purity})}$$

V1= the volume of concentrated acid to be taken for dissolving in water.

V2= the total of given volume of dilute acid of normality N1 to be prepared.

Problem:

TITRATION

Titration is a volumetric technique by which concentration of an unknown solution (usually an acid or base) is determined by reacting with a corresponding neutralizing solution of known strength (called standard solution). The end point of neutralization is marked by the change of colour of an indicator added to the titrating system.

Procedure:

(i) Prepare the burette for the solution. The burette should be clean, free from chips or cracks and stopcock lightly. To grease a clean stopcock, apply a bit of grease with the fingertip down the two sides of the stopcock away from the capillary bore. Then insert

the stopcock in the burette and rotate it until a smooth covering of the whole stopcock is obtained. If the burette is equipped with a Teflon plug (plastic), the stopcock need not be lubricated. Rinse the burette with a little amount of the titrant, which will later be used to fill the burette. Rinsing is done by rolling the titrant over the inside wall and discarding the rinsed solution through the stopcock. During rinsing, watch if the burette is clean. A clean burette will drain without any solution clinging to its sides; if the burette is dirty, there will be droplets of liquid clinging to the sides.

(ii) Fasten the burette clamp to the burette stand.

(iii) Fill the burette slowly and carefully with the titrant (unknown). Do not allow air bubbles to form inside the burette. A small beaker may be used during pouring and allow the titrant to flow down the inside wall of the burette. Fill the titrant past the zero mark of the burette, then bring the miniscus exactly to the zero mark by draining. Use the stopcock to control the flow of the titrant. Collect the drained titrant in the beaker used for filling the burette.

(iv) Measure the second solution (standard) into a Erlenmeyer flask (or beaker) with a volumetric pipette. Take extreme care to measure the volume accurately. Add the required amount of the indicator solution into the flask. If the volume is low, you can add 5 to 10 ml of the distilled water to dilute the volume of the fluid in the flask. The volume of diluent is not critical, since it does not enter into the reaction or affect the volumes of the solutions that are being titrated.

(v) Before starting the titration, inspect the burette carefully for any air bubble trapped within the burette column or inside the stopcock or on the tip. Presence of air bubbles adds air-volume to the volume of the titrant as an error. Keep the tip of the burette well within the Erlenmeyer flask, which contains the fluid to be titrated (standard); clamp the burette at this position. Put one hand at the stopcock and the other is used to swirl the flask.

Titration is done by adding the solution in the burette to the Erlenmeyer flask by rotating the stopcock carefully. For accurate reading, titration is done at least three times. The titration readings of three replicates must be close; the second and the third readings should be almost identical.

Clean up the burette following titration by thoroughly rinsing the burette several times with tap water followed by rinsing with distilled water. Dry the burette by holding it on the burette stand.

OBJECTIVE: DETERMINANTION OF CONCENTRATION OF A GIVEN SOLUTION OF SODIUM HYDROXIDE USING A STANDARD SOLUTION OF 0.1 N OXALIC ACID.

DATE:

PRINCIPLE**:** The concentration of a given solution of an alkali can be determined by titrating it against a standard solution of acid. For example, if the given solution is NaOH, it is required to be titrated against standard solution of oxalic acid.

For this, the formulae $N_BV_B = N_AV_A$ is to be used. Where N_B and N_A are the strength of alkali and acid respectively and V_B and V_A are the volume of alkali and acid respectively.

MATERIALS REQUIRED:

a. GLASSWARES: Funnel, Burette, Burette stand, Beaker (250 ml) and conical flask (50 ml).
b. SOLUTIONS AND CHEMICALS: 0.1 N Oxalic Acid, given solution of NaOH, Phenolphthalein.

PROCEDURE:

1. Take the given solution of NaOH in beaker.
2. Place the funnel over the top of burette. And fill the burette with the given solution of NaOH using beaker. Remove the air gap inside the tip of burette. Bring the lower meniscus of NaOH solution to the mark of "0".
3. Take 10 ml of 0.1 N Oxalic acid along with two drops of phenolphthalein in 50 ml conical flask.
4. Place the flask below the tip of burette. Allow the NaOH solution to fall into the oxalic acid drop by drop in very slow speed. Once the pink color appears, the flask is required to be shaken and the persistence of the pink color is to be observed. If the pink color fades away, the process is to be continued else to be stopped.
5. The point of burette reading where the oxalic acid solution develops the persistent pink color is called the end-point. That point is to be considered equal to V_B.
6. The titration is to be repeated thrice. The average value of second and third reading is to be considered for the calculation.

RESULT:

Initial Burette Reading (A)	Final Burette Reading (B)	V_B (A—B)	Average V_B

CALCULATIONS:

OBJECTIVE: **ESTIMATION OF PLASMA/SERUM PROTEIN**

METHOD: **Biuret method**

PRINCIPLE:

Biuret is the most commonly used method for total plasma or serum protein estimation. Biuret reagent consists of alkaline copper sulphate solution containing sodium-potassium tartarate. The cupric ions form a co-ordination complex with four – NH groups present in peptide bonds giving an absorption maximum at 540 nm. The method is reliable, reproducible and useful in the clinical field. Its main disadvantage is its lack of sensitivity, being unsuitable for the assay of proteins at concentration much less than 1 mg/ml.

REAGENTS:

1. Biuret reagent – Prepare 200 ml of 2% (w/v) of NaOH solution. Put this solution in 500 ml volumetric flask. Add 4.5 grams of sodium potassium tartarate and 15 grams of copper sulphate pentahydrate and 1 gram of potassium iodide. Mix the contents continuously until complete dissolution. Add distilled water till the mark of 500 ml in the volumetric flask.

2. Bovine serum albumin (BSA) standard – 6 g/100 ml NSS.

PROCEDURE:

	Test (a)	**Standard (b)**	**Blank (c)**
Distilled water (ml)	-	-	0.02
Serum/plasma (ml)	0.02	-	-
Standard (ml)	-	0.02	-
Biuret reagent (ml)	1	1	1

Incubate all the tubes at room temperature for 10 min. Read at 555 nm. Adjust reagent blank at 0.

Calculation:

$$\textbf{Serum total protein } (\textbf{g/dl}) = \frac{\textbf{OD of test}}{\textbf{OD of Standard}} \times (\textbf{Concentration of standard})$$

OBJECTIVE: **ESTIMATION OF PROTEIN IN BIOLOGICAL FLUID**

METHOD: **Lowry (Folin – Ciocalteau) method**

PRINCIPLE:

The phenolic group of tyrosine residues in a protein will produce a blue/purple colur, with maximum absorption in the region of 660 nm with Folin and Ciocalteau reagent, which consist of sodium tungstate, molybdate and phosphate. The method is sensitive down to about 20 μg/ml and is probably the most widely used protein assay in spite of the fact that it is only a relative method.

REAGENTS:

1) Alkaline reagent – 20 g sodium carbonate + 0.5 g Na – K tartarate in 1 lit of 0.1 N NaOH.

2) Copper sulphate solution (1% w/v)

3) Alkaline coper sulphate solution [mixing 45 ml of (1) + 0.1 ml of (2)].

4) Folin – Ciocalteau reagent (2N): Dilute it to 1 N.

5) Standard BSA (0.25, 0.125, 0.0625, 0.03125, 0.015625 mg/ml)

PROCEDURE:

Test tube	**Test (T)**	**Standard (S)**	**Blank (B)**
Biological fluid (ml)	0.1	-	-
Standard (ml)	-	0.1	-
Distilled water (ml)	0.9	0.9	1
Alkaline copper reagent (ml)	5	5	5
Incubate at room temperature for 10 min.			
1 N Folin-Ciocalteau reagent (ml)	0.5	0.5	0.5
Immediately mix and incubate at room temperature in DARK for 30 min. Read at 650 nm.			

Calculation: Calculate the amount of protein using regression analysis taking the OD of standard as y-variable and concentration as x-variable.

OBJECTIVE: DETERMINATION OF PK_A VALUE OF PHOSPHATE BUFFER

What is pKa value of an acid?

The pKa value of an acid is that value of pH in which 50% of acid molecules are in ionized form and rest 50% in unionized form

$$HA \rightleftharpoons H^+ + A^-,$$

$$K_{eq} = \frac{[H^+][A^-]}{[HA]} = K_a$$

$$pK_a = \log \frac{1}{K_a} = -\log K_a$$

In case of strong acids, the K_a value is larger and pK_a is smaller. As per the Henderson-Hasselbalch equation,

$$pH = pK_a + \log \frac{[\text{proton acceptor}]}{[\text{proton donor}]}$$

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

The $[A^-]$ is ionized form of acid and HA is the unioized form of acid. Thus, in the situation when the concentration of ionized form of acid is equal to unioized form of acid or $[HA]$ equals $[A^-]$,

Equation mentioned above becomes

$$pH = pK_a + \log 1 = pK_a + 0 = pK_a$$

So, from the above equation, it can be said that pK_a is that value of pH when the concentration of ionized form of acid is equal to unioized form of acid.

So, for the calculation of pK_a of phosphate buffer, we need to take equal concentration of monobasic sodium phosphate (unionized acid) and di-basic sodium phosphate (conjugate base). The pH of the resulting solution would be the pK_a value of phosphate buffer.

Write the calculation here.

Find out what should be the ratio of dibasic sodium phosphate and monobasic sodium phosphate for maintaining the pH of 7.0, 7.2, 7.3 and 7.4 in phosphate buffer.

OBJECTIVE: Agarose gel electrophoresis

The term electrophoresis describes the migration of a charged particle under the influence of an electric field. Many important biological molecules such as amino acids, peptides, proteins, nucleotides and nucleic acids, possess ionisable groups and therefore, at any given pH, exist in solution as electrically charged species either as cation or anions. Under the influence of an electric field these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge. The equipment required for electrophoresis consists basically of two items, as power pack and an electrophoresis unit.

For the majority of DNA samples, electrophoretic separation is carried out in Agarose gels. This is because the DNA molecules and their fragments that are analyzed routinely are considerably larger than proteins and therefore, because most DNA fragments would be unable to enter a polyacrylamide gel, the larger pore size of an Agarose gel is required. For example, the commonly used plasmid pBR322 has a molecular weight of 2.4×10^6. However, rather than use such large numbers it more convenient to refer to DNA size in terms of the number of base pairs. So, the plasmid pBR322 has the size of 4.36 kb (4360 base pairs).

As all the DNA molecules are negatively charged at neutral and alkaline pH, under the electric field, the DNA molecules would be moving towards the anode. The separation in Agarose gels is achieved due to resistance to their movement caused by the gel matrix. The largest molecules will have the most difficulty passing through the gel pores (very large molecules may even be blocked completely), whereas the smallest molecules will be relatively unhindered. Consequently the mobility of the DNA molecules during gel electrophoresis will depend on the size, the smallest molecules moving fastest. This is analogous to the separation of proteins in SDS.

Once the gel has been run, the DNA in the gels needs to be stained and visualized. The reagent most widely used is the fluorescent dye ethidium bromide. The gel is rinsed gently in solution of ethidium bromide (0.5µg per ml) and then viewed under ultraviolet light (300 nm wavelength)*. The ethidium bromide is a cyclic planer molecule that bind between stacked base-pairs of DNA (i.e. it intercalates). The ethidium bromide concentration therefore buildup at the site of DNA bands and under ultraviolet light the DNA bands fluoresce orange-red. Al little as 10 ng of DNA can be visualized as a 1 cm wide band.

** Generally the ethidium bromide is added into the gel during its preparation. The concentration of ethidium bromide is used @ 0.5µg per ml of gel.*

STOCK SOLUTION:

Agarose Gel Electrophoresis buffer: For Agarose gel electrophoresis, either 1×TAE or 0.5×TBE can be used. For better resolution of DNA bands TAE is preferred to TBE. However, for routine gel runs TBE is preferred due to its long keeping quality. The stock solutions of TAE and TBE are 50×TAE and 10×TBE.

A. Tris Acetate EDTA (TAE) 50X buffer

Tris base	: 24.2 g
Glacial acetic acid	: 5.71 ml
0.5M EDTA (pH 8.0)	: 10 ml

Make the volume up to 100 ml with double distilled water. Autoclave and store at RT.

Tris Borate EDTA (TBE) 10X buffer,

Tris base	: 10.8 g
Boric acid	: 5.5 g
0.5M EDTA	: 4 ml (pH 8.0)

Make the volume up to 100 ml with double distilled water. Autoclave and store at RT

B. 6X Gel loading buffer:

Bromophenol blue	: 0.25%
xylene cyanol	: 0.25%
Sucrose	: 40%

Store at 4°C.

C. Ethidium bromide solution (10 mg/ml) (Stock solution)

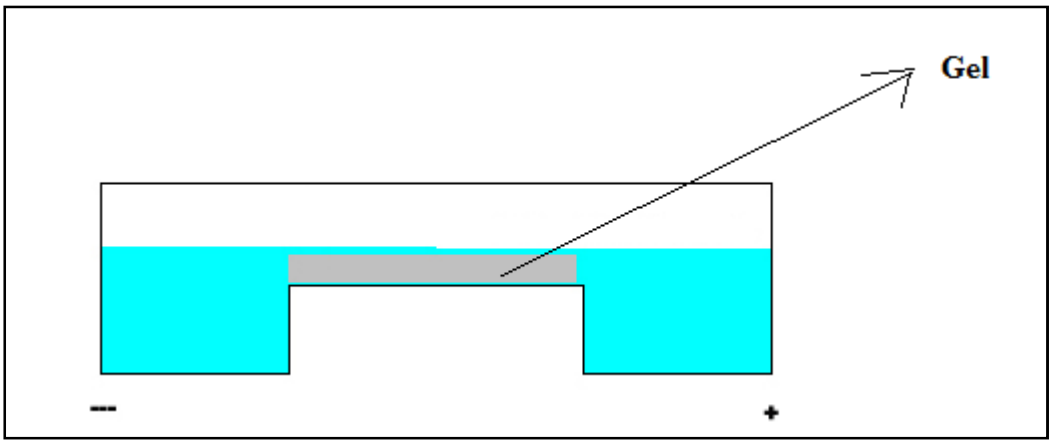
Ethidium Bromide	: 10 mg
Triple glass distilled water	: upto 1 ml

Working solution of EtBr is 1 mg per ml that is obtained by diluting the stock solution ten times.

The Ethidium Bromide is carcinogenic in nature. So, it is to be handled using gloves.

PROCEDURE FOR THE AGAROSE GEL ELECTROPHORESIS:

1. Preparation of Agarose gel (0.8%) 100 ml:
    a. Weigh 0.8 g of Agarose gel base and pour it into the 250 ml flat bottom flask.
    b. Add 1XTAE upto 100 ml.
    c. Heat the suspension with continuous stirring till a clear solution is obtained.
    d. Add working solution of EtBr (0.5µg per ml gel) when the gel solution has the temperature of 60°C and mix the gel solution by stirring.
    e. Caste the gel on the horizontal gel electrophoresis casting unit with the comb placed in it.
    f. Mix the DNA solution in 6X loading dye in the ratio of 2µl of dye with 10 µl of DNA solution.
    g. Load the dye-mixed DNA solution into the well of gel.
    h. Run the electrophoresis @ 5 volts per cm.
2. Viewing the DNA bands.
    a. The DNA bands can be visualized by placing the gel in U.V transilluminator.



Horizontal electrophoresis assembly

OBJECTIVE: Poly-Acrylamide Gel electrophoresis (PAGE)

SDS-PAGE (sodium dodecyl sulphate- Poly-Acrylamide Gel electrophoresis) is the most widely used method for analysing protein mixtures qualitatively. It is particularly useful for monitoring protein purification and because the method is based on the separation of proteins according to size. The method can also be used to determine the relative molecular mass of protein. SDS is an anionic detergent. Samples to be run on SDS-PAGE are firstly boiled for five minutes in sample buffer containing beta-mercapto-ethanol and SDS. The beta-mercapto-ethanol reduces any disulphide bridges present that are holding together the protein tertiary structure and SDS binds strongly to and denatures the protein. Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod shaped structure and with a series of negatively charged SDS molecules along the polypeptide chain. On average, one SDS molecule binds for every two amino acids residues. The original native charge on the molecule is therefore completely swamped by the negatively charged SDS molecules. The rod-like structure remains, as that tends to fold up the protein chain would result in repulsion between negative charges on different parts of the protein chain, returning the conformation back to the rod shape. The sample buffer also contains an ionisable tracking dye, usually bromophenol blue, that allows the electrophoretic run to be monitored and sucrose or glycerol, which gives the sample solution density thus allowing the sample to settle easily thorough the electrophoresis buffer to the bottom when injected into the loading well. Once the samples are all loaded, an electric current is passed through the gel. The samples are to be separated are not in fact loaded directly in to the main separating gel. When the main separating gel has been poured between the glass plates and allowed to set , a shorter (approximately 1 cm) stacking gel is poured on the top of separating gel and it into this gel that the wells are formed and the proteins loaded. The purpose of the stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel.

This is achieved by utilising difference in ionic strength and pH between the electrophoresis buffer and the stacking gel and involves a phenomenon known as *isotachaphoresis* (Explained in the page number 8).

When the dye reaches the bottom of the gel, the current is turned off, and the gel is removed from between the glass plates and shaken in an appropriate stain solution (usually Coomassie Brilliant Blue) for a few hours and then washed in destain solution overnight. The destain solution removes unbound background dye from the gel leaving stained proteins visible as blue bands on a clear back ground.

Staining of the gel is usually done with Coomassie Brilliant Blue R-250 (CBB). Staining is usually carried out

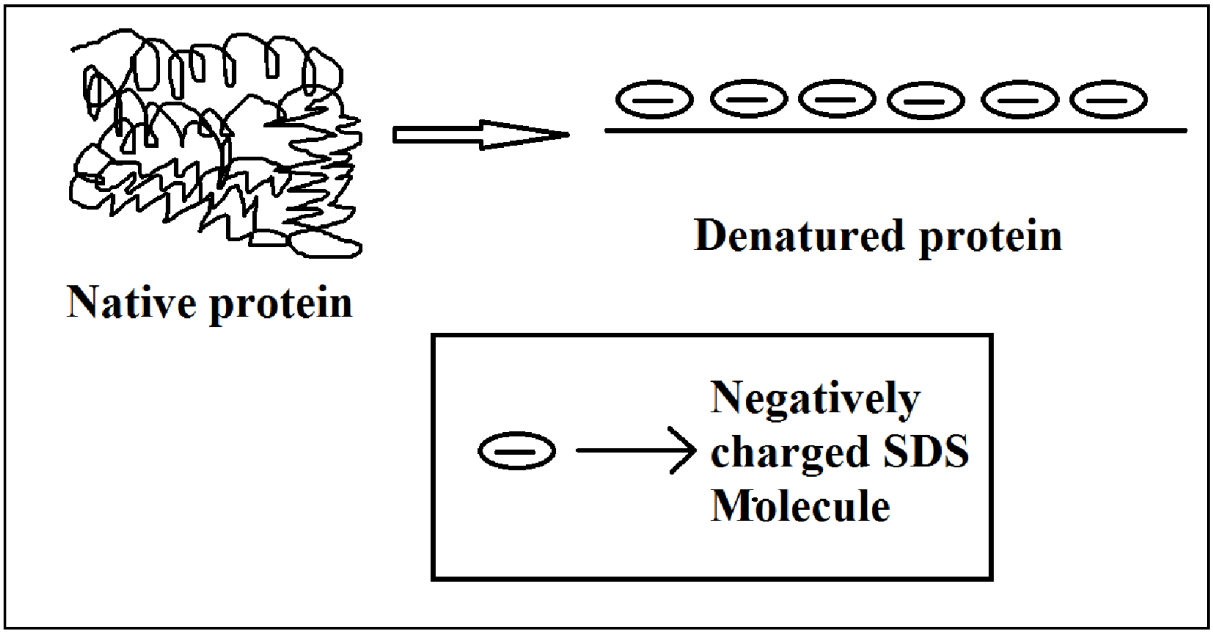


Fig. The native protein is denatured by SDS molecules into straight chain of polypeptides.

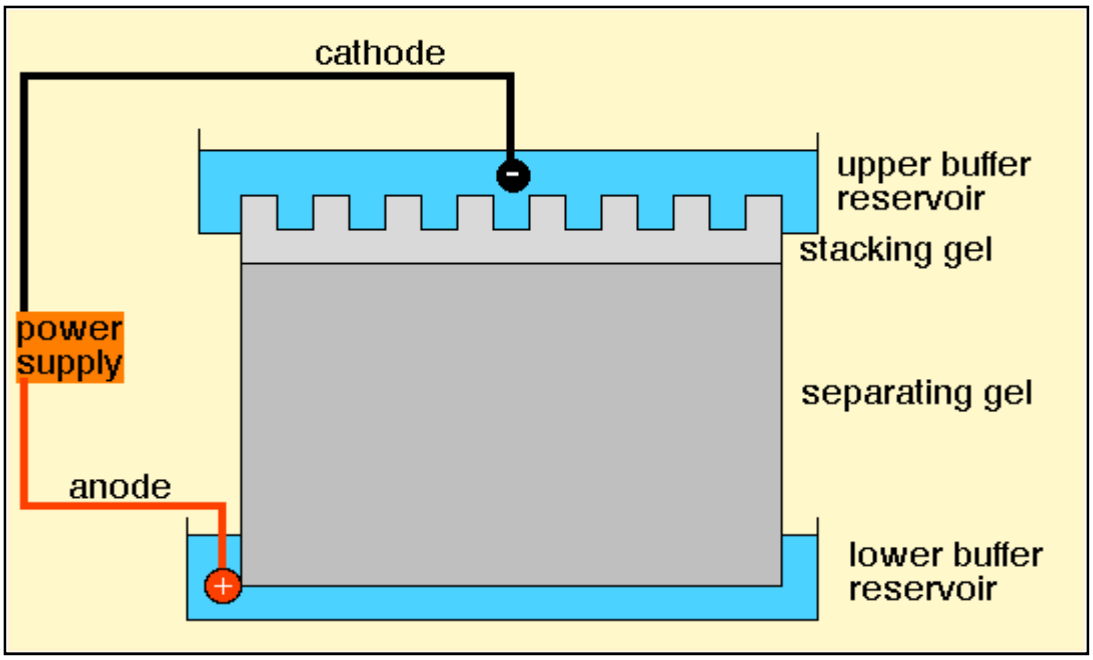


Fig. Schematic diagram of SDS-PAGE assembly.

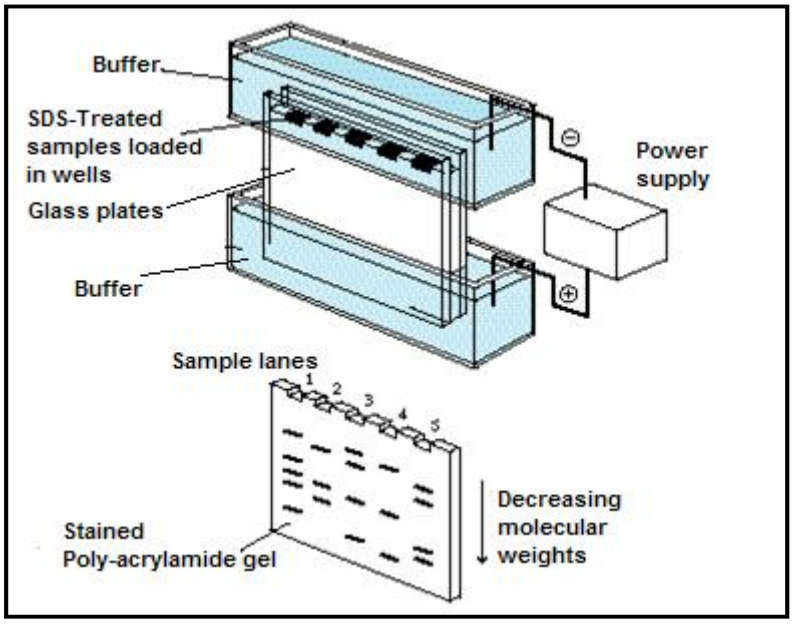


Fig. Complete SDS-PAGE assembly

THE PHENOMENON OF ISOTACHAPHORESIS

The stacking gel concentrate the protein sample into a sharp band before it enters the main separating gel. The electrophoretic mobility of glycinate ions, SDS-protein complex and Cl^- ions are not same. Their electrophoretic mobility

is in the order of glycinate<SDS-Protein< Cl^{-}. Now if all the chemical species do not move in same speed the electric circuit will break. Thus all three will have to move in same speed that is possible if glycinate ions decrease its concentration than SDS-Protein complex and Cl^{-} ions. And on the other hand the SDS-Protein complex and Cl^{-} will have to increase their concentration to decrease the electrophoretic mobility. The electrophoretic mobility of ionic species is inversely proportional to their concentration. This phenomenon is called isotachaphoresis.

$$Electrophoretic\ mobility \propto \frac{1}{Concentration}$$

SDS PAGE solutions

1. Sample buffer (2X) for 20 ml

INGRIDIENTS	VOLUME IN ml
Stacking gel buffer (pH 6.8)	0.2
10% SDS	8
1 % Bromophenol Blue	4
Glycerol	4
Beta mercaptoethanol	2
Water	1.8

Vortex the suspension and keep it in 4 degree Celsius.

2. Seperating gel buffer

Tris base 1.5 M
pH 8.8
Adjust pH by adding conc HCl

3. Stacking gel buffer

Tris base 1.0 M
pH 6.8
Adjust pH by adding conc HCl

4. Ammonium per sulphate: 10%

5. Electrode Buffer (5X)

Tris 15.1 g
Glycin 94 g
SDS 5.0 g
pH 8.3
Make final volume upto 1000 ml by adding triple glass distilled water
Prepare 5X stock by dissolving 15.1 g Tris base and 94 g Glycin in 900 ml of deionized water. Then add 5g of electrophoresis grade SDS and adjust the volume to 1000 ml with water.

6. Staining solution

Coomassie Brilliant Blue R-250 0.25 g
Methanol 50 ml
Acetic Acid 10 ml
Water 40 ml

7. Organic Destaining solution

Methanol	50 ml
Acetic Acid	10 ml
Water	40 ml

8. Stock Acrylamide solution (30%)

Acrylamide	29 %
Bis-Acrylamide	1 %

9. Seperating gel (8 ml)

	12 % gel	15% gel
Water	2.632 ml	1.84 ml
Acrylamide solution (30%)	3.2 ml	4 ml
Seperating gel buffer (pH 8.8)	2 ml	2 ml
10% SDS	80 µl	80 µl
10% APS	80 µl	80 µl
TEMED	4 µl	4 µl

10. Stacking gel (5ml) 5%

Water	3.4 ml
Acrylamide solution (30%)	830 µl
Stacking gel buffer (pH 6.8)	630 µl
10% SDS	50 µl
10% APS	50 µl
TEMED	5 µl

PROCEDURE FOR THE PREPARATION OF SDS-PAGE GELS

1. Assemble the glass plates of SDS-PAGE assembly.
2. Determine the volume of the gel mold to be prepared.
3. Take a clean and dry beaker and prepare the separating gel as mentioned in the table no. 9. Mix the component in the order shown. The polymerization will begin as soon as the TEMED has been added. Immediately proceed to the next step after addition of the TEMED.
4. Pour the separating gel solution into the gap between the glass plate. Leave sufficient space for the stacking gel.
5. Overlay the separating gel with the triple-glass distilled water.
6. After the polymerization of separating gel is complete, remove the overlay and wash the top of the separating gel with triple-glass distilled water.
7. Prepare the stacking gel as mentioned in table no. 10.
8. Pour the stacking gel solution immediately after addition of TEMED.
9. Place the comb over the separating gel and alow it to polymerize.

Procedure for the sample for the SDS-PAGE

1. Take the protein solution in a plastic centrifuge tube (1.5 ml).
2. Add equal volume of 2×SDS-PAGE sample buffer and mix it properly.
3. Place the tube in boiling water bath for five minutes.
4. Centrifuge the sample at 10,000×g for five minutes.
5. Take the supernatant in separate tube.

PROCEDURE FOR LOADING THE SAMPLE FOR THE SDS-PAGE

1. Load 15µl of the sample into the well of stacking gel.
2. Run the electrophoresis at the voltage of 8 Volts per cm.

3. After the dye front has reached the separating gel, increase the voltage to 15 Volts per cm.
4. Run the gel until the tracking dye reaches the bottom of the gel.

PROCEDURE FOR STAINING THE SDS-PAGE GEL

1. Prepare the staining solution as mentioned in table no. 6.
2. Remove the gel from the two glass plates carefully and place into the staining solution.
3. Keep the gel in staining for about 3 hours.
4. Prepare the destaining solution as mentioned in table no. 7.
5. Pour off the staining solution and add the destaining solution.
6. Keep the gel in destaining solution for overnight.
7. The gel will show the protein bands after overnight destaining.