EXPERIMENT NO: 1

DATE:

PREPARATION OF BUFFER

AIM:

To prepare the buffer at required pH.

PRINCIPLE:

The pH meter measures at electrical potential developed by pair of electrode pins in a solution. For measurement of pH, an electrode system sensitive to change in H+ ion concentration of solution is taken. The electrode system consists of sequence of electrode whose potential raise with pH (H+ concentration of the solution).

PROCEDURE:

1. ACETIC ACID- SODIUM ACETATE BUFFER:

REAGENTS REQUIRED:

Acetic Acid 0.2M: 1.5 ml of glacial acetic acid is made upto 100ml with distilled water.

Sodium Acetate Solution: 0.64 gm of sodium acetate or 2.72gm of sodium acetate trihydrate is dissolved in 100ml Distilled water.

PROCEDURE:

Pipette out exactly 36.2ml of sodium acetate solution into 100ml of standard flask and add 14.8ml of glacial acetic acid, make the volume 100ml using distilled water using distilled water. This gives 0.2 M of acetic acid and sodium acetate buffer. The pH is measured with pH meter.
The pH meter is first standardised with pH buffer. Wash electrode with distilled water and introduced into 0.2M acetic acid-sodium acetate buffer prepared, the pH of solution is 4.6.

RESULT:

36.2ml Sodium acetate and 14.8 ml glacial acetic acid were mixed and buffer was prepared. pH was measured initial reading observed was 4 which made upto 4.6 with 5N NaOH.

2. BARBITONE BUFFER:

REAGENTS REQUIRED:

- Diethyl barbituric acid.
- Sodium diethyl barbiturate

PROCEDURE:

Dissolve 2.85gm of diethyl barbituric acid and 14.2gm of sodium diethyl barbiturate in distilled water and upto 1 liter. This gives the barbitone buffer.

The pH meter is first standardised with pH buffer. Wash electrode with distilled water and introduced into barbitone buffer prepared, the pH of solution is 6.8.

3. CITRATE BUFFER:

REAGENTS REQUIRED:

- Citric acid: Dissolve 2.101 gm of citric acid in 100ml distilled water.
- Sodium citrate solution 0.1 M: Dissolved 2.941gm of sodium citrate in 100ml distilled water.
PROCEDURE:

46.5ml of citric acid with 3.5ml of sodium citrate solution and upto 100ml with distilled water. It corresponds to 0.1 M citrate buffer and standardised with pH meter and measures the pH of the prepared solution. This gives citrate buffer at pH 2.5.

RESULT:

Citrate buffer was prepared and the pH observed was 4.8 which was adjusted to 2.5 using 1N HCl and 5N NaOH.

4. CARBONATE- BICARBONATE BUFFER:

REAGENTS REQUIRED:

- Sodium carbonate solution 0.2M: Dissolve 2.12gm of anhydrous sodium carbonate in 100ml Distilled water.
- Sodium bicarbonate solution: Dissolve 1.68gm of sodium bicarbonate in 100ml of distilled water.

PROCEDURE:

Pipette out exactly 27.5ml of sodium carbonate (Na₂CO₃) solution. To this add 22.5ml of sodium bicarbonate solution and made upto 100ml with distilled water which corresponds to 0.2 M sodium carbonate and bicarbonate buffer.

Standardise pH meter and measure the pH of required buffer. This gives the Carbonate- bicarbonate buffer pH 10.2.
RESULT:

Carbonate bicarbonate buffer was prepared and pH observed was 7.5 which was adjusted to 10.2 using 1N Hcl and 5N NaoH.

5. PHOSPHATE BUFFER:

REAGENTS REQUIRED:

- Monobasic: Dissolve 2.78gm of sodium dihydrogen phosphate in 100ml of distilled water.

- Dibasic sodium phosphate (0.2M): Dissolve 5.3gm of disodium hydrogen phosphate or 7.17 gm sodium hydrogen phosphate in 100ml distilled water.

PROCEDURE:

39 ml of dihydrogen sodium phosphate is mixed with 61 ml of disodium hydrogen phosphate. This made up to 200ml with distilled water. This gives phosphate (Po₄)₂ buffer of 0.2M.

Standardized pH meter with standard buffer. Washed electrode with distilled water and introduced it into phosphate buffer prepared. The pH of the solution is 6.8.

RESULT:

Phosphate buffer was prepared and pH was observed 8.5 which was made up to 6.8 using 1N Hcl and 5N NaoH.

6. POTASSIUM PHOSPHATE BUFFER:

REAGENTS REQUIRED:
- Dipotassium hydrogen phosphate
- Potassium dihydrogen phosphate

**PROCEDURE:**

174.18 g/mol dipotassium hydrogen phosphate and 136.09 g/mol potassium dihydrogen phosphate was taken and made up to 200ml using distilled water. This gives the potassium buffer.

Standardised pH meter with standard buffer. Washed electrode with distilled water and introduced it into potassium buffer prepared. The pH of the solution is 6.5.

**RESULT:**

Dipotassium hydrogen phosphate (K2HPO4) and potassium dihydrogen phosphate (KH2PO4) solution were prepared and the pH was measured to be 9.87 and 4.23 respectively, the solution were made using 1N HCl and 5N NaoH respectively and the pH was found to be 6.5.
EXPERIMENT NO: 2

DATE:

ESTIMATION OF PROTEIN BY LOWRY’S METHOD

AIM:

To estimate the amount of Protein present in given unknown solution.

PRINCIPLE:

Alkaline CuSO₄ catalyses the oxidation of aromatic amino acids with subsequent reduction of sodium potassium molybdate tungstate of Folin’s reagent giving a purple colour complex the intensity of the colour is directly proportional to the concentration of the aromatic amino acid in the given sample solution.

REAGENTS REQUIRED:

1. Stock Solution:

   Bovine Serum albumin of 100 mg is weighed accurately and dissolved in 100 ml of distilled water in a standard flask (concentration 1 µg/ml).

2. Working Standard:

   The Stock Solution of 10 ml is distilled to 100 ml with distilled water in a standard flask (concentration 100 mg/ml).

3. Folin’s Phenol Reagent:

   Folin’s Phenol Reagent is mixed with distilled water in the ratio 1:2.

4. Alkaline copper reagent:

   Solution A: 2% sodium carbonate in 0.1 N sodium hydroxide.
   Solution B: 0.5% copper sulphate in 1% sodium potassium tartrate.

   Solution A, B, C is mixed in the proportion of 50:1:0.5.

Unknown Preparation:

The unknown protein is made up to 100 ml with distilled water.
**PROCEDURE:**

Working standard of 0.2 -1ml is pipette out into clean test tube and labeled as S1-S5. Test solution of 0.2ml is taken into test tube and labeled as T1. The volume is made upto 1ml of distilled water. Distill water of 1ml serve as blank.

To all the test tube 4.5ml of alkaline CUSO4 reagent is added and incubated at room temperature for 10 minutes. All the test tube 0.5ml of folin’s phenol reagent is added. The contents are mixed well and the blue colour developed is read at 640 rpm after 15 minutes. From the standard graph the amount of protein in the given unknown solution is calculated.

**RESULT:**

The amount of protein present in the given unknown solution is \( \) mg (\( \mu \)g of protein).
EXPERIMENT NO: 3
DATE:

ESTIMATION OF REDUCING SUGAR USING BENEDICT’S METHOD

AIM:
To estimate the amount of glucose present in the given unknown solution using Benedict’s quantitative reagent.

PRINCIPLE:
Benedict’s quantitative reagent is a modification of qualitative aspects. It contains copper sulphate-sodium acetate and sodium carbonate. It also contains potassium thiocyanate and small amount of potassium ferricyanide. The inclusion of acetate prevents the precipitation of copper carboning by chelating Cu$^{3+}$ ions. The thiocyanate causes with the precipitation of white cuprous thiocynate rather than red cupric oxide. On the reduction of Cu$^{3+}$ ions, which inhibits the end point of the titration digest the transition from blue to white to be readily observed. Methylene blue will be used as an additional indicator. The small amount of potassium ferricyanaide prevents the pre oxidation of copper. The non-stiochemetric reaction is on which not follow a defined pathway and cannot be described by an equation either quantitatively or qualitatively. The reduction of Cu$^{3+}$ ions by sugar is a non-stiochemetric equation and is only constant over a small range of sugar concentration. To obtain accurate results the volume of sugar added must be within 6-12ml for 10ml of Benedict’s regent.

REGENT REQUIRED:
(i).Standard glucose solution:
200mg of glucose was weighed accurately and made up to 100ml with distance water. (Conc. 2mg/ml).
(ii) **Benedict’s quantitative reagent:**

100mg of sodium citrate and 62.5gm potassium thiocynate were dissolved in 300ml of distilled water by warming gently and filtered. 18% of copper sulphate is dissolved in 50ml of water, added with continuous stirring. 2.5ml of 5% potassium ferricyanide is added and volume is made up to 500ml with water.

(iii) **Anhydrous sodium carbonate**

**PROCEDURE:**

5ml of Benedict’s reagent was pippeted out into a clean conical flask. About 600mg. of anhydrous sodium carbonate was added to provide the required alkalinity with a few porcelain bits and heated to boiling over a moderate flame. Standard glucose solution is taken in a burette. When the Benedict’s solution boils continuously, glucose solution is added drop by drop (1 drop/sec) till last trace of blue colour disappears. The volume of glucose rundown is noted and the titrations are repeated to concordant values.

The given unknown sugar solution was made up to 100ml in a standard flask with distilled water. Then the burette was filled with unknown sugar solution and the Benedict’s reagent was titrated as before. The volume of sugar solution rundown was noted and titrations are repeated for concordant values.

**RESULT:**

The amount of glucose present in 100ml of given unknown solution is ____ mg.
EXPERIMENT NO: 4

DATE:

LIPID ANALYSIS - ESTIMATION OF CHOLESTROL

AIM:

To estimate the amount of Cholesterol (lipid) in the given unknown sample by Folch Method.

REAGENTS REQUIRED:

1. Chloroform: Methanol - 2:1 v/v
2. Saline: 0.9% saline.

PROCEDURE:

The tissue was washed with ice-cold saline and dried between the folds of a filter paper. A weighed amount of tissue (500 mg) was mixed well and homogenized with 10 ml of chloroform–methanol mixture. The homogenate was filtered through whatman filter paper (No.42) into a separating funnel. The filtrate was mixed with 0.2 ml of physiological saline and the mixture was kept overnight undisturbed. The lower phase containing the lipid was drained off into pre weighted beakers. The upper phase was reextracted with more of chloroform–methanol mixture and the extracts were pooled and evaporated under vacuum at room temperature. The lipid extract was redissolved in 3 ml of chloroform–methanol (2:1) and the aliquots were taken for the estimation.

ESTIMATION OF CHOLESTEROL:

Cholesterol content was estimated by the method of Parekh and Jung (1970).

REAGENTS REQUIRED:

1. Ferric chloride–Uranyl acetate reagent: 500 mg of crystalline ferric chloride was dissolved in 10 ml of water; 3 ml of concentrated ammonia was added to it and centrifuged. The precipitate was washed several times with distilled water and dissolved in 1 litre of glacial acetic acid. 100 mg of Uranyl
acetate was added to the mixture and the contents were shaken well and kept over night. The reagent was stable for 6 months.

2. Sulphuric acid–ferrous sulphate reagent: 100 mg of ferrous sulphate was dissolved in 100 ml of glacial acetic acid and 100 ml of sulphuric acid. After cooling to room temperature the volume was made up to 1 litre with concentrated sulphuric acid. The reagent was stable for 6 months.

3. Cholesterol standard: 200 mg of cholesterol (recrystallized from ethanol) was dissolved in 100 ml of acetic acid. 1 ml of the stock solution was diluted to 100 ml to obtain a working standard containing a concentration of 20 micro g/ml.

PROCEDURE:

About 0.1 ml of the aliquot of the total lipid extract evaporated to dryness, 3 ml with ferric chloride–Uranyl acetate reagent was added. Standard (20–60 micro g) were made to 3 ml with ferric chloride–Uranyl acetate reagent. Blank consisted of 3 ml of the reagent. To all these tubes, 2 ml of sulphuric acid–ferrous sulphate reagent was added to all the tubes and the contents were mixed well and kept in ice bath. After 20 min the colour developed was read at 540 nm using a Shimadzu UV spectrophotometer.

RESULT:

Amount of Cholesterol (lipid) in the given unknown serum sample is µg/ml.
EXPERIMENT NO: 5
DATE: 

ESTIMATION OF DNA

AIM:
To estimate the amount of DNA present in the given unknown solution by diphenylamine method.

PRINCIPLE:
When DNA is treated with diphenylamine under the acidic condition a bluish green colored complex is formed which has an absorption peak at 595nm. This reaction is given by 2 deoxypentose in general. In acidic solution deoxypentose are converted into a highly reactive β hydroxyl leavulinic aldehyde which reacts with diphenylamine gives bluish green colored complex. The colour intensity was measured using a red filter at 595nm.

REAGENT REQUIRED:
1. Stock Standard Solution:
   50mg of DNA was dissolved in 50ml of Saline Sodium citrate buffer. Concentration 1mg/ml
2. Working Standard Solution:
   5ml of stock solution solution was diluted to 50ml with distilled water. Concentration 100µg/ml
3. Diphenylamine Reagent:
   10g of pure diphenylamine was dissolved with 25ml of concentration sulphuric acid which was made up to 1ml with glacial acidic acid the solution must be prepared freshly.
4. Buffered Saline ph 7.4:
   0.14N Sodium chloride and 0.02M sodium citrate.
5. Unknown Solution:
The given unknown solution is mad up to 100ml with distilled water.
PROCEDURE:
1. 0.5-2.5ml of working standard solution is pipetted out into 5 test tubes labeled as s1-s5 where concentration ranging from 50-250µg.
2. 1ml and 2ml of unknown solution is pipetted out into two test tube u1 and u2.
3. The volume in all test tubes is made up to 3ml with distilled water and 3ml of distilled water alone serve as a blank.
4. 4ml of diphenylamine reagent was added to all the tubes. The tubes were kept in a boiling water bath at 36°C for 20min. The tubes were than cooled and the bluish colour developed is read at 595nm.
5. A standard graph is drawn taking concentration of DNA on x-axis and absorption of y-axis. From the standard graph the amount of DNA present in the unknown solution is calculated.

RESULT:
The amount of DNA present in the given unknown solution is found to be
AIM:
SDS-PAGE was performed to separate and observe the protein pattern of the sample by the method of Lammeli (1970).

PRINCIPLE:
SDS-PAGE was performed to accomplish the following:
   a) To observe the protein pattern of the enzyme mixture.
   b) To determine the homogeneity of the purified enzyme mixture.
   c) To determine the molecular weight of the purified enzyme.

REAGENTS REQUIRED:
1. Preparation of stock solution and buffers:
   30% acrylamide
   a) Acrylamide: 29.2g
   b) N, N-methelyne–bis–acrylamide: 0.8g Added water, dissolved and made upto 100mL and filtered with Whatman no.1 filter paper.
2. Separating gel buffer:
   a) Tris-HCl: 1.5M, pH 8.8
   18.171g of Tris was dissolved in 60mL of water and adjusted the pH to 8.8 with HCl and finally made upto 100mL with water.
3. Stacking gel buffer:
   a) Tris-HCl: 1M, pH 6.8
   6.057g of Tris was dissolved in 60mL water and adjusted the pH to 6.8 with HCl and upto 100mL with water.
4. 10% SDS solution: 1g of SDS in 10mL of distilled water.
5. N,N,N’N’-Tetramethylene diammine (TEMED)
6. 10% Ammonium per sulphate (APS): 1g of APS in 10mL of distilled water.

7. Electrophoresis Buffer:
   a) Tris: 25mM, pH 8.3
   b) glycine: 250mM, pH 8.3
   c) SDS: 0.1%: Dissolved in minimum amount of water (500mL) and then added SDS.
   Allowed to settle and dissolve. This was finally made up to 2.5 liters.

8) Sample buffer 4x: 5.0mL
   a) Tris (1M, pH 6.8): 2.1mL
   b) 2% SDS: 100mg
   c) Glycerol (100%): 1.0mL
   d) b-mercaptoethanol: 0.5mL
   e) Bromophenol blue: 2.5mg
   f) Distilled water: 0.4mL

9) Staining solution (100mL):
   a) Alcohol: 40%
   b) Acetic acid: 10%
   c) Comassie Brilliant Blue (CBB): 259mg
   d) Distilled water: 50%

10) Destaining solution (100mL)
    a) Alcohol: 50%
    b) Acetic acid: 10%
    c) Distilled water: 40%
**PROCEDURE**

**Preparation of gel:**

The glass plates were washed in warm detergent solution, rinsed subsequently in tap water, deionised water and ethanol and dried. The unnotched outer plates were laid on the table and Vaseline (or grease) was coated. Spacer strips were arranged approximately at the sides and bottom of the plates. The notched inner plates were laid in position, resting on the spacer strips and the arrangement was mounted vertically. Sealing was done properly to avoid leakage. The volume of the gel solution required for making separating gel was calculated as follows (the reagents in the following table yield 20mL of solution after the addition of APS and TEMED)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>8%</th>
<th>10%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O (ml)</td>
<td>9.3</td>
<td>7.9</td>
<td>4.6</td>
</tr>
<tr>
<td>30% acrylamide mix (ml)</td>
<td>5.3</td>
<td>6.7</td>
<td>10.0</td>
</tr>
<tr>
<td>1.5M Tris (pH 8.8) (ml)</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>10% SDS (ml)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>10% APS (ml)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>TEMED (ml)</td>
<td>0.012</td>
<td>0.008</td>
<td>0.008</td>
</tr>
</tbody>
</table>

APS and TEMED were added just prior to the pouring of gel. The solution was mixed well and poured into the space between the two plates leaving an inch of the upper space unfilled. Water was carefully laid over the surface of the poured gel mixture to avoid air contact, which reduces the polymerization reaction. The gel mixture was allowed to polymerize, undisturbed at room temperature for 60 minutes. In the mean time gel mixture for stacking gel was prepared. (The reagents in the following table yield 10mL of solution after the addition of APS & TEMED)
After the separating gel was polymerized the overlaid water was removed carefully with filter paper and an appropriate comb was inserted between the plates. 0.1mL of 10% APS and 10 l of TEMED were added to the stacking gel mixture. It was mixed well and poured immediately (to the brim) over the separating gel. The stacking gel was allowed to polymerize. Additional gel mixture was added when gel retracted significantly.

Preparation of protein samples:

The required volume of sample buffer was added to protein samples and they were loaded (the final concentration of sample buffer in the prepared sample should come to 1x. If the protein was dried suspend it in 1x buffer). The samples were incubated for 2min in a boiling water bath prior to loading. When the polymerization was completed the comb was removed and the lower spacer strip was carefully removed. The Vaseline (or grease) from the bottom was removed with a piece of tissue paper. The gel was attached to the electrophoresis tank using appropriate clips/clamps. The lower reservoir was filled with 1x electrophoresis buffer, using a bent Pasteur pipette or syringe needle to remove any air bubble trapped beneath the bottom of the gel. The protein samples were loaded using a micropipette and the wells were completely and carefully filled with 1x electrophoresis buffer. The

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>6.8</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>1.7</td>
</tr>
<tr>
<td>1M Tris-Cl, pH 6.8</td>
<td>1.25</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.1</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01</td>
</tr>
</tbody>
</table>
upper reservoir was also carefully filled with 1x electrophoresis buffer. The electrodes were connected to a power pack.

The gel was run at constant current (20 milli ampere 100 volts) for 4-6 hrs at room temperature. Electrophoretic mobility of the samples was determined by bromophenol blue front. At the end of the run the power pack was switched off. The gel and plates were laid flat on the table and a corner of the upper glass plate was lifted up and the gel was carefully removed.

**Staining of the gel:**

After the completion of the electrophoresis, the gel was fixed with 10% trichloroacetic acid for 5 minutes and stained with CBB. The CBB staining solution was prepared using methanol, acetic acid and double distilled water in the ratio of 4:1:5 and 0.25gm of CBB was added and the gel was stained over night.

**Destaining of the gel:**

The destaining of CBB stained gel was done by using methanol, acetic acid and double distilled water in the ratio of 5:1:4 till the appearance of clear bands on the gel.

**RESULT:**

The sample proteins are separated by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis. The proteins appeared as discrete bands in the gel. The relative molecular weights of the protein with respect to their bands were observed in Kilo Daltons.
EXPERIMENT NO: 7
DATE:

WESTERN BLOTTING

AIM:

Western Blotting was performed by the rapid method of Towbin et al., (1979) to detect the expression pattern of a protein. To detect the antigens blotted on a nitrocellulose membrane with the use of an antibody.

PRINCIPLE:

Western blotting (also known as protein blotting or immunoblotting) is a rapid and sensitive assay for detective and characterization of proteins. Western blotting technique exploits the inherent specificity by polyclonal or monoclonal antibodies.

It is an analytical method wherein a protein sample is electrophoresed on an SDS-PAGE and electrotransferred onto nitrocellulose membrane. The transferred protein is detected using specific primary antibody and secondary enzyme labeled antibody and substrate. A protein sample is subjected to polyacrylamide gel electrophoresis. After this the gel is placed over a sheet of nitrocellulose and the protein in the gel is electrophoretically transferred to the nitrocellulose. The nitrocellulose is then soaked in blocking buffer (3% skimmed milk solution) to "block" the nonspecific binding of proteins. The nitrocellulose is then incubated with the specific antibody for the protein of interest. The nitrocellulose is then incubated with a second antibody, which is specific for the first antibody. For example, if the first antibody was raised in mouse, the second antibody might be termed "goat anti-mouse immunoglobulin". What this means is that mouse immunoglobulins were used to elicit an antibody response in goats. The second antibody will typically have a covalently attached enzyme which, when provided with a chromogenic substrate, will cause a color reaction. Thus the molecular weight and amount of the desired protein can be characterized from a complex mixture (e.g. crude cell extract) of other proteins by western blotting.
**REAGENTS AND MATERIALS:**

1. Nitrocellulose membrane
2. Plastic staining box
3. Electroblotting apparatus
4. Whatmann No.1 filter paper
5. Transfer buffer (500 ml, pH 8.3)
   - Tris–HCl -25 mM
   - Glycine -192 mM
   - Methanol-20%
6. 10X Tris buffered saline (TBS) (100 ml, pH 7.6)
   - Tris -2.4 g
   - NaCl -8 g
   They were dissolved in low amount of double distilled water, the pH was adjusted to
7. And the total was made upto 100 ml with double distilled water.
8. Blocking solution (50mL)
   - 5% Non-dry fat milk powder - 0.25g
   - 1X TBS (pH 7.6) - 50mL
   - 0.1% Tween- 20 - 0.05mL
9. Washing buffer (100mL) (TBS)
   - 1X TBS (pH 7.6) -100 ml
   - 0.1% Tween - 20 - 0.1 ml
10. Preparation of primary antibodies
11. Preparation of secondary antibodies
12. Colour indicator solution

0.05% 0f 3’3- diaminobenzidine tetra hydrochloride (DAB) substrate and
0.01% of H2O2 were dissolved in 1X PBS (pH 7.6). This chromogen substrate was prepared just prior to the treatment.
13. Ponceau S red solution (100mL)

- Ponceau S red - 0.5 g
- Glacial acetic acid - 5%

PROCEDURE:

1. After SDS-PAGE, the gel was equilibrated in blotting buffer for 20 min at room temperature. While the gel was equilibrating, a piece of nitrocellulose membrane was cut into the same dimension as the gel it was wet slowly by sliding it at 45° angle into transfer buffer and was soaked for 20 min.

2. The pieces of Whatmann No.1 filter paper, four pads were also soaked in transfer buffer for 20 min.

3. Then, the pads, filter paper, nitrocellulose membrane and gel were assembled in the semi-dry blot apparatus in the following order: The two presoaked pads were placed at the bottom and a glass pipette was rolled over the surface of the pad to remove air bubbles. Then, the Whatmann No.1 filter paper was placed followed the nitrocellulose membrane. Carefully, the equilibrated gel was placed on top of the nitrocellulose membrane. The second Whatmann No.1 filter paper and followed it, the second set of pad were placed on top of the gel. (After each step care was taken to remove the bubbles). The transfer cell and plug was assembled and the gel transferred for 2h at 25V/130.

After the transfer, protein were visualized by staining in ponceau S solution for 5 min, destained in the distilled water and the molecular marker was marked with indelible ink and destained for 10 min. The membrane was blocked in blocking buffer for 1h at room temperature. Then, the membrane was washed again with washing buffer and incubated with primary antibody overnight at 4°C. The next day, the membrane was washed again with washing buffer and incubated with HRP-conjugated secondary antibody for 2 h at room temperature. The membrane was washed and DAB solution was added and incubated at room temperature and watched for colour development, which is usually completed within 5–10 min. The membrane was rinsed with distilled water to stop the reaction of DAB. It was
then placed on filter paper to air dry. Dilutions of the primary and secondary antibody were standardized after several trials. The specific protein was detected as a band in the nitrocellulose membrane.

**RESULT:**

The presence of specific protein or the presence of antigen or specific antibody was visualised as a bluish grey colored band.
EXPERIMENT NO: 8
DATE:

SOUTHERN BLOTTING

AIM:

To transfer the electrophoresed DNA from Agarose gel to a nitrocellulose membrane by capillary action.

PRINCIPLE:

Southern blotting refers to the transfer of electrophoresed DNA from an Agarose gel into nitrocellulose membrane. The gel is mounted on a filter paper wick dips into the transfer buffer. The nitrocellulose filter is sandwiched between the gel and the stack of paper towel which serves to draw the transfer buffer through the gel by capillary action. The DNA molecules are carried out of the gel by the buffer flow and immobilized on the membrane.

REAGENTS REQUIRED:

1. DNA fragments electrophoresed in Agarose gel.
2. depurination solution – 0.25N HCL
   Slowly add 10.5ml of concentration HCL to 489.5ml of sterile distilled water.
3. Denaturation solution- 1.5M Sodium chloride and 0.5 M Sodium hydroxide.
   Dissolve 43.85g of sodium chloride and 10g of Naoh in 300ml of sterile distilled water made-up the volume to 500ml (Autoclave and store at 4°C).
4. Neutralisation solution- 1M Tris and 1.5M Nacl
   Dissolve 60.55g of Tris base and 43.80g of Nacl in 300ml of distilled water. Adjust the pH to 7.5 using concentration HCL.
   (Autoclave and store at room temperature)
5. Alkaline transfer buffer – 20X ssc buffer:-
   Dissolve 87.6g of Nacl and 44.1g of sodium citrate in 300ml of distilled water. Adjust pH to 7 with in 1N NaoH and make.
   (Autoclave and store at 4°C)

6. Distilled water.

7. 6X ssc buffer.

**PROCEDURE:**

1. DNA fragments electrophoresed in Agarose gel was viewed under UV Transilluminator.

2. Gel containing DNA bands were cut ensuring the gel measures about 4-4.5 cm, according to the size of the nitrocellulose membrane and filter paper.

3. The gel piece is placed in a tray containing depurination solution. The tray was rocked gently for 15 minutes (bromophenol blue yellow indicating that acid treatment is complete)

4. The acid solution was decanted and the gel was rinsed, to twice with distilled water.

5. Then the gel is placed in a tray containing 20 gel volumes of Denaturation solution. This was gently shaken to 20 minutes.

6. This solution was removed and replaced with fresh Denaturation solution.

7. This was incubated for 20 minutes with gentle shaking.

8. Denaturation solution was decanted and neutralization solution was added.

9. The gel was incubated in this solution with shaking for 20 minutes.

10. Neutralization was repeated with solution for another 20 minutes.

11. 20 x ssc buffer was poured and the filter paper wick was placed on the support.
12. About 6 filter papers cut were arranged on the wick after dipping in the transfer buffer and whatman 2 filter paper were placed in it.
13. Invert the gel such that the sample faces towards the support.
14. Nitrocellulose membrane was cut and kept in distilled water. This was then immersed in transfer buffer for 5 minutes.
15. Gel was placed in two pieces of whatman No1 filter paper was placed on it and this was smoothed but with a glass rod removes air bubbles.
16. The stacks of filter papers were cub and placed in whatman filter paper. A glass plate was placed in top of the stack and was weighed down with 500g weight.
17. DNA transfer was allowed for 8 to 24 hours.
18. The stacks of filter were removed and the nitrocellulose paper were turned over and laid, gel side up on a day sheet of whatman filter paper.
19. The gel was checked for complete DNA transfer before discarding and the nitrocellulose membrane was soaked in 6x ssc buffer for 5 minutes at room temperature.
20. The membrane was sand witched between two dry whatman filter paper and haked for 3 minutes to 2 hour at 80°C in a vacuum oven.

RESULT:

DNA bands were blotted from the Agarose gel from nitrocellulose membrane.
EXPERIMENT NO: 9 (A)

DATE:  

DNA ISOLATION

1. Bacterial culture was pelleted.
2. Pellet was washed twice with TE (pH 8.0)
3. 500µl of TE buffer was added.
4. Cells were lysed by adding 50µl of 20% SDS (tubes were invert mixed).
5. 50µl of proteinase k(20µg/ml) was added.
6. Incubated at 37 c for 1 hour.
7. 500µl of equal volume of phenol.Choloroform mixture was added and centrifuged for 2 min at 5000rpm.
8. Aqueous tap phase was separated and 10µl of 3M sodium acetate was added.
9. After mixing 60µl of isopropanol was added (allowed the DNA to dissolve).
10. Tubes were spin for a few minutes supernant discarded and pellet dissolved in 50-100 µl TE buffer.

MATERIAL REQUIRED:

1. TE BUFFER:
   
   i. 10mM Tris Hcl pH 7.4
   ii. 1mM EDTA pH 8.0

2. TE BUFFER STOCK:
   
   i. Tris Hcl 1M stock pH 7.4
   ii. EDTA 0.5 M stock pH 8.0

3. WORKING-RT:
   
   i. 1ml of 1M Tris Hcl
   ii. 200µl of 0.5 M EDTA
   iii. 98.9 ml of water.
EXPERIMENT NO: 9 (B)

DATE:

AGAROSE GEL ELECTROPHORESIS

AIM:

To analyze the given nucleic acid using Agarose Gel Electrophoresis.

MATERIALS REQUIRED:

1. Agarose solution,
2. Ethidium bromide,
3. Electrophoresis buffer,
4. 6x gel buffer,
5. DNA sample,
6. DNA size standard.

PRINCIPLE:

Agarose gel electrophoresis used to analyze and quantitate nucleic acid. The Agarose for Agarose gel electrophoresis is purified from agar. Agarose is a linear polymer made up of repeating units of 1,3-Linked β D galactopyranose and 1,4-linked 3,6 anhydro a L galactopyranose [ P-D –gal (1-4)-3,6 anhydro – a L Gal (1-3) ]n Agarose has an average MW of 12,000 and contains about 35-40 agarobiose units. Agarose in solution exist as left handed double helices. About 7 to 11 such helices form bundles which extend as long rods and appear to intertwine with one another, further strengthening the frame work of the gel. The cross links are held together by hydrogen and hydrophobic bonds. By changing the gel concentration the pre size can be altered. Higher the concentration of Agarose smaller the pre size and vice versa. Because of large pore size even at low concentration, Agarose gels are widely used for separation of DNA and RNA.
Effect of Agarose concentration on separation ranges

The following table describes the relationship between Agarose concentration and separation range of nucleic acid.

<table>
<thead>
<tr>
<th>AGAROSE CONCENTRATION (%)</th>
<th>SEPARATION RANGE (KB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>5 to 60</td>
</tr>
<tr>
<td>0.6</td>
<td>1-20</td>
</tr>
<tr>
<td>0.8</td>
<td>0.8 to 10</td>
</tr>
<tr>
<td>1.0</td>
<td>0.4 to 8</td>
</tr>
<tr>
<td>1.2</td>
<td>0.3 to 6</td>
</tr>
<tr>
<td>1.5</td>
<td>0.2 to 4</td>
</tr>
</tbody>
</table>

Factors which affect the rate of migration of nucleic acids in Agarose gels

Rate of migration of nucleic acids in Agarose gels depends mainly on four important parameters.

a. Agarose concentrations

Higher concentration of gels are used for the separation of lower molecular weight DNA and RNA fragments and vice-versa

b. Molecular weight

A duplex DNA fragment migrates at rates inversely proportional to the log Molecular weight. A plot of logM.W vs. Mobility gives a straight line

c. Conformation

Supercoiled DNA moves fastest followed by linear forms and relaxed open circular forms.

d. Applied Voltage

At low voltage (<5V/cm) the rate of migration is directly proportional to the applied voltage. However, if the voltage is increased, mobility of high molecular weight DNA fragments increased differentially.
e. Base composition and temperature
Base composition and running the gel between 4 and 30C do not change the nobilities.

Preparation of stock solutions for DNA gel electrophoresis
To different buffer systems are widely used for separation of nucleic acids by agarose gel electrophoresis. Their composition are given in the table

<table>
<thead>
<tr>
<th>TBE buffer</th>
<th>10X buffer/lit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tris borate (1X TBE)</strong></td>
<td></td>
</tr>
<tr>
<td>89mm Tris base</td>
<td>108.0g</td>
</tr>
<tr>
<td>89mm Boric acid</td>
<td>55.0g</td>
</tr>
<tr>
<td>25mm Na2-EDTA</td>
<td>9.3g</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TAE buffer</th>
<th>10X buffer/lit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tris acetate (1X TBE)</strong></td>
<td></td>
</tr>
<tr>
<td>50mm Tris base</td>
<td>302.5g</td>
</tr>
<tr>
<td>25mm acetic acid</td>
<td>71.4g</td>
</tr>
<tr>
<td>1mm Na2-EDTA</td>
<td>18.6g</td>
</tr>
</tbody>
</table>

Sterilize the stock solutions by autoclaving

Preparations of ethidium bromide (stock solution)

Weigh 10 mg ethidium bromide into a sterile tube and dissolve in 10 ml sterile distilled water. The stock is stored at 4C

Preparation of sample loading dye Glycerol & bromophenol blue (6x)

3ml glycerol (30%), 25mg bromophenol blue (0.25%) dH2O to 10mL

Preparation of agarose solution for casting the gel

Dissolve the Agarose by placing the flasks in boiling water both cool to Luke warm. Cover the sides of a tray using cellotape and place the comb about 1 cm from the top of the tray.
Pour the Agarose with out making any bublics, cool it for 20 mins and take off the combs and uncover the cellotapes

The DNA sample (100 to 200 ng) is mixed with the loading dye (for 5 µl of DNA sample 1µl of 6x dye is used) and loaded in to the well carefully, using a pipetman or capillary tube. Once the sample is loaded in to the well, the cathode (Black negative terminal) is connected towards the top end of the gel and the anode (Red positive terminal is connected towards the bottom end of the gel. The maximum volume that can be loaded on to a well formed from a 1.5 mm thickness tooth of the comb is 30 µl. The electrophoresis is started by switching on the D. C. Powerpack. The gel is run at 5v/cm. As the bromophenol blue(the tracking dye) has moved 1 cm above the bottom end, the current is switched off, the power supply is disconnected and the gel along with the platform is stained in the plastic tray containing 0.5 µg/ml ethidium bromide in the sterile distilled water( use gloves when handling ethidium bromide). After about 30-45 min, the platform and the gel is rinsed with distilled water and by keeping the platform in a slanting position, the gel is gently pushed onto the UV Transilluminator. (As UV rays are dangerous for the eye, protect your eyes by wearing a UV face shield, goggles or using glass plate). Now the UV light is switched on and the DNA bands are seen and Photographed at f 5.6 for 10 seconds with an orange filter.

**PHENOL: CHLOROFORM EXTRACTION**

1. Mix the DNA solution with equal volume of phenol: chloroform (1:1 v/v).
2. Centrifuge at 10,000 rpm 5 min.
3. Transfer the aqueous phase to a fresh tube, add equal volume of chloroform, and centrifuge 10,000 rpm for 5 min.
4. Transfer the aqueous phase to a fresh tube and mix with 1/10th volume of 3 M NaOAc and 2.5 volume of ethanol. Leave at -20°C for 1 h for precipitation.
5. Centrifuge the samples at 10,000 rpm for 10 min; decant the supernatant.
6. Add 1 ml of 70% ethanol to the pellet, vortex and centrifuge at 10,000 rpm for 5 min.

7. Air-dry the pellet and dissolved in appropriate volume of 0.1X TE buffer.

RESULT:

After electrophoresis DNA bands can be visualized under UV light and they appeared as orange fluorescence.
EXPERIMENT NO: 10
DATE:

EVALUATION OF ENZYME KINETIC PARAMETER

AIM:

To determine the maximum velocity of reaction $V_m$ and $K_m$ value of given enzyme to compare the $V_m$,
   i) Longmuir plot (Hanes woolf plot)
   ii) Line weaver –Burk plot
   iii) Eadie hofter plot.

PRINCIPLE:

For many enzymes, the initial rate $V$ varies hyperbolically with substrate concentration $[S]$ for find concentration of enzyme, at low substrate concentration the occupancy. The occupancy of the active sites on the enzyme molecules in low and the reaction rate is related directly to the number of site occupied this approximate to first order kinetics in that the rate is proportional to the substrate concentration. At high substrate concentration effectively all of the active site are occupied and the reaction becomes independent of the substrate concentration, since no more enzyme substrate $[ES]$ complex are formed and no observed under or saturation kinetics are observed under these conversion of the products from the enzyme.

The mathematical equation expressing this hyperbolic relationship between initial rate and substrate concentration is known as Michael’s Menten equation.

$$ V = \frac{V_m [S]}{K_m + [S]} $$

This equation can be used to calculate $K_m$ and $V_m$ and its use is subject to error owing to the difficulty of experimentally measuring initial state at higher substrate concentration and hence of interoperating the hyperbolic curve to give an
accurate value of $V_{\text{max}}$ linear transformations of the Michael’s Menten equation are preferred the most popular of these in the line weaver-Burk equation.

\[
\frac{1}{V} = \frac{\frac{K_m}{V_m}}{1} \cdot \frac{1}{[S]} \quad + \quad \frac{1}{V_m}
\]

A plot of $1/v$ Vs $1/[S]$ gives a straight line of slope $K_m$ with an intercept on the $1$ axis of $-1$

\[
\frac{1}{[S]} \quad \frac{1}{K_m}
\]

Alternative plots are based on the hangmeir or hanes equation.

\[
\frac{V_m[S]}{K_m + [S]} = \frac{V}{V}
\]

\[
\frac{[S]}{V} \quad \frac{K_m}{V_m} \quad \frac{[S]}{V_m}
\]

And the Eadie-hofshee equation

\[
V \cdot [K_m + [S]] = V_m \cdot [S]
\]

\[
V = V_{\text{max}} \quad \Rightarrow \quad V = V_{\text{max}} - \frac{K_m + V}{[S]}
\]

LIST OF REAGENTS & INTERMEDIATE:-

A. Equipments:

1. Test tubes,
2. Pipettes,
B. Reagents:
   i) Substrate starch solution 1% (buffered using PH 7.0 phosphate buffer).
   ii) - amylase (25mg/100ml)
   iii) dinitrosalicylic acid reagent solution 1%
   iv) Potassium sodium tartarate solution 40%.

PROCEDURE:
   i. Prepare 1% substrate starch by using phosphate buffer.
   ii. Take 0.5 ml to 3.0 ml of 1% substrate starch in Test tubes labeled S1, S2, S3, S4, S5 and S6.
   iii. Make it up to 3ml with distilled water.
   iv. 3ml of distilled water alone serves as blank.
   v. Add 3ml of - amylase solution to all the test tube.
   vi. Incubate the test tubes in water bath at 40°C for 10 mins.
   vii. After 10 mins discard 3ml of the contents from the test tubes.
   viii. Conc. the test tubes with piece of paraffin film.
   ix. Place all the test tubes in water bath at 90°C for 10 to 15 Mins until the red brown colour develops.
   x. Add 1ml of a 40% potassium sodium tartarate Rochelle salt solution to stabilize the colour.
   xi. After cooling to room temperature is cold water bath record the absorbance with a spectrometer at 575nm.

RESULT:
   The Michaelis-Menten parameter Vm and Km was calculated.
EXPERIMENT NO: 11

DATE: 

ABO BLOOD GROUPING

AIM: 
To identify the monoclonal antibodies for phenotyping of human red blood cells.

PRINCIPLE: 
To study hemagglutination of human blood grouping mouse monoclonal antibody raised against blood group A and B obtained by immunizing a mouse with red cell of blood group A and B and fusing the splenocytes of the mouse with myeloma cells. Red blood cell antigen A or B when mixed with their respective antibodies leads agglutination determines the group of tested blood.

CHARACTERISTICS OF ABO ANTIGENS:

ABO antigens are glycolipid in nature, meaning they are oligosaccharides attached directly to lipids on red cell membrane. These antigens stick out from red cell membrane and there are many antigens sites per red blood cell (approximately 800,000).

Besides their presence on red blood cells, soluble antigens can be present in plasma, saliva, and other secretions. These antigens are also expressed on tissues other than red cells. This last fact is important to consider in organ transplantation.

ABO antigens are only moderately well developed at birth. Therefore ABO-HDN not as serve as other kinds of hemolytic disease of the newborn.

CHARACTERISTICS OF ABO ANTIGENS:

1. These are expected naturally occurring antibodies that occur without exposure to red cells containing the antigen. (There is some evidence that similar antigens found in certain bacteria, like E.coli, stimulate antibody production in individuals who lack the specific A and B antigens.)

2. Immunoglobulin M antibodies, predominantly
3. They react in saline and readily agglutinate. Due to the position of the antigen and the IgM antibodies it is not necessary to overcome the zeta potential.

4. Their optimum temperature is less than 30°C, but reactions do not take place at body temperature.

5. Not only are these antibodies expected and naturally occurring, they are also commonly present in higher titer, 1/128 or 1/256.

6. They are absent at birth and start to appear around 3-6 months as result of stimulus by bacterial polysaccharides. (For this reason, newborn blood is only forward typed.)

**ABO INHERITANCE**

**INHERITANCE TERMINOLOGY:**

**GENE:**
Determine specific inherited trait (ex. Blood type)

**LOCUS:**
Unit of inheritance carries genes 23 pairs of chromosomes per person, carrying many genes. One chromosome inherited from mother, one from father.

**ALLELE:**
Alternate choice of genes at a locus (ex A or B; C or c, Lewis a or Lewis b)

**HOMOZYGOUS:**
Alleles are the same for any given trait are different on each chromosome (ex. A/A)
HETEROZYGOUS:

Alleles for a given trait are different on each chromosome (ex. A/B or A/O)

PHENOTYPE:

Observed inherited trait (ex. Group A or Rh Positive)

GENOTYPE:

Actual genetic information for a trait carried on each chromosome (ex. O/O or A/O)

DOMINANT:

The expressed characteristic on one chromosome takes precedence over the characteristic determined on the other chromosome (ex. A/O types as A)

CO-DOMINANT:

The characteristic determined by the genes on both chromosomes are both expressed – neither is dominant over the other (ex. A/B types as AB)

RECESSIVE:

The characteristic determined by the allele will only be expressed if the same allele is on the chromosome also (ex. Can type as only when genotype is O/O)

ABO PHENOTYPES AND GENOTYPES:

1. Group A phenotype = A/A or A/O genotype

2. Group B phenotype = B/B or B/O genotype

3. Group O phenotype = O/O genotype

4. Group AB phenotype = A/B genotype
**PRODUCTION OF A, B AND H ANTIGENS**

The production of A, B and H antigens are controlled by the action of transferases. These transferases are enzymes that catalyze (or control) addition of specific sugars to the oligosaccharide chain. The H, A or B genes each produce a different transferases, which adds a different specific sugar to the oligosaccharide chain.

**PROCEDURE:**

1. Label two glass slides with name or number of the patient and make two circles on each slide. Label the circle as A, B and Rh.
2. Add one drop of monoclonal antibody A in circle A, monoclonal antibody O in circle Rh. Add one drop of patients’ whole blood or its each circle.
3. Mix the red cells and the antibody immediately with an applicator stick and spread it over an area of about one sequence in the circle.
4. Gently till the slides forward and backward at room temperature for a maximum of two minutes. Read the slides for heamagglutination.
RESULT:
The RBC cells agglutination in the given sample indicates

<table>
<thead>
<tr>
<th>Sample Red cells reacted with</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mediclone A</td>
<td></td>
</tr>
<tr>
<td>Mediclone B</td>
<td></td>
</tr>
<tr>
<td>Mediclone O</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
| -                             |         | A Group
| -                             |         | B Group
| +                             |         | AB Group
| -                             |         | O Group
| -                             |         | A or B Group

The table above shows the results of the RBC cells agglutination test. The sample reacts positively (+) or negatively (-) with different reagents to determine the blood group.
EXPERIMENT NO: 12 (A)

DATE:

IMMUNODIFFUSION

AIM:

To learn the technique of Ouchterlony double diffusion.

PRINCIPLE:

Immunodiffusion in gels encompasses a variety of techniques, which are useful for the analysis of antigens and antibodies. An antigen reacts with a specific antibody to form an antigen-antibody complex, the composition of which depends on the nature, concentration and proportion of the initial reactants.

Immunodiffusion in gels are classified as single diffusion and double diffusion. In ouchterlony double diffusion, both antigen and antibody are allowed to diffuse into the gel. This assay is frequently used for comparing different antigen preparation. In this test, different antigen preparations, each containing single antigenic species are allowed to diffuse from separate wells against the antiserum. Depending on the similarity between the antigens, different geometrical patterns are produced between the antigen and antiserum wells. The patterns of lines form can be interpreted to determine whether the antigens are same or different as illustrated below:

![Diagram of Ouchterlony double diffusion](image)

**Pattern of identity: A**

The antibodies in the antiserum react with both the antigen resulting in a smooth line of precipitate. The antibodies cannot distinguish between the two antigens. i.e.) the two antigens are immunologically identical.
**Pattern of identity: B**

In the ‘pattern of partial identity’, the antibodies in the antiserum react more with one of the antigens than the other. The ‘spur’ is thought to result from the determinants present in one antigen but lacking in the other antigen.

**Pattern of identity: C**

In the ‘pattern of non-identity’, none of the antibodies in the antiserum react with antigenic determinants that may be present in both the antigens (i.e., the two antigens are immunologically unrelated as far as that antiserum is concerned).

**MATERIALS REQUIRED:**

1. Agarose
2. 10X assay buffer
3. Antiserum (A, B, C)
4. Test antigens
5. Glass plate
6. Gel punch with syringe
7. Template
8. Assay buffer: PBS

**PROCEDURE:**

1. 25 ml of 1.2% Agarose (0.3g/25ml) was prepared in 1X assay buffer and Agarose was dissolved completely by boiling.
2. The solutions were cooled at 50-60°C and poured 4ml/plate onto 5 grease-free glass plates placed on a horizontal surface. The gel was allowed to set for 30 minutes.
3. Wells were punched by keeping the glass plate on the template.
4. The wells were filled with 10 µl each of the antiserum and the corresponding antigens.
5. The glass plates were kept in a moist chamber overnight at 37°C.
6. After incubation, opaque precipitin lines between the antigen and antiserum wells were observed.
RESULT:

1. Reaction of identity:

   This occurs between identical antigenic determinants. The line of precipitation is given as a continuous arc.

2. Reaction of non-identity:

   When they do not contain any common antigenic determinant the two lines are found independently and were without any interaction.

3. Reaction of partial-identity: This has 2 components:

   i. Those antigenic determinants which are common to both give a continuous line of identity.

   ii. The unique determinant recognition is one of the antigen, in addition a line of non-identity, so that a spur is formed.
EXPERIMENT NO: 12 (B)

DATE: RADIAL IMMUNODIFFUSION

AIM: To learn the techniques of Radial immunodiffusion.

PRINCIPLE: Single radial immunodiffusion (RID) is used extensively for the quantitative estimation of antigens. The antigen-antibody precipitation is made more sensitive by the incorporation of antiserum in the agarose. Antigen (Ag) is then allowed to diffuse from wells cut in the gel in which the antiserum is uniformly distributed. Initially, as the antigen diffuses out of the well, its concentration is relatively high and soluble antigen-antibody adducts are formed. However, as Ag diffuses farther from the well, the Ag-Ab complex reacts with more amount of antibody resulting in a lattice that precipitates to form a precipitin ring.

Thus, by running a range of known antigen concentrations on the gel and by measuring the diameters of their precipitin rings, a calibration graph is plotted. Antigen concentrations of unknown samples, run on the same gel can be found by measuring the diameter of precipitin rings and extrapolating this value on the calibration graph.

MATERIALS REQUIRED:
1. Agarose
2. 10x assay buffer
4. Test Antigen(1 &2)
5. Antiserum
6. Gel punch with syringe
7. Glass plate
8. Template
PROCEDURE:
1. 10ml of 1.0% Agarose (0.1g/10ml) in 1x Assay buffer was prepared and heated slowly till Agarose dissolves completely. Take care not to scorch or froth the solution.
2. Molten Agarose was allowed to cool to 55°C.
3. 120 µl of antiserum to 6ml of Agarose solution was added. Mix by gentle swirling for a uniform distribution of antibody.
4. Agarose solution containing the Antiserum was poured onto a grease free glass plate and set on a horizontal surface. Leave it undisturbed to form a gel.
5. Cut wells using a Gel Puncher using the template provided.
6. 20 µl of the given Standard Antigens and Test Antigens were added to the wells.
7. The gel plate were kept in a moist chamber (box containing wet cotton) and incubate overnight at room temperature.
8. Mark the edges of the circle and measure the diameter of the ring.
9. Plot a graph of diameter of ring (on Y-axis) versus concentration of antigen (on X-axis) on a semi-log graph sheet.
10. Determine the concentration of unknown by reading the concentration against the ring diameter from the graph.

RESULT:

The Concentration of antigen of the test samples were found to be