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Practical Handbook of Biochemistry

Dr. Rupali Khandekar - Shinde

For Students of B. Sc. Zoology
(As per syllabus of Shivaji University, Kolhapur)

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PRACTICAL HANDBOOK OF BIOCHEMISTRY

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(As per syllabus of Shivaji University, Kolhapur)

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PREFACE

I am happy to hand over this "Practical handbook of biochemistry" to the students of B. Sc. The field of biological sciences demands not only theoretical knowledge but also practical proficiency in various laboratory techniques. Understanding and mastering these techniques are essential for any aspiring biologist. With this objective in mind, we have compiled a series of practical exercises that cover a wide array of essential methodologies. This book is written strictly according to syllabus of Shivaji University, Kolhapur implemented to first year, second year and third year of B. Sc. as the all practicals considered in biochemical work. Various reference books have been referred while preparing this book. All the procedures are done in lab and results are analyzed. For easy understanding of the students' simple language, easy protocols, chemical preparation methods and neat labeled diagram are used.

I am also very much thankful to Dr. V. R. Chavan, Principal, Shri Vijaysinh Yadav college, Peth Wadgaon, Dr. S. V. Maske, Dr. R. H. Atigre, Mr. Jagtap. D. J. Shri Vijaysinh Yadav college, Peth wadgaon for their necessary guidance and encouragement.

I hope this book will helpful to all B. Sc. Zoology students and teachers of zoology to carry out experiments in laboratory. I also request to readers to point out any mistakes and make suggestions if any. The mistakes will be corrected and the suggestions will be incorporated in next edition. It is our fervent hope that this Practical Handbook will not only serve as a guide but also inspire curiosity and enthusiasm for the fascinating world of biological sciences among our students.

- Dr. Rupali Khandekar – Shinde

DEDICATED TO

OUR RESPECTED TEACHER

Prof. Dr. D. V. Muley

Former Registrar,
Dr. Babasaheb Amdedkar Marathwada University,
Aurgangabad, Maharashtra
&
Shivaji University,
Kolhapur, Maharashtra

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PRACTICAL I: BIOCHEMICAL METHODS

1. Qualitative Detection of Carbohydrates

Unknown Sample

Molish Test

Procedure:

2 ml unknown sample + 2 ml Molish reagent + 2 ml conc. sulphuric acid

Result: Red violet colour (indicates Carbohydrates)

Iodine Test

Procedure:

2 ml of unknown sample + 2 drops of I2 solution

Results:

- Blue colour (indicates Starch)
- Colourless (indicates Mono/ Disaccharides)
- Red colour (indicates Glycogen)

Barfoed's Test

Procedure:

1 ml of Unknown sample + 5 ml of Barfoed's reagent, boil and allow to stand.

Results:

- No ppt (indicates Sucrose)
- Red Orange ppt (after 5-7 min) (indicates Monosaccharides)
- Precipitate (after 7-12 min) (indicates Disaccharides)

Fearson's Test

Procedure:

4 ml. O. S. + 4 drops of 10 % methylamine hydrochloride, boil for 30 seconds, add 5 drops of 20% NaOH solution

Result: Yellow colour appears which turns red

Seliwanoff Test

Procedure:

1 ml.O. S.+ 3 ml Seliwanoff's reagent, boil for 30 seconds

Results:

- Colourless or faint (indicates Glucose or Galactose)
- Red – orange colour (indicates Fructose)

2. Quantitative detection of carbohydrates

A. Estimation of total Glucose from blood sample

Aim – Quantitative estimation of the glucose in the given blood sample by DNSA method

Principle – Sugars are polyhydroxy derivatives of aldehydes and ketones hence they acts as strong reducing agents. In DNSA method, reduction of 3,5 dinitrosalicylic acid (DNSA) takes place to 3 amino 5 nitro salicylic acid. Glucose gets oxydised into gluconic acid.

This reduced form of DNSA has absorption maxima (Xmax) at 530nm. The intensity of colour is directly proportional to number of aldehyde groups. i.e. glucose concentration which can be determined quantitatively.

Chemicals –Standard glucose solution, DNSA reagent, distilled water, colorimeter

Procedure –

Preparation of Unknown sample solution- Take blood as sample solution. Centrifuge it for 10 minutes at 2000 rpm. Take clear solution (serum) as unknown sample.

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml Std glucose solution in a clean and dyr test tubes
2. Adjust the volume of each test tube to 1 ml by distilled water.
3. Add 2.5 ml DNSA solution in each test tube.
4. Keep all the tubes in boiling water bath for 10 minutes and allow it to cool.
5. Find the optical density of solution at 530nm.
6. Plot the std. graph of optical density at 530nm vs µg of glucose solution.
7. Pipette out 0.5 ml of unknown sample and repeat the same procedure as above.
8. Find out the optical density at 530 nm of unknown sample by using this value.

Determine the unknown glucose concentration by using standard graph.

Observation Table –

Standard glucose concentration = 500 µg/ml

Sr. No.	**Std. glucose (ml)**	**Distilled water (ml)**	**DNSA (ml)**		**µg of glucose**	**O. D. at 530 nm**
1	0.0	1.0	2.5	Keep in boiling water bath for 10 minutes	00	
2	0.2	0.8	2.5		100	
3	0.4	0.6	2.5		200	
4	0.6	0.4	2.5		300	
5	0.8	0.2	2.5		400	
6	1.0	0.0	2.5		500	
7	Unknown A-0.5	0.5	2.5		-	
8	Unknown B-0.5	0.5	2.5		-	

Graph- Plot a graph of Optical density Vs Concentration of standards. Coincide the OD of unknown sample to the Conc. of standard. This is Conc. of unknown sample

Result-

1. The concentration of glucose in given unknown A sample =
2. The concentration of glucose in given unknown B sample =

B. Estimation of total Glucose from urine sample by Titration method-

Aim – Quantitative estimation of the glucose in the given urine sample by Titration method.

Principle –The Benedict's test is quantitative tool to detect glucose from sample which consists of potassium ferrocynade, potassium thiocyanate, sodium citrate, sodium carbonate and copper sulphate. Glucose reduces cupric ions in the form of cuprous ions, which react with potassium thiocyanate to form white coloured cuprous thiocyanate. As the white precipitate is formed, there is reduction of all blue colour indicating complete reduction of cupric ions.

Chemicals – Benedict's quantitative reagent, unhydrous sodium carbonate, urine sample

Procedure-

1. Pipette out 5 ml of Benedicts quantitative reagent in a porcelain dish.
2. Add 2-3 gm unhydrous sodium carbonate and mix well.
3. Heat the mixture till boiling.
4. Add urine dropwise by using a 5 ml graduated pipette. Keep constant stirring by using a glass rod till the blue colour disapppears completely and white precipatate is formed.
5. Note the titration reading.

Calculations –

$$\text{Urinary glucose (mg/dl)} = \frac{10 \text{ X } 10}{\text{Titration reading (ml)}}$$

C. Estimation of total Glycogen from tissue by Anthrone reaction-

Aim – To Estimate of total Glycogen from tissue by Anthrone reaction-

Principle –The glycogen in the tissue is coverted to glucose allowing the glucose to react with anthrone which gives a green colour. Depending upon the concentration of glycogen the colour form intensitifies.

Chemicals –Antrone reagent, Standard stock solution –Dissolve 1 gm glucose in 100 ml distilled water. Working solution – 10 ml of stock solution dilute in 100 ml distilled water.

Procedure-

1. Digest 1 gm of tissue in 2 ml boiling 30% KOH for 20 minutes
2. Keep tissue in KOH till it gets digested completely.
3. Allow to cool and add 2-3 ml ethanol, boil again for 1-2 minutes cool and centrifuge.
4. Discard supernatant and again add 2-3 ml of 95% ethanol.
5. Then keep at refrigerator for 30 minutes in order to precipitate the glycogen with cotton plug.
6. Again centriguge and discard the supernatant. Dilute the ppt in 5 ml distilled water.
7. Add 1 ml of aliquot in each sample tube.
8. Add 1ml of standard solution and 1 ml of distilled water in blank tube.
9. Then add 4 ml of Anthrone reagent in each test tube, Keep in an ice bath.
10. Immediately transfer the tubes to boiling water bath for 4 minutes.
11. Cool immediately and read O. D at 620nm.

Calculations-

$$\textbf{mg. of Glycogen / gm. of tissue} = \frac{\text{O. D. of test}}{\text{O. D. of std}} \text{ X Conc. Std X } \frac{\text{Vol. of extract}}{\text{gram of tissue}} \text{ X 100 X 0.9}$$

3. Quantitative estimation of amino acids by using Ninhydrin reaction

Aim – To detect the presence of amino acids in the given samples by Ninhydrin test**.**

Principle –Ninhydrin is act as powerful oxidising agent due to its presence, amino acid undergoes oxidative determination releasing ammonia, CO2 and corresponding aldehyde and reduced ninhydrin is formed. The reduced form (hydrindantin) of ninhydrin react with ammonia from amino group of amino acid to give blue substrate diketohydrin (ruhemann's purple); while in case of imino acid like proline, bright yellow colour is formed. Aspargine having amide group reacts to give a brown coloured product.

Reagents –Boiling water bath, Ninhydrin: 0.2 % solution prepared in acetone,

Test solution – Prepare solutions containing 0.5% of different amino acids.

Procedure –

1. Add 1 ml of test solution in a test tube.
2. Add 2-5 drops of ninhydrin solution keep for 5 minutes in boiling water bath
3. Observe the development of pink, purple or violet – blue colour.

Observation Table:

Sr. No.	Sample	Initial Observation	Final Observation	Interpretation
1	0.5 % Glycine			
2	0.5 % BSA			
3	0.5 % Proline			
4	0.5 % Aspergine			

Results and Conclusion-

4. Quantitative estimation of total protein in given solutions by Lowry's method

Aim – To determine total concentration of protein by Lowry method

Principle –The copper (cupric) ion reacts with 5 or 6 peptide bond of protein in alkaline solutions to form blue coloured complex. Tartarate is used as stabilizer and iodide which prevent further reaction of the alkaline copper complex. The colour intensity is directly proportional to the protein concentration when measured at 660 nm.

Chemicals –

1. Reagent A – 2% sodium carbonate in 0.1 N sodium hydroxide.
2. Reagent B – 0.5 % copper sulphate in 1 % Potassium Sodium Tartarate. Prepare fresh by mixing stock solutions.
3. Reagent C – Mix 50ml of distilled water in volumetric flask add 1 ml of reagent B prior to use.
4. Reagent D – dilute Folin – Ciocalteau reagent with an equal volume of 0.1 N NaOH
5. Preparation of Standard – Dissolve 50 mg BSA in 50 ml of distilled water in a volumetric flask. Take 10 ml of this stock solution and dilute to 50 ml in another flask for working standard solution. One ml of this solution contains 200 µg proteins.
6. Preparation of Unknown sample - Homogenize the 5 mg tissue in 50 ml saline solution. Centrifuge at 3000 rpm for 15 minutes. Take supernatant as unknown sample.

Procedure –

1. Take 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard in to the labelled test tubes.
2. Add 0.8, 0.6, 0.4, 0.2 and 1 ml of distilled water to make up volume of 1 ml in each test tube.
3. Blank – to prepare blank add 1 ml of distilled water in test tube.
4. Unknown sample – add 1 ml of unknown sample in another test tube.
5. Add 5 ml of reagent C in all test tubes.
6. Incubate at room temperature for 10 minutes.
7. Add 0.5 ml of reagent D in all test tube. Shake it well and incubate for 30 minutes at dark room temperature.
8. Now record the absorbance at 660 nm against blank.
9. The plot the standard curve by taking concentration of protein along X axis and absorbance at 660 nm along Y axis.
10. With the help of standard curve calculate the concentration of protein in given unknown sample.

Observation Table

Volume of standard (ml)	**Volume of distilled water (ml)**	**Conc. of Protein (µg)**	**Volume of reagent C (ml)**		**Volume of reagent D (ml)**		**Absorbance at 660 nm (Optical Density)**
0.0	1.0	00	5	**Incubate at Room temp. for 10 minutes**	0.5	**Incubate at dark room temp. for 30 minutes**	0.00
0.2	0.8	40	5		0.5		
0.4	0.6	80	5		0.5		
0.6	0.4	120	5		0.5		
0.8	0.2	160	5		0.5		
1.0	0.0	200	5		0.5		
Unknown 1.0	0.0	?	5		0.5		

Result –

The given unknown sample contains ……. µg protein.

5. Qualitative test for Lipids

Test	Observation	Inference
Emulsification test: 2 ml. O. S. + 3 ml of water shake well	Droplets are observed	Oil confirmed
Sudan III test: 3ml O. S. + 2 drops of Sudan III stain. Take out one to two drops on a clean and dry slide. Observe under microscope	Brick red coloured droplets	Oil confirmed
Saponification test 1 ml O. S. + 3 ml of alc. 10% NaOH boil, cool & excess of Na2 SO4	Soap is formed & it rises to the surface	Oil confirmed

6. Quantitative detection of total Lipids

A. Estimation of Lipid by Sulpho-phosphovanillin reagent

Aim – To estimate total Lipid by Sulpho-phosphovanillin reagent from tissue.

Principle – Lipids react with vanillin in the presence of sulphuric and phosphoric acids to form a pink coloured complex.

Chemicals – Total Lipid Standard – 300mg cholesterol in 50ml Glacial acetic acid

Vanillin reagent, chloroform, methanol, sulphuric acids, Sodium sulphite

Homogenate – 100 mg chilled tissue was homogenized in 10 ml Folch mixture (chloroform: methanol = 2: 1). A pinch of sodium sulphite was added and the solution was filtered.

Procedure –

1. Take 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard in to the labelled test tubes.
2. Unknown sample – Take homogenized 1 ml of sample was taken
3. Boil the standard tubes and unknown sample for 2 hours and dried it.
4. 1 ml of concentrated H2SO4 was added in all tubes. Prepare blank test tube having 1ml conc. H2SO4 and boiled for 5 minutes and cool it.
5. In cooled solution, 2 ml of Vanillin reagent was added. Pink colour developed.
6. The optical density was measured at 530 nm.

Observation Table

Volume of standard Cholesterol (ml)	**Conc. of Cholesterol (µg)**		**Volume of Conc. H2SO4 (ml)**		**Volume of Vanillin reagent (ml)**	**Absorbance at 530 nm (Optical Density)**
0.2	1200	**Boil for two hours and dry it**	1	**Boil for 5 min. and cool it**	2	
0.4	2400		1		2	
0.6	3600		1		2	
0.8	4800		1		2	
1.0	6000		1		2	
Unknown 1.0	?		1		2	
Blank -	00		1		2	

Graph- Plot a graph of Optical density Vs Concentration of standards. Coincide the OD of unknown sample to the Conc. of standard. This is Conc. of unknown sample

Result –

The given unknown sample contains ……. **µg** lipid.

B. Estimation of Serum Total Cholesterol by Watson's Calorimetric Technique

Aim – To Estimate of Serum Total Cholesterol by Watson's Calorimetric Technique

Principle –Cholesterol reacts with acetic anhydride in the presence of glacial acetic acid and concentrated sulphuric acid to form green coloured complex. Intensity of colour is proportional to the cholesterol concentration.

Chemicals- Cholesterol standard (Dissolve 200mg Cholesterol in 100 ml of Glacial acetic acid), Conc. H_2SO_4, Cholesterol reagent.

Procedure-

Volume of standard Cholesterol (ml)	**Conc. of Cholesterol (µg)**	**Volume of Cholesterol reagent (ml)**		**Volume of Conc. H_2SO_4 reagent (ml)**		**Absorbance at 575 nm (Optical Density)**
0.2	400	2.5	**Mix well and cool to cold water bath**	0.5	**Keep in water bath at room temp for 10 min.**	
0.4	800	2.5		0.5		
0.6	1200	2.5		0.5		
0.8	1600	2.5		0.5		
1.0	2000	2.5		0.5		
Serum 0.2	?	2.5		0.5		
Blank - 0.2ml DW	00	2.5		0.5		

Calculations-

By Graph - Plot a graph of Optical density Vs Concentration of standards. Coincide the OD of unknown sample to the Conc. of standard. This is Conc. of unknown sample.

By Formula-

$$\text{Serum total Cholesterol (mg/dl)} = \frac{\text{O. D. Test}}{\text{O. D. Std (0.2ml)}} \quad \text{X } 200$$

PRACTICAL II. HAEMATOLOGICAL TECHNIQUES

1. Preparation of Haemin crystals

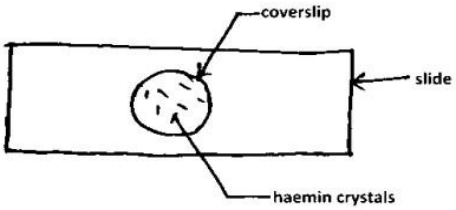
Aim – To prepare haemin crystals from own blood sample

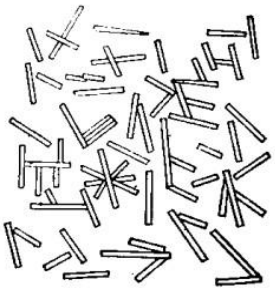
Principle –Hemoglobin is aglobin protein conjugated with a red pigment, haeme or hematin. The hematin is a porphyrin iron complex. Hydrochloride of the hematin forms the hemin crystals.

Chemicals – Drops of fresh blood, glacial acetic acid, spirit lamp

Procedure-

1. Sterlize the finger with alcohol. Prick the finger with lancet and take 2-3 drops of blood on slide.
2. Add 2-3 drops of Glacial acetic acid.
3. Heat gently, on a flame of spirit lamp.
4. Cool and observe dark brown haemin crystals under the microscope.





Rhomboidal haemin crystals

Result – The haemin crystals look like rod, prism and shining rhombie plate structures.

2. Determination of Bleeding time

Aim – To determine bleeding time by Duke's method.

Principle – Determination of bleeding time helps to investigate vascular and platelet disorders. The length of time required to stop bleeding, to be recorded. Normal range is 1 to 5 min.

Procedure –

1. Sterlize the finger with alcohol. Prick the finger with lancet to make a deep incision.
2. Take a first drop of blood on filter paper and record it as 0 seconds.
3. Take a blood drop at 20 sec. interval of time sequentially on filter paper.
4. Note the time at which bleeding completely stops and there is no any blood spot on paper.
5. Count the time at which blood stop. This is bleeding time.

Result – My own bleeding time is ……

3. Determination of Blood Clotting time

Aim – To determine blood clotting time by capillary method.

Principle –The blood is collected in a capillary tube forms a fibrin string after some time which can be observed by breaking the capillary tube at regular intervals and recording the time at the formation of first fibrin string. Normal range is 4 to 9 min.

Procedure –

1. Sterlize the finger with alcohole. Prick the finger with lancet make a deep incision.
2. Wipe off the first blood drop and collect the blood in a capillary tube up to 2/3 of its length.
3. After every half a minute break down about 1 cm of the capillary piece and find out whether fibrin is formed or not.
4. When the fibrin string appears in a capillary piece, then stop to break capillary. Record the total time required for clotting.

Result – My own clotting time is ……

4. Identification of ABO and Rh blood group.

Aim – To identify ABO and Rh group in human blood.

Principle – the human blood having 8 groups of ABO system. These groups are specified by presence and absence of antigen on plasma membrane and antibodies in blood plasma.

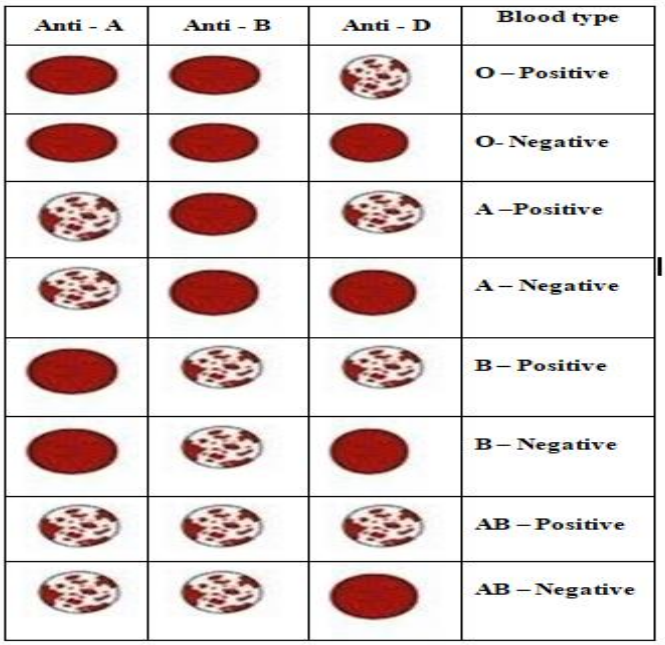
Requrements- Spirit or 70% alcohol, lancet, antisera kit, slide, pin etc.

Procedure –

1. Take a clean slide, mark A, B and D with glass mark on slide.
2. Sterlize the finger with spirit or alcohol and prick with lancet.
3. Take three blood drops on slide marked as A, B and D.
4. Add a drop of antisera A, B and D in respective mark.
5. Mix well antisera and blood with separate pin.
6. Observe agglutination under microscope.

Observations –

1. Agglutination with antisera indicates positive test.
2. No agglutination indicates negative test.
3. Agglutination with antisera D indicates Rh + ve test.
4. No agglutination with antisera D indicates Rh - ve test.



Anti - A	Anti - B	Anti - D	Blood type
			O – Positive
			O- Negative
			A –Positive
			A – Negative
			B – Positive
			B – Negative
			AB – Positive
			AB – Negative

Result – My blood group is …….

5. Total Erythrocyte count

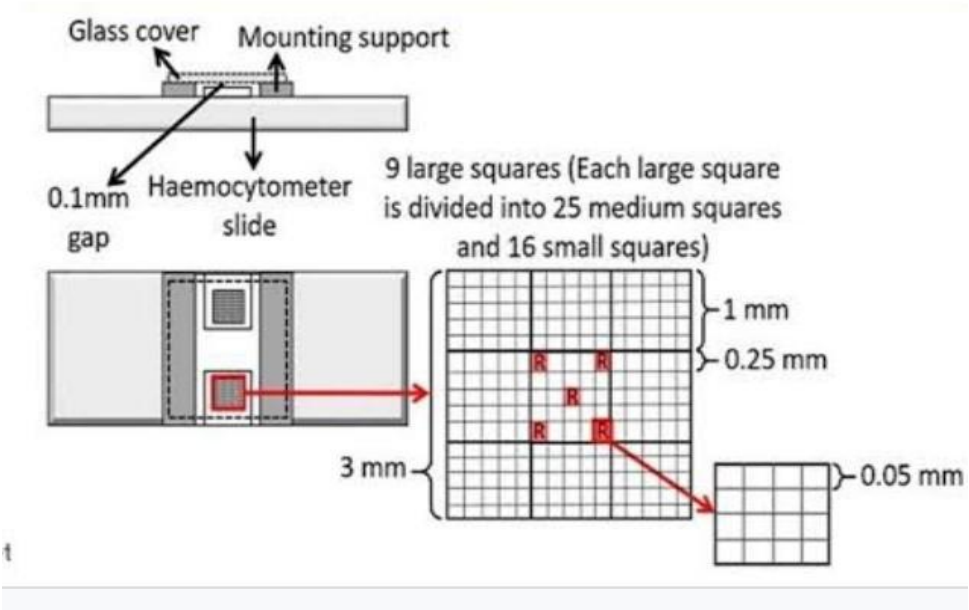
Aim – To determine total erythrocyte count.

Principle – By using RBC diluting fluid, the blood is diluted up to 1:200 times and cells are counted under high power (40X) with the help of hemocytometer.

Requirements- Microscope, RBC pipette. Neubauer chamber (hemocytometer), RBC diluting fluid.

Procedure-

1. Wash and clean finger with alcohole/spirit and prick gently by sterlized lancet.
2. Wipe out 1 -2 drops of oozing blood.
3. Take clean RBC pipette and suck the blood in a pipette upto 0.5 mark without any air bubble.
4. Clean the blood sticking outside the pipettte by spirit.
5. Now suck the diluting fluid up to 101 mark. Mix the content by rotating with thumb and fingers for 5 min. It provides 200 times dilution of the blood.
6. Discard first few drops and fill the Neubauer counting chamber.
7. Diluted blood solution is filled up between the coverslip and counting chamber of hemocytometer by capillary action.
8. Leave the hemocytometer to stand for 3-5 min. to settle the RBCs.
9. The RBCs are identified by their slightly transparent bluish appearance.
10. Now count in 5 squares.



Calculations-

$$\text{RBC per cu.mm} = \frac{\text{Number of RBC counted X Dilution X 4000}}{\text{Number of small squares counted}}$$

Total RBC for 5 small squares = A+B+C+D+E

RBC in 80 smallest squares would be A+B+C+D+E

Therefore, RBC in smallest square = A+B+C+D+E / 80

$$\text{RBC in 400 smallest squares} = \frac{\text{400 X A+B+C+D+E =5(A+B+C+D+E)}}{80}$$

But the height of blood film = 0.1mm

Dilution of blood =200

So that 1 cu mm volume of blood containing total RBC

= 200X 10 X 5(A+B+C+D+E)

= 10,000 (A+B+C+D+E)

Result – Total RBC count = …… cells/cu mm

6. Total Leucocyte count

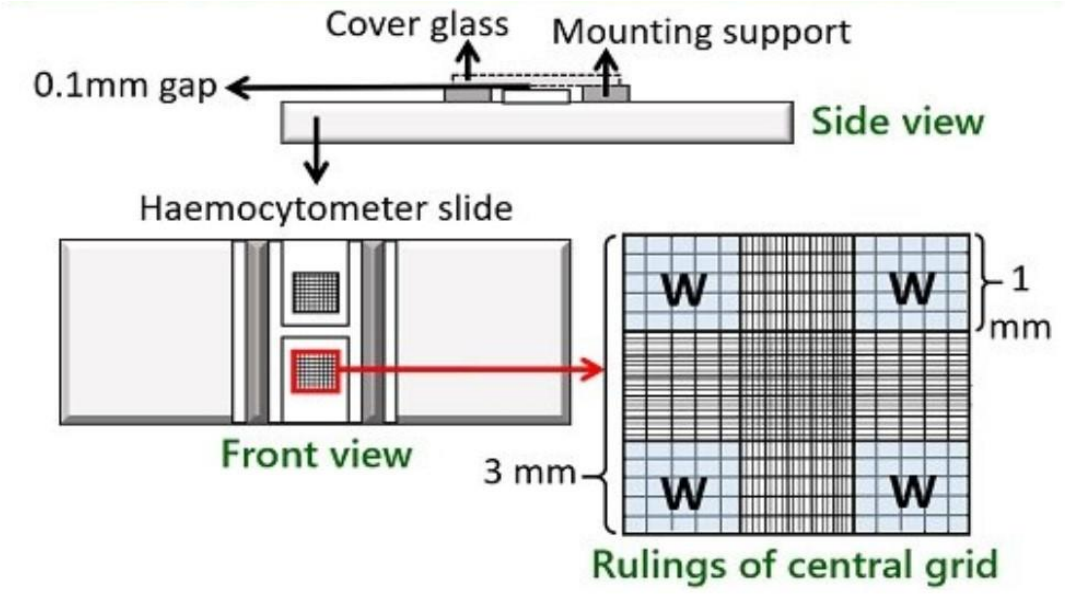
Aim – To determine total leucocyte count.

Principle – The glacial acetic acid lyses the RBC while gentian violet slightly stains the nuclei of the leucocytes. The blood is diluted up to 1:20 in WBC diluting fluid and ells are counted under low power of the microscope by using counting chamber.

Requirements- Microscope, WBC pipette. Neubauer chamber (hemocytometer), WBC diluting fluid.

Procedure-

1. Wash and clean finger with alcohole, spirit and prick gently by sterlized lancet.
2. Wipe out 1 -2 drops of oozing blood.
3. Take clean WBC pipette and suck the blood in a pipette upto 0.5 mark without any air bubble.
4. Clean the blood sticking outside the pipettte by spirit.
5. Now suck the diluting fluid up to 11 mark. Mix the content by rotating with thumb and fingers for 5 min. It provides 20 times dilution of the blood.
6. Discard first few drops and fill the Neubauer counting chamber.
7. Diluted blood solutions is filled up between the coverslip and counting chamber of hemocytometer by capillary action.
8. Leave the hemocytometer to stand for 3-5 min. to settle the WBCs.
9. The WBCs are identified by their slightly transparent bluish appearance.
10. Now count in 4 squares.



Calculations-

$$\text{WBC per cu.Mm} = \frac{\text{Number of WBC counted X Dilution X 10}}{\text{Number of 1 sq.mm counted}}$$

The no. WBC in 4 outer squares is x.

The dilution is 20 times and cubic capacity of the area counted is 1/10 cu.

So the number of WBC in 1/200 cu mm is x.

Therefore the number of WBC in 1 cu. Mm = x X 200 X ½ = y

Then Total WBC amount to = y cu. Mm

Result- The total WBC count is ……./cu. Mm

7. Differential Leucocyte count

Aim – To determine differential WBC count per 100 cells.

Principle –The polychromatic staining solutions contain methylene blue and eosin. These basic and acidic dyes react with nuclei and cytoplasm respectively and induce multiple colours when applied to WBC. In this way acidophilic, eosinophilic, basophilic and neutrophilic are differentiated. Methanol acts as a fixative as well as a solvent. The fixative does not allow any change in the cells and make them to adhere on surface of a glass slide.

Requirements- Leishmann stain\ Giemsa stain, methanol, buffer solution

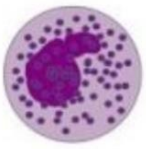
Procedure-

1. Take clean and dry slide, make smear of blood on slide.
2. Dry the blood smear at room temperature. Keep it away from dust.
3. Fix in methanol for 2-3 min.
4. Cover the smear with the staining solution by adding 10 -15 drops on the smear. Wait for 5 min.
5. Add equal number of drops of buffer solution, mix both the solutions by blowing the pipette. Wait for 10 min.
6. Wash the smear with some drops of tap water. Stand the slide obliquely on draining position to get dry completely.
7. Put a drop of glecerin and mount a smear with a clean coverslip.
8. Examine under low power first, select a region of smear, put a drop of oil immersion. Count at least 100 leucocyte.

Calculations-

$$\text{Percentage of different types of leucocytes} = \frac{\text{Number of type of cells}}{\text{Total number of leucocytes}} \times 100$$

Leucocyte count

Lecocyte type	**Percent**	**Cytological features**	**Diagram**
Neutrophils	50-75	Polynucleated granulocytes	
Eosinophils	1-4	Granulocytes, orange large granule	
Basophils	0.5-1.0	Granulocytes, Blue large granules	
Lymphocytes	20-45	Pale blue cytoplasm, large nucleus	
Monocytes	2-8	Kidney shaped nucleus	

8. Determination of Hemoglobin by Sahli's Hemoglobinometer Method

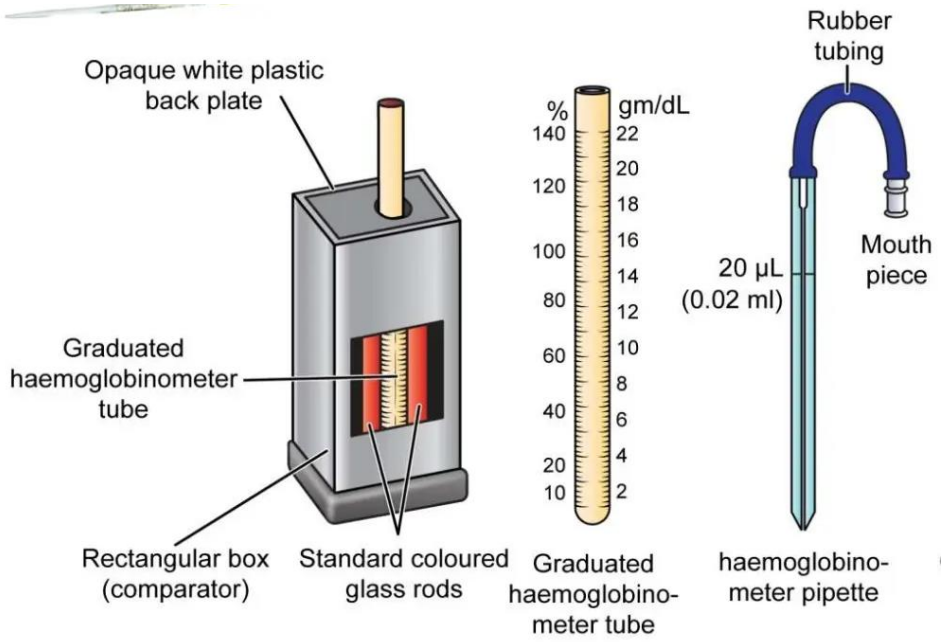
Aim - To determine Hemoglobin by Sahli's Hemoglobinometer Method.

Principle- Hydrochloric acid converts hemoglobin to brown colored acid hematin which is after dilution compared with brown glass reference blocks.

Requirements – EDTA containing anticoagulating venous blood, Sahli's hemoglobinometer, graduated tube, Hb – pipette, 0.1 N HCl.

Procedure –

1. Add 0.1 N HCl in a tube up to the lowest mark.
2. Draw the blood by Hb pipette up to 20 mark and adjust the blood column carefully without any air bubble.
3. Transfer the blood to the acid in the graduated tube and allow to stand the mixture for 10 minutes.
4. Dilute the solution with distilled water by adding drop by drop at a time carefully and mixing the reaction mixture with capillary until the colour matches to the glass plate in the comparator.
5. The matching must against the natural light.
6. Note the level of the fluid at its lower miniscus and the reading corresponding to this level on the scale is recorded in g/dl.



Result – My haemoglobin count is ……

PRACTICAL III. STAINING TECHNIQUES

1. Histological slide preparation

1. Premicrotomy process

a. Anesthetizing and narcotizing the animals

b. Dissection in saline solutions

c. Fixation in suitable fixative

d. Dehydration through alcohol grades

e. Clearing, infilteration and embedding

f. Block preparation and trimming

2. Microtomy process

3. Post microtomy process

1. Premicrotomy process

a. Anesthetizing and narcotizing the animals –

Different anesthetizing agents are used for different group of animals to narcotize for dissection. Trematodes are anaesthetized by menthol for 30 min. Soap emulsion can be used to killing the insects. Mostly chloroform soaked cotton ball can be used to kill insects, arachnids and small mammals like mice or rat in insect killing bottles or jars.

b. Dissection in saline solutions –

The anaesthesized animals can be dissected in the saline solution i. e. isotonic media in order to maintain normal condition of the cells otherwise the cells may swell or shrink in hypotonic or hypertonic dissection media.

c. Fixation in suitable fixative –

After dissection immediate fixation of the organs or tissue is essential to prevent autolysis and post martem changes. Different fixatives like formaline, bouins fixative(aqueous), Bouin Duboscq fixative(alcoholic) and Carnoys fixative are mostly used. After dissection tissues are transferred for fixation, but after 24 hours change fixative media with new one which can fix tissue for long term.

d. Dehydration through alcohol grades-

After fixation, next step is dehydration of the material. This process is aimed to remove excess water from the tissue. The process of dehydration is carried out by keeping the tissue in ascending order of alcohol grades beginning with 30%, 50%, 70%, 90% and absolute alcohol for period of 10 to 30 minutes as per size of tissue. Generally two changes of either 30 minutes or 1 hour of absolute alcohol are recommended for complete removal of water**.**

e. Clearing, infilteration and embedding –

Next step after dehydration, is the clearing method. The ckearing fliud clears opacity of dehydrated tissue and makes it almost transparent, hard and crystalline.

The clearing agents are three hydrocarbons- xylene, benzene and toluene among which xylene is widely used as clearing agent. After dehydration transfer the tissue in alcohol + xylene as 1: 1 concentration for 5 minutes then transfer it in pure xylene for 4- 5 minutes.

Then there is process of tissue embedding having to steps as cold impregnation and hot impregnation. In cold impregnation the paraffin wax is completely dissolve in xylene. After clearing in xylene, tissues are to cold impregnation for 30 minutes then transferred to hot impregnation.

In hot impregnation, the wax is melted in oven overnight at 56-58 ^{0}C. After cold impregnation, transfer tissue in melted wax for 15 minutes so as to dissolve excess wax which is being attached to tissue.

Then transfer in fresh melted wax for 30 min. then second change is done for 20 minutes then observe the bubbling from the tissue as it surely minimises and tissue become transparent, it is completely embedding of tissue in paraffin wax.

f. Block preparation and trimming

It is the last step in which small rectangular paper pockets, metal rectangles or any available staing container is used. At first inner surface of container is coated with glycerol. Then the melted paraffin is put in into embedding container in such amount that material should be immersed completely and desired orientation can be maintained, for solidification, the embedding container is kept for 1 -2 hours.

After solidification as per orientation, with help of scarpel trim the bock and mount on wooden block holder for sectioning.

2. Microtomy process

With the help of microtome, the sections of constant thickness without damaging the tissue are prepared. Attach the block holder to the microtome. Tighten the block holder clamp, at the proper angel, insert knife, clear the surface of the block and take the sections at desirable thickness (4 -6µ).

3. Post microtomy process

Make thin smear of albumen on glass slide. Cut the ribbon at desirable length so that these pieces of the ribbon can easily mounted on about three forth length of the slide and leaving one forth space for identification mark. Take a drop of water on albuminised slide, put the piece of ribbon and warm gently so as to fix the section on slide. Then these slides are ready for staining.

2. Haemotoxylin – Eosin staining tachnique

Procedure-

1. Transfer the slides to xylin for 10 – 30 min.
2. Transfer to absolute, 90%, 70%, 50%, 30% for 3-5 minutes in descending order.
3. Transfer to distilled water for 5 minutes.
4. Put drop of hemotoxylin for 10 – 15 minutes, wash with running water and observe under microscope. If stain is over then give 2 -3 dips in 1% HCl.
5. Transfer the slides to 30%, 50% and 70% alcohol then stain with alcoholic eosin for 1 – 2 minutes. Wash with 70% alcohol, transfer it in 90% alcohol for 5 minutes.
6. Observe under microscope for proper staining then keep in absolute alcohole for 10 minutes.
7. Transfer it in alcohol : xylene (1:1) for 15 minutes.
8. Clear in xylene for 1 -2 minutes. Put Canada balsam or DPX by glass rod on xylene wet sections mouunt coverslip without allowing air bubbles.

Results - Nuclei –deeply blue, cytoplasm - pink.

3. Alcian Blue staining technique at pH – 1

Procedure –

1. Deparaffinize in xylene for 15 minutes. Hydrate in descending orders in alcohol grades i.e from absolute alcohol, 90%, 70%, 50%, 30% and distilled water for 5 minutes.
2. Stain it with alcian blue solutions for 30 minutes.
3. Rinse in running tap water.
4. Counterstain in nuclear fast red for 10 minutes.
5. Wash in running tap water for 1 minute.
6. Dehydrate in alcohol grades in ascending water for 5 minute each and 10 minutes in absolute alcohol.
7. Clear in xylene and mount in DPX.

Results – Acid mucin – blue, Proteoglycans and hyaluronic acid – blue, Nuclei – red

4. Alcian Blue staining technique at pH – 2.5

Procedure –

1. Deparaffinize in xylene for 15 minutes. Hydrate in descending orders in alcohol grades i.e from absolute alcohol, 90%, 70%, 50%, 30% and distilled water for 5 minutes.
2. Place in 3% acetic acid solution for 5 minutes Stain it with alcian blue solutions, pH 2.5 for 30 minutes.
3. Rinse with distilled water
4. Counterstain with nuclear fast red.
5. Rinse with running tap water for 1 minute.
6. Dehydrate in alcohol grades, clear in xylene and mount with DPX.

Results – Weakly acidic sulfated mucosubstances, hyaluronic acid, sialomucins are stained dark blue, nuclei are stained red or pink

5. PAS (Periodic Acid Schiff) staining protocol

Procedure –

1. Deparaffinize in xylene and hydrate in alcohol grades.
2. Oxidize in 0.5% periodic acid solution for 5 minutes.
3. Rinse in distilled water.
4. Place in schiff's reagent for 15 minutes.
5. Wash in tap water for 5 minutes.
6. Counter stain in Mayers hematoxylene for 1 minute.
7. Wash in tap water for 5 minute. Then rinse with distilled water.
8. Dehydrate through alcohol grades, clear in xylene and mount with DPX.

Result – Glycogen, Mucin, and some basement membranes – red/purple.

6. Whole mount Stained preparation of Chick Embryo

Procedure –

1. Break the egg shell from the air space by hammering with handle of scissor. Remove the egg shell soo as the white albumen was drained out slowly.
2. Submerge the egg in physiological saline at 37^0C in a finger bowl or petri plate and chick embryo will seen floating on upper surface of the yolk.
3. Remove excess yolk around the embryo carefully. Give a cut around the vascular mass of embryo and transfer the embryo on albumenized slide.
4. Put Bouins/ carnoys or 10 % formalin on the embryo. Leave the fixative overnight.
5. Remove the fixative by adding distilled water by pipette for washing the embryo.
6. Stain with borax caramine or eosin for 5 minutes.
7. Dehydrate carefully by putting sufficient amount of 70%, 90% and absolute alcohol for 10 minutes in each.
8. Clear in xylene for 5 minutes as per size of embryo.
9. Mount in CB\DPX

Result –Embryo – Pink

7. Cytological stained preparation of Mitochondria from oral mucosa

Aim – To prepare stained slide of mitochondria from oral mucosa.

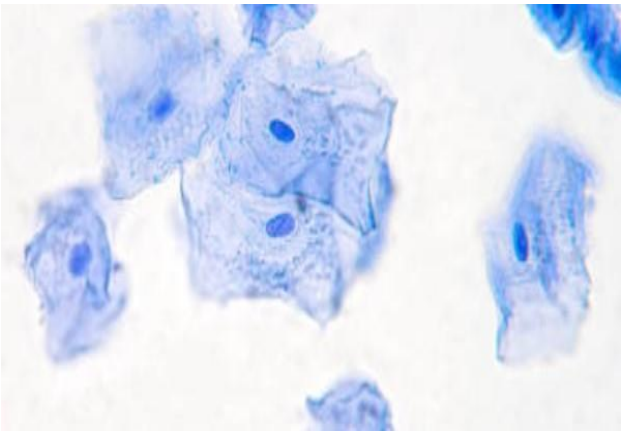
Requirements – Slide, coverslip, Janus green B

Procedure –

1. Clean mouth, take smear of oral mucosa on dry slide.
2. Dry the slide, Stain with dilute Janus green B stain for 5 minutes.
3. Observe under microscope under low power then high power.

Results - Mitochondria appears bluish green stained granules.

Diagram –



8. Stained preparation of Polytene Chromosome in Salivary gland of Drosophila

Aim – To prepare stained slide of Polytene chromosome in salivary gland of Drosophila

Requirements – Drosophila larvae, slide, coverslip, acetocarmine stain

Procedure –

1. Take larva of drosophila, with the help of forecep remove the salivary gland.
2. Keep the salivary gland on slide, squash with coverslip.
3. Stain with acetocaramine for 10 minutes.
4. Observe under low power then high power in microscope.

Result – Polytene chromosome are appear pinkish in colour.

PRACTICAL IV. ECOLOGICAL PRACTICALS

1. Estimation of dissolved O_2 from given water sample.

Aim – To estimate dissolved O_2 from given water sample.

Principle – Oxygen from the given water sample can be estimated by Wrinkler's method. This technique is based on the fact that when MnSO4 is added in the known volume of water in presence of alkali, the Mn $(OH)_2$ and $NaSO_4$ will be formed in the reaction mixture. The dissolved oxygen present in the given amount of water reacts with $Mn(OH)_2$ get oxidized with non oxidising agent like HCl, H_2SO_4 which reacts with KI, equivalent amount of iodine remains in reaction bottle when starch solution is added to it. It reacts with liberated iodine and blue colour develops when titrated with this standard sodium thiosulphate (N\80) blue colour disappears due to formation of Sodium ion which is end point of reaction.

Chemicals- Wrinkler's solution A, Wrinkler' solution B, starch solution, N/80 standard sodium thiosulphate solution (0.025N), Conc. H_2SO_4.

Procedure –

1. Fill the BOD bottles with given water sample untill it overflows. Avoid air bubbles.
2. Add 1 ml of Wrinkler's solution A, shake it well. Add 1 ml of Wrinkler's solution B, shake it well.
3. The precipitate is developed, allow it to settle down.
4. Now add 2 ml of conc. H_2SO_4 to dissolve the precipitate to get clear brown coloured liquid.
5. Take 100 ml of clear solution in conical flask without vigorous shaking and 2-3 drops of starch solution to it as indicator, solution turns blue in colour.
6. Titrate this against 0.025N sodium thiosulphate till blue colour disappears, note burette reading. Repeat this experiments for three times for each sample.

Calculations –

$$\text{Dissolved oxygen (mg/l)} = \frac{\text{M. B. R. X N X 1000}}{V \; \frac{V1 - V2}{V1}}$$

Where, V = Volume of sample =100ml; V1 = Volume of sample of BOD bottles = 300ml

V2 = Volume of titrant = 2ml; N = Normality of Na_2SO_4 = 0.025N

Result -

2. Estimation of free CO_2 from given water sample.

Aim – To estimate free CO_2 from given water sample

Principle – Free CO_2 present in water can react with the sodium carbonate to form $NaHCO_3$. In this reaction phenolpthalein is suitable indicator. It takes pink colour in absence of CO_2 i.e. presence of $NaHCO_3$ and remain colourless in presence of CO_2.

Chemicals - Phenolpthalein indicator, standard Na_2CO_3 (0.0454N)

Procedure –

1. Take 100 ml of water sample in conical flask without vigorous shaking.
2. Add few drops of phenolpthalein indicator if pink colour develops then free CO_2 absent if solution remains colourless free CO_2 present.
3. Then titrate it against (Na_2CO_3). Repeat procedure at least three times.

Calculation-

$$\text{Free } CO_2 \text{ mg\lit} = \frac{\text{M.B.R X N X 22000}}{\text{Volume of water sample}}$$

Where, N = Normality of sodium carbonate = 0.0454

Volume of water sample = 100

Result -

3. Estimation of alkalinity of given water

Aim – To estimate alkalinity of given water sample

Principle – Alkalinity of water is the capacity to neutralize strong acid. it is characterised by presence of hydroxyl ion. Generally, the alkaline nature of fluid is because of high concentration of hydroxyl ions. It gives alkalinity of the solutions.

Chemicals – 0.1N HCl, conc. HCl, Sodium carbonate solution (0.1N), methyl orange indicator, phenolpthalein indicator.

Procedure-

1. Take 100ml of water sample in conical flask, add 2-3 drops of phenolpthalein indicator.
2. If colour changes to pink after adding of phenolpthalein, titrate it against 0.1N HCl untill colour disappears. This is phenolpthalein alkalinity.
3. Now add 2-3 drops of methyl orange to the same sample and continue the titration untill yellow colour changes to orange.

Calculation-

$$\text{Total alkalinity of } CaCO_3 \text{ in given} = \frac{\text{M. B. R. X N X 1000 X 50}}{\text{Volume of water sample}}$$

Where, N = Normality of sodium carbonate = 0.1

Volume of water sample = 100

Result -

4. Estimation of Hardness of water sample

Aim – To estimate hardness of water sample.

Principle – Hardness is property of water which prevents foam formation with soap and increasing the boiling point of water. The major cations imparting calcium (Ca++), magnessium (Mg++) and the anions responsible for bicarbonates, carbonates, sulphates and chlorides etc. The major hardness of water which effect the quality of water in comparison to normal.

Chemicals- EDTA solution, Buffer solution A, Buffer solution B, Erichrome black T.

Procedure –

1. Take 50ml of water sample in conical flask
2. Add 1ml of buffer solution in it.
3. Add 100mg of erichrome black T as indicator, solution turns to red wine colour.
4. Titrate it against 0.01 N EDTA solution.
5. At the end colour changes from red wine to blue.

Calculation –

$$\text{Hardness of water mg/lit} = \frac{\text{M.B.R X 1000}}{\text{Volume of water sample}}$$

Where, N = Normality of EDTA = 0.01

Volume of water sample = 50

Result –

5. Estimation of biological oxygen demand (BOD) of given water sample

Aim – To estimate biological oxygen demand (BOD) of given water sample

Principle- The rate of removal of oxygen and consumption of oxygen by an organism and degradation of the dissolved particulates organic matter in the water is the biological oxygen demand (BOD). BOD is used as index of an organism / an organic matter present in the water is more than the amount of oxygen require to degrade the biological demand of oxygen. BOD is evolved by measuring water concentration in sample before and after incubation in dark at 20^0C for 3 or 5 days or 27^0C for 3 days preliminary dilution during at sample by necessary 4 days ensure that not an oxygen is consumed during incubation.

Chemicals – Phosphate buffer, magnessium chloride, feric chloride solution, sodium hydroxide, conc. H_2SO_4, alkaline KI, 0.025N sodium thiosulphate, 1% starch

Procedure –

1. Take 1 liter of aerated water in glass and add 1 ml of phosphate buffer, magnessium sulphate, calcium chloride and ferric chloride.
2. Take polluted water usually necessary to prepare a suitable dilution lastly neutralised to the polluted water.
3. Fill two sets of BOD bottles and keep it in dark place for 27^0C immediately and carry out procedure like estimation of O_2 of two water samples.
4. Add 2 ml of $MnSO_4$ and 2 ml of alkaline KI in BOD bottles mix the reagent properly brown colour form. Dissolve ppt with conc. H_2SO_4 and we use dissolve solution to O_2 estimation. If titration is carried out by few days then keep the sample.
5. Take 50 ml sample in conical flask and add 3-4 drops of starch indicator, blue colour develops. Titrate this against 0.025N sodium thiosulphate untill the blue colour disappears.

Calculations –

$$\text{Dissolved oxygen (mg/l)} = \frac{\text{M. B. R. X N X 1000}}{V \; \frac{V1 - V2}{V1}}$$

Where, V = Volume of sample =100ml

V1 = Volume of sample of BOD bottles = 300ml

V2 = Volume of titrant = 2ml

N = Normality of Na_2SO_4 = 0.025N

Result –

6. Estimation of chemical oxygen demand (COD) of given water sample

Aim – To estimate chemical oxygen demand (COD) of given water sample

Principle – Chemical Oxygen demand is a measure of oxygen consumption during oxydisable organic matter by a strong oxydising agents like Potassium dichromate in presence of sulphuric acid both these are used as oxydising agents in determination of COD. Sample is refluxed with K2Cr2O7 and H2SO4 in presence of mercury sulphate to neutralize effects of fluorides into sulphate. The excess K2Cr2O7 is titrates against ferrous ammonium sulphate by using ferron as in indicator. Amount of potassium dichromate is in the proportion to oxydising organic matter present in the sample.

Apparatus – Chemical oxygen demand reflux assembly containing COD flasks, condensation rubber, rubber tube stand, heating plate titration assembly, burette, measuring cylinder, pipette.

Chemicals – Potassium dichromate (0.25N), ferous ammonium sulphate (0.1N), ferrron as indicator, conc. H2SO4, mercury sulphate (solid), silver sulphate (solid).

Procedure –

1. Take 20 ml sample in COD flask expected to have COD more than 50 mg/lit.
2. Then add 10 ml of 0.25N $K_2Cr_2O_7$, in case of less COD (50mg/lit) then add 2ml of $K_2Cr_2O_7$ extreme care should be taken in low COD sample because small size of organic matter in the glass ware may contribute significant air.
3. Add a pinch of silve sulphate to the flask (if the sample contains more than 2 chlorides in higher amount then mercury sulphate is to be added in the ration of 100:1 to the fluoride).
4. COD can not be determined accurately if sample contain more than 2000mg/lit of fluoride. Add 30 ml of conc. H_2SO_4 and reflux at least for 2 hour on water bath or hot plate.
5. Remove the flask, cool and add 2-3 drops of ferron indicator and titrate against 0.1N ferrous ammonium sulphate. If $K_2Cr_2O_7$ of 0.25N is used end of titration is dull green or blue.

Calculation –

$$\text{COD mg/lit} = \frac{\text{(b-a) X N of titrate X 1000 x 8}}{\text{ml of sample}}$$

Where, a = 84 ml titrate with sample; b = 112 ml titrate with sample; N = 0.1; Sample = 20

$a = \frac{\text{Total volume X M. B. R.}}{\text{Volume of sample of titrate (10)}}$	$b = \frac{\text{Total volume X M. B. R}}{\text{Volume of sample titrate}}$

Result –

8. Qualitative and quantitative analysis of Zooplankton

Aim - Qualitative and quantitative analysis of Zooplankton

Collection – The sample is collected from appropriate site.

Preservation-

1. Collect the sample by using plankton net and preserve in 4% formalin.
2. Take a drop of sample on the zooplankton counting chamber make a coverslip over the chamber and observe under microscope.
3. Calculate the percentage of spreads.

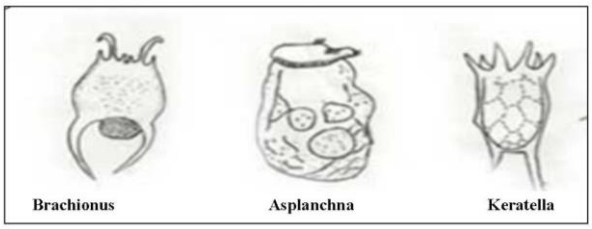
Zooplankton-

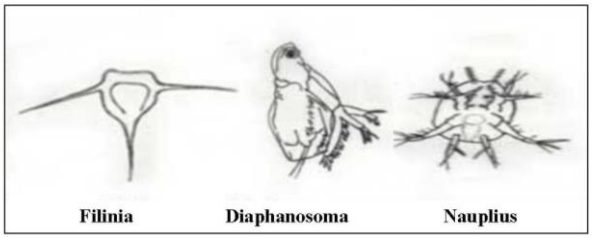
These are microscopic organism including larval stage. The zooplanktons are present in aquatic system sometime spend total life in aquatic media. They generally feed on organic matter, plant material and mostly phytoplankton.

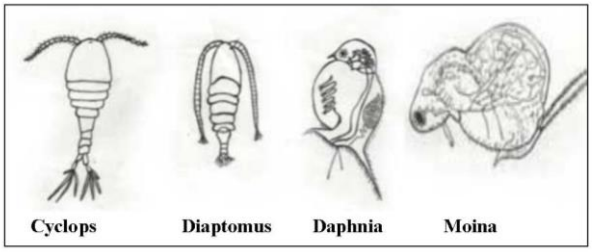
Formula –

$$\text{Percentage of species} = \frac{\text{Number of individual}}{\text{Total no. of individuals}} \times 100$$

Zooplankton Identification Key







PRACTICAL V. OTHER PRACTICALS

1. DNA isolation from plant material

Aim – To isolate plant genomic DNA.

Principle – The plant cells contain genetic material in the form of DNA and RNA. This genetic material can be easily degraded or denatured by enzymatic action of DNAase, bytreatment with cold temperature or heating at high temperature. Due to boiling DNA get denatured. This can be precipitated by treating

Material – Test tube, pipette, morter and pestle, refrigator, electronic balance, funnel etc.

Chemicals – Table salt, ice cold ethanol, distilled water, washing liquid etc.

Sample collection – Plant material: fresh or frozen peas.

Procedure –

1. Prepare hypotonic solution by dissolving 800 mg in 90 ml distilled water. To this add 10 ml dilute lipid detergent and mix gently.
2. Take peas and crush them in morter and pestle. Mix the pulp into a beaker containing salty washing solution.
3. Stand beaker in a water bath at 60^0C for 30 min. Cold the mixture by placing the beaker in an ice water bath for 10 minutes and stir frequently.
4. Filter the mixture into another beaker through four layered muscling cloth or centrifuge if possible.
5. Take 5 ml extract in a test tube. Add ice cold ethanol to this tube slowly and carefully by inclining the test tube. It forms a layer of ethanol on the top of the pea extract.
6. Leave the tubes undisturbed for few minutes. The nucleic acid DNA will be precipitate into upper ethanol layer as white coloured precipitate.
7. Remove the precipitate and isolate the DNA from RNA by electrophoresis if needed.

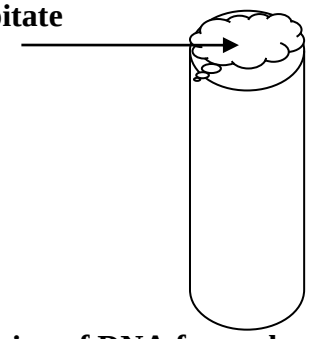


Figure: Isolation of DNA from plant material

2. DNA isolation from animal material

Aim: To isolate animal genomic DNA.

Principle: The animal cells contain genetic material in the form of DNA. The extraction of DNA includes rupturing of tissues, cell membrane, nuclear membrane followed by deproteinization and finally precipitetion of the nucleic acid by using ethyl alcohol. The process of isolation of DNA occurs in step by step manner and each step the reagents help in the isolation.

Materials: Tissue homogenizer, test tubes, auto pipette set, pasteur pipette, measuring cylinder, incubator, microcentrifuge tubes, electronic balance, DNA spooling glass rod etc.

Chemicals: Phosphate buffer solution (PBS), Proteinase K, phenol, chloroform, isoamyl alcohol, ethenol, lysis buffer (1 M tris, 5 M NaCl, 0.5 M EDTA,10% SDS dissolve in 500ml distilled water)

Procedure:

1. Prepare cell suspension in eppendroff tube with mincining in distilled water. Centrifuge for a minute at 1000 rpm.
2. Respended the cell pellet in 100 µl cold PBS and mix by pipetting up and down repeatedly.
3. Add 100 µl of cell lysis buffer and 2 µl of 20 mg/ml proteinase K mix it by vortexing.
4. Incubate at 56^0C water bath for 1 hour and vortex.
5. Add an equal volume of phenol/chloroform/isoamyl alcohol (25:24;1) mix well.
6. Spin at max. speed for 5 minutes, carefully transfer the upper aqueous layer to fresh eppendroff tube.
7. To precipitate DNA add 1 ml of 100% ethanol (room temp.), close the tubes and invert untill DNA precipitate forms.

3. Study of activity of salivary amylase under optimum conditions.

Aim- Study of activity of salivary amylase under optimum conditions.

Principle- The salivary amylase is starch digestive enzyme which broken down polyaccharide into dextrins and finally into disaccharides. Iodine is used as indicator to determine the action of salivary amylase. The iodine turns to brown or red colour when starch is broken up to dextrin and it becomes pale brown or yellow when reaction is completed.

Extraction of Saliva – Clean the teeth, gargle with a mild antiseptic and rinse the mouth thoroughly with water. Hold a piece of sour food stuff in front of the tongue. Collect the saliva under the tongue with a pipette. Filter it and use it as salivary amylase for its action.

Material- Starch solution (1%), standard iodine solution, distilled water, test tubes, hot water bath

Procedure-

As per shown in the table, label the 5 test tubes as A to E. Add original solution and other reagents as per the instructions in these test tubes. Observe the changes in colour during experiment and note down the observations.

Sr. No.	Test Tube	Test	Observation	Inference
1	Test tube- A	5 ml O. S. + I2 solution	Blue colour suspension	Starch present
2	Test tube – B	5 ml O. S. + 5 ml saliva, keep at room temp. and after some time add I2 solution	Blue colour suspension	Starch present (Starch do not digested)
3	Test tube – C	5 ml O. S. 5 ml saliva incubate in water bath at 370C for 15 minutes		
4	Test tube - D	Half of the incubated solution from test tube C -+ I2 solution few drops	No Blue colour suspension	Starch absent
5	Test tube - E	-Half of the remaining incubated solution from Test tube + 5 ml Benedict's solution boil	Red ppt	Reducing sugar present

Result –

1. In test tube B Salivary amylase does not act on starch at room temperature which is indicated ase colour solution
2. In test tube D – starch is digested into reducing sugar at 370c by the action of salivary amylase hence the blue colour is disappeared
3. In the test tube E – reduced sugars are present and confirmed by appearance of red ppt.

4. Effect of Temperature on activity of salivary amylase.

Aim – Study of effect of temperature on salivary amylase.

Principle – The salivary amylase is starch digesting enzyme found in saliva. The activity of salivary amylase is optimum at specific temperature. At very low and very high temperature the enzyme denatures and losts its activity. The effect of temperature can be studied by keeping temperture variation during the activity.

Material –Starch solution (1%), standard iodine solution, distilled water, test tubes, hot water bath, etc

Procedure-

As per shown in the table, label the 4 tubes as A to D. Add 5 ml original solution and 5 ml saliva solution in these test tubes. Keep the test tubes at 4, 20 37 and 50^0C and observe the change in colour and note down the observations. Wright the conclusion based on temperature specific action of salivary amylase.

Sr. No.	Test tube	Temp.	Observation	Inference
1	Test tube A	4^0C	Blue coloured suspension	No enzyme action
2	Test tube B	20^0C	Blue coloured suspension	No enzyme action
3	Test tube C	37^0C	Blue coloured disappeared	Enzyme action
4	Test tube D	50^0C	Blue coloured suspension	No enzyme action

Result –

1. In test tube A, B and D salivary amylase does not act on starch at 4, 20 and 50 ^{0}C temperature which is indicated by blue colour of solution.
2. In test tube C starch is digested into reducing sugars at 37^0C by the action of salivary amylase hence the blue colour disappeared.

5. Effect of pH on activity of salivary amylase.

Aim – Study of effect of pH on activity of salivary amylase

Principle –The salivary amylase is starch digesting enzyme found in saliva. The activity of salivary amylase is optimum at specific pH. At very low and very high pH the enzyme denatures and lost its activity. The effect of pH can be studied by keeping pH variation during the activity.

Material- Starch solution (1%), Standard iodine solution, buffers of different pH, distilled water, test tubes, hot water bath, etc.

Procedure –

As per shown in the table, label the 4 tubes as A to D. Add 5 ml original solution and 5 ml saliva solution in these test tubes. Add buffer solution of different pH in these test tubes as shown in the table. Keep the test tubes at 37^0C and observe the change in colour and note down the observations. Wright the conclusion based on pH specific action of salivary amylase.

Sr. No.	Test tube	pH	Observation	Inference
1	Test tube A	2.0	Blue coloured suspension	No enzyme action
2	Test tube B	5.0	Blue coloured suspension	No enzyme action
3	Test tube C	7.0	Blue coloured disappeared	Enzyme action
4	Test tube D	10.0	Blue coloured suspension	No enzyme action

Result –

1. In test tube A, B and D salivary amylase does not act on starch at 2, 5 and 10 pH which is indicated by blue colour of solution.
2. In test tube C starch is digested into reducing sugars at 7.0 pH and 37^0C by the action of salivary amylase hence the blue colour disappeared.

6. Effect of salinity on activity of salivary amylase.

Aim - Effect of salinity on activity of salivary amylase.

Principle - The salivary amylase is starch digesting enzyme found in saliva. The activity of salivary amylase is optimum at specific salinity in digestive tract. At very low and very high salinity the enzyme denatures and lost its activity. The effect of salinity can be studied by keeping salinity variation during the activity.

Material - Starch solution (1%), Standard iodine solution, saline solution of different salinity, distilled water, test tubes, hot water bath, etc.

Procedure –

As per shown in the table, label the 4 tubes as A to D. Add 5 ml original solution and 5 ml saliva solution in these test tubes. Add saline solution of different salinity in these test tubes as shown in the table. Keep the test tubes at 37^0C and observe the change in colour and note down the observations. Wright the conclusion based on salinity specific action of salivary amylase.

Sr. No.	Test tube	Salinity (NaCl)	Observation	Inference
1	Test tube A	0.2	Blue coloured suspension	No enzyme action
2	Test tube B	0.5	Blue coloured suspension	No enzyme action
3	Test tube C	0.9	Blue coloured disappeared	Enzyme action
4	Test tube D	2.0	Blue coloured suspension	No enzyme action

Result –

1. In test tube A, B and D salivary amylase does not act on starch at 0.2,0. 5 and 2.0 salinity which is indicated by blue colour of solution.
2. In test tube C starch is digested into reducing sugars at 0.9 salininity and 37^0C by the action of salivary amylase hence the blue colour disappeared.

7. Abnormal constituents of Urine and pathological significance.

A) Preliminary Tests

Sr. No.	Test	Observation	Inference
1	Sample + water Heat II) Take few ml in test tube and observe the colour	Coagulation of Sample Milky white appearence	Protein present Protein present
2	Take few ml in test tube and observe the colour	Clear solution appears	Glucose present
3	Take few ml in test tube and observe the colour	Yellow – greenish colour appearance	Bile present
4	Take few ml in test tube and observe the colour	Reddish appearence	Blood present

B) Tests for Albumen

Sr. No.	Test	Observation	Inference
1	**Xanthoprotein test** Few ml. sample + 5 ml. conc. HNO3. Boil the solution carefully. Cool and add 40% NaOH slowly	Yellow colour appears and changes to red	Albumen present
2	**Boiling Test** Urine samle few ml. Boil the content over spirit lamp	Turbidity appears	Albumen present
3	Few ml. sample + few ml 10% NaOH – heat gently + 2 drops of CuSO4	Reddish violet colour	Albumen present

C) Tests for Glucose

Sr. No.	Test	Observation	Inference
1	**Benedict's Test-**5ml of Benedict's solution + Urine sample few ml. Keep the test tube in water bath for 10 min. at 37^0C	Brown colour appears	Glucose present
2	**Barford's Test-** 3 ml of sample + 3 ml of Barford's reagent. Boil for 2 min. and allow to stand	Red ppt appears	Glucose present
3	**Fehling's test**- Boil 5 ml of Fehlling's solution, neither colour norprecipitate appears. Add 1 ml sample solution and boil again.	Yellow or brick red ppt	Glucose present

D) Test for Bile

Sr. No.	Test	Observation	Inference
1	**Hay's Test-** Take few ml sample of urine in test tube and sprinkle sulphur powder on the surface	Sulphur powder falls through the surface to bottom	Bile Present
2	5 ml of urine sample + equal volume of conc. HNO3	Various coloured rings develop	Bile Present
3	5 ml of urine sample + 2 drops of methylene blue	Green colour develops	Bile Present

E) Test for Blood

Sr. No.	Test	Observation	Inference
1	**Benzidine test-** Take knife point of benzidine in a dry test tube + 5 ml acetic acid + H2O2 few ml. mix well and add few ml of urine sample	Deep blue or green colour	Blood present
2	**Microscopic Test-** Place a drop of urine sample on a slide and observe under microscope	RBCs are observed	Blood present

8. Paper Chromatography

Aim – To separate amino acids from solution.

Principle – Paper chromatography is a very simple chromatographic method used to separate amino acids, sugars and other substances from solution. The solution has two immiscible phases, one stationary and other mobile. The mobile phase moves over stationary phase and the component of mixture migrate at different ratedue to their more or less affinity towards the stationary or mobile state. Those having more mobile affinity migrate faster than those having more affinity towards the stationary phase. The distribution of components (solute molecules) of a mixture (solution) can be visualised in the form of widely separated coloured spots or bands.

Requirements – Cellulose paper, capillary, glass jar, holder, solution containing different amino acids, solvent system, colouring agent etc

Solvent system –

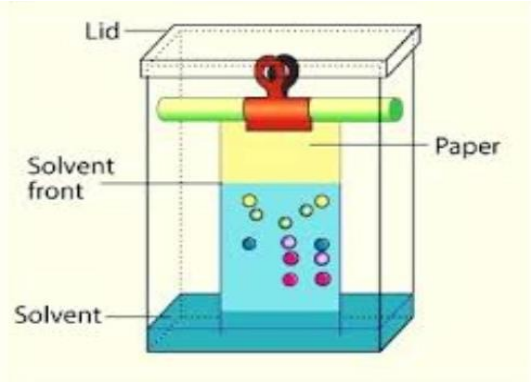
1. Butanol + acetic acid + DW = 40 + 10 + 50 = Aminoacids
2. Butttanol + pyridine +DW = 50 + 28 + 22 = Mono or disaccharides
3. Chloroform + petroleum ether = 4 + 96 = plant pigments

Procedure –

1. Take cellulose paper and make thin line at about 1.5cm from any marginal end.
2. Take spot of amino acids on the marginal line with the hepl of capillary and air dry every time.
3. Put the paper as the paper is dipped only in solvent below the marginal line. Hold the paper with holder and the system for running up to 20 to 25 minutes.
4. Remove the paper dry it in hot lamp or air dry it.
5. Colour developing solution of ninhydrin reagent is sprayed.
6. The separated spot of amino acids are seen.

Calculation – Rf = retardation factor

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$



Appendix

- **Preparation of Chemical Solutions**

1. Preparation of 1% solution – Dissolve 1 gram of chemical in 100 ml distilled water

Eg.a) 1% of NaCl – Dissolve 1 gm of NaCl in 100 ml distilled water

b) 0.9% NaCl - Dissolve 0.9 gm of NaCl in 100 ml distilled water

2. Preparation of Normal Soluttion – Dissolve gram equivalent weight in 1000ml distilled water.

Eg.

a) 1 N of NaOH - Molecular wt. of NaOH is 40 gm (23+16+1=40 Valency =1) So dissolve 40 gm of NaOH in 1000 ml Distilled water

b) 0.1 N of NaOH - dissolve 4 gm of NaOH in 1000 ml Distilled water

c) 10 N of NaOH - dissolve 40 gm of NaOH in 1000 ml Distilled water

d) 1 N H2SO4 - Molecular wt. of H2SO4 is 98 gm (2+32+64=98 Valency =2) So dissolve 98/2=49 gm of H2SO4 in 1000 ml Distilled water

3. Preparation of Molar Solution –Dissolve gram molecular mass in 1000 ml of distilled water

Eg

a) 1 M of NaOH - Molecular wt. of NaOH is 40 gm (23+16+1=40) So dissolve 40 gm of NaOH in 1000 ml Distilled water

b) 0.1 M of NaOH - dissolve 4 gm of NaOH in 1000 ml Distilled water

c)10 M of NaOH - dissolve 40 gm of NaOH in 1000 ml Distilled water

d) 1 M H2SO4 - Molecular wt. of H2SO4 is 98 gm (2+32+64=98) So dissolve 98 gm of H2SO4 in 1000 ml Distilled water

- **Gradation of Alcohols**

Alcohol Grade	**Absolute alcohol/Rectified spirit**	**Distilled Water**
30%	30/33	70/67
50%	50/55	50/45
70%	70/77	30/23
90%	90/100	10/00
100%	100Absolute alcohol	00

- **Isotonic Saline solution of NaCl for different groups**

Mammals- 0.9%

Birds – 0.75%

Reptiles- 0.8%

Amphibians -0.64%

Insects – 0.6-0.8%

Other invertebrates - 0.75%

- **Separation of Serum**

Collect the blood in tube and allow to cloat room temperature. After 15-20 minutes transfer separated supernatant (serum) in another test tube. Centrifuge at 1500rpm for 10 minutes. Transfer the supernatant in clean dry tube store at 4^0C till it is used.

- **Separation of Plasma**

Take 5-7 blood in a tube containing anticoagulating, mix by shaking for some time and centrifuge at 1500rpm for 10 min. Transfer the liquid layer of plasma to a clean and dry test tubes and store at 4^0C till it is used.

- **Preparatiom of Sodium Phosphate buffer solution (100 ml)**

pH	**Volume of 1 M of Na2HPO4(ml)**	**Volume of 1 M of NaH2PO4(ml)**
5.8	8	92
6.0	12	88
6.2	17.8	82.2
6.4	25.6	74.6
6.6	35.2	64.8
6.8	46.4	53.8
7.0	57.8	42.4
7.2	68.4	31.6
7.4	77.4	22.6
7.6	84.6	35.6
7.8	89.6	10.4
8.0	93.2	6.8

- **Chemical recipes**

Alcian blue, pH 1 – Dissolve 1 gm of alcian blue (8GX) in 100 ml 0.1 M HCl solution.

Alcian blue, pH 2.5 – Dissolve 1 gm of alcian blue (8GX) in 100 ml 3% acetic acid solution.

Barfoed's reagent – Dissolve 13.3 gm of neutral crystalline copper acetate in 200 ml of distilled water, filter and add 1.8 ml of Glacial acetic acid.

Benedicts quantitative reagent – Dissolve Sodium citrate (200gm), Anhydrous sodium carbonate(75gm), Potassium thiocyanate(125gm) in 600ml distilled water. Heat the solution to dissolve all contets, cool at room temp. and filter then add Copper sulphate (18gm), 5% Potassium ferrocyanide, aqueous (5ml). Mix well make the solution upto 1 liter by adding required quntity of distilled water.

Buffer solution A – Dissolve 16.9 gm of ammonium chloride in 143 ml of conc. ammonium hydroxide

Buffer solution B – Dissolve 1.179 gm of sodium EDTA and 0.750 gm of MgSO4 in 50 ml distilled water. Mix A and B solution dilute upto 250 ml with distilled water.

Cholesterol Reagent – Mix 5.6g of 2,5 dimethyl benzene sulphonic acid in 200ml of Glacial acetic acid and 300ml of acetic anhydride. Store in amber coloured bottle at room temperature; stable for more than one year.

Iodine solution – 1.27gm of Iodine crystals + 3 gm Potassium iodide mix and dissolve in 100 ml distilled water.

Leishman stain – Dissolve 0.15 gm of Leishman stain powder in 100 ml of methyl alcohol.

Liquid washing solution for DNA isolation – Dissolve 0.7 gm of NaCl in 100 DW, add pinch of washing powder or SDS.

Methyl orange indicator – Dissolve 0.5 gm methyl orange in 100ml distilled water

Molisch Reagent – 10% alcoholic alpha napthol solution

Nuclear fast red – Aluminium sulphate – 5gm, nuclear fast red – 0.1 gm, distilled water 100ml.

Phenolpthalein indicator – 5 gm phenolpthalein in 100 ml of 95% ethanol.

Seliwanoffs reagent – Dissolve 50 gm of conc. HCl and dilute upto 100 ml.

Standard sodium carbonate – 0.0454N prepared by dissolving 0.2gm of sodium carbonate in 250ml of CO2 free dissolved water

Sudan III – 10% Sudan III stain in 90ml alcohole

Vanillin reagent – 0.64g vanillin in 350ml distilled water, Orthophosphoric acid 600ml, Distilled water 50, Keep this bottle in Dark place for maturation at least 30 days.

Wrinkler's solution A – 40 gm of MnSO4 in 100 ml of distiled water.

Wrinkler's solution B – 30 gm of NaOH + 10 gm of KI in 100 ml distilled water.

------- **Notes** -------

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