University of Mysore

YUVARAJA'S COLLEGE

(Autonomous)

Mysuru - 570005

FIVE YEAR INTEGRATED M.Sc.

MOLECULAR BIOLOGY

A

E - BOOK

FOR

LAB - IX

MACROMOLECULES AND ANIMAL PHYSIOLOGY

Editor

Dr. H.B. MAHESHA

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Department of Sericulture

Yuvaraja's College, Mysuru-570005

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Editor acknowledges all scientists who have pioneered and developed methods described in this e-book

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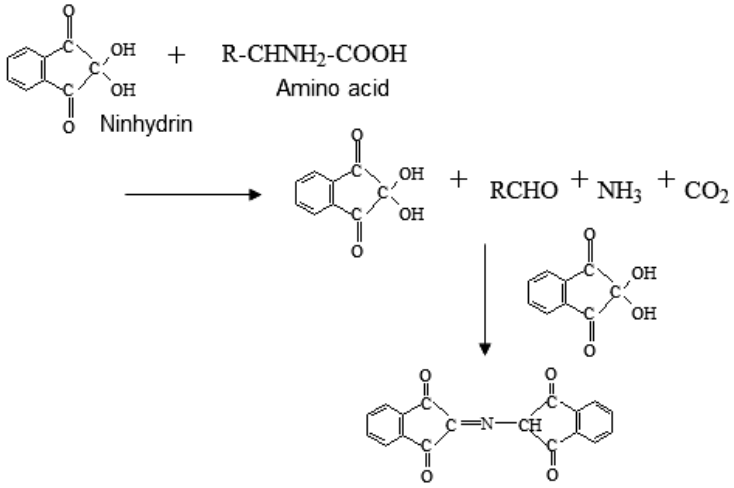
Experiment No. 1: Estimation of Amino Acid by Ninhydrin Method.

Introduction: The amino acids are colourless ionic compounds that form the basic building blocks of protein. Apart from being bound as proteins, amino acids also exist in the free form in many tissues and are known as free amino acids. They are mostly water soluble in nature. Very often in plants during disease conditions, the free amino acid composition exhibits a change hence, the measurement of the total free amino acids gives the physiological and health status of the plants.

Aim: To estimate amino acid concentration by ninhydrin reaction

Principle: Ninhydrin, a powerful oxidizing agent, decarboxylates the alpha-amino acids and yields an intensely coloured bluish purple product is colorimetrically measured at 570nm.

Reaction:



Ruhemann's Purple

Reagents Required:

1. **Ninhydrin:** Dissolve 0.8 g stanious chloride ($SnCl_2.2H_2O$) in 500 mL of 0.2 M citrate buffer (pH 5.0). Add this solution to 20 g of ninhydrn in 500 mL of methyl cellosolve (2 methoxyethanol).
1. **0.2 M Citrate Buffer pH 5.0:**

   Solution A: 0.2 M Citric acid

   Solution B: 0.2 M Sodium citrate

   Mix 20.5 mL of solution A with 29.5 mL of solution B and check pH.
2. **Diluent Solvent:** Mix equal volumes of water and n-propanol and use.

3. **Standard Amino Acid:** Dissolve 50 mg leucine in 50 mL of distilled water in a volumetric flask. Take 10 mL of this stock standard and dilute to 100 mL in another flask for working standard solution.

Apparatus and Glass wares required: Test tubes, Pipettes, Colorimeter, *etc*.,

Procedure:

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard amino acid in to the series of labeled test tubes.
2. Pipette out 1 mL of the given sample in another test tube.
3. Make up the volume to 1 mL in all the test tubes. A tube with 1 mL of distilled water serves as the blank.
4. Now add 3 mL of ninhydrin reagent to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes.
6. Then cover all the test tubes with marble / paper.
7. Place all test tubes in boiling water bath for 15 minutes.
8. Now cool the contents in cold water and add 5 mL of diluent solvent to each test tube and mix well.
9. Now record the absorbance at 570 nm using a Colorimeter.
10. Then plot the standard curve by taking concentration of amino acid along X-axis and absorbance at 570 nm along Y-axis.
11. Then from this standard curve calculate the concentration of amino acid in the given sample.

Observations and Calculations

Volume of standard amino acid (mL)	Volume of distilled water (mL)	Concentration of amino acid (µg)	Volume of ninhydrin reagent (mL)	Incubate in boiling water bath for 15 min and cool to room temperature	Volume of Solvent (mL)	Incubate at Room Temperature for 10 min	A_{570}
0.0	1.0	00	1		5		0.00
0.2	0.8	20	1		5		
0.4	0.6	40	1		5		
0.6	0.4	60	1		5		
0.8	0.2	80	1		5		
1.0	0.0	100	1		5		
1.0 UK	0.0	To be estimated	1		5		

Result: The given unknown sample contains ----µg amino acid/mL.

Experiment No. 2: Estimation of Protein by Biuret Method.

Aim: To estimate the protein using Biuret method.

Principle: The –CO-NH- bond (peptide) in polypeptide chain reacts with copper sulphate in an alkaline medium to give a purple colour which can be measured at 540 nm.

Reagents Required:

1. Biuret Reagent: Dissolve 3 g of copper sulphate ($CuSO_4.5H_2O$) and 9 g of sodium potassium tartarate in 500 mL of 0.2 mol/liter sodium hydroxide; add 5 g of potassium iodide and make up to 1 liter with 0.2 mol/liter sodium hydroxide.

2. Protein Standard: 5 mg BSA/mL.

Apparatus and Glass wares required: Test tubes, Pipettes, Colorimeter, *etc*.,

Procedure:

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard in to the series of labeled test tubes.
2. Pipette out 1 mL of the given sample in another test tube.
3. Make up the volume to 1 mL in all the test tubes. A tube with 1 mL of distilled water serves as the blank.
4. Now add 3 mL of Biuret reagent to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and warm at 37 ºC for 10 min.
6. Now cool the contents and record the absorbance at 540 nm against blank.
7. Then plot the standard curve by taking concentration of protein along X-axis and absorbance at 540 nm along Y-axis.
8. Then from this standard curve calculate the concentration of protein in the given sample.

Observations and Calculations

Volume of standard BSA (mL)	Volume of distilled water (mL)	Concentration of Protein (mg)	Volume of Biuret reagent (mL)	Incubate At 37ºC for 10 Min & Cool	A_{540}
0.0	1.0	00	3		0.00
0.2	0.8	1	3		
0.4	0.6	2	3		
0.6	0.4	3	3		
0.8	0.2	4	3		
1.0	0.0	5	3		
1.0 UK	0.0	To be estimated	3		

Result: The given unknown sample contains ----mg protein/mL.

Experiment No. 3: Estimation of Protein by Lowry's Method.

Aim: To estimate the protein using Lowry's method.

Principle: The –CO-NH- bond (peptide) in polypeptide chain reacts with copper sulphate in an alkaline medium to give a blue colored complex. In addition, tyrosine and tryptophan residues of protein cause reduction of the phosphomolybdate and phosphotungstate components of the Folin-Ciocalteau reagent to give bluish products which contribute towards enhancing the sensitivity of this method.

Reagents Required:

1. **Reagent A:** 2% sodium carbonate in 0.1 N sodium hydroxide.
2. **Reagent B:** 0.5% copper sulphate ($CuSO_4.5H_2O$) in 1% potassium sodium tartarate. Prepare fresh by mixing stock solutions.
3. **Alkaline copper solution (Reagent C):** Mix 50mL of reagent A and 1 mL of reagent B prior to use.
4. **Diluted Folin's reagent (Reagent D):** Dilute Folin-Ciocalteau reagent with an equal volume of 0.1 N NaOH
5. **Standard:** Dissolve 50mg BSA in 50mL of distilled water in a volumetric flask. Take 10mL of this stock standard and dilute to 50 mL in another flask for working standard solution. One mL of this solution contains 200 µg proteins.

Apparatus and Glass wares required: Test tubes, Pipettes, Colorimeter, *etc*.,

Procedure:

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard in to the series of labeled test tubes.
2. Pipette out 1 mL of the sample in another test tube.
3. Make up the volume to 1 mL in all the test tubes. A tube with 1 mL of distilled water serves as the blank.
4. Now add 5 mL of reagent C to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and allow to stand for 10 min.
6. Then add 0.5 mL of reagent D rapidly with immediate mixing well and incubate at room temperature in the dark for 30 min.
7. Now record the absorbance at 660 nm against blank.
8. Then plot the standard curve by taking concentration of protein along X-axis and absorbance at 660 nm along Y-axis.
9. Then from this standard curve calculate the concentration of protein in the given sample.

Observations and Calculations

Volume of standard BSA(mL)	Volume of distilled water (mL)	Concentration of Protein (µg)	Volume of reagent C (mL)	Incubate at Room Temp for 10 min	Volume of reagent D (mL)	Incubate at dark room temp. for 30 min	A660
0.0	1.0	00	5		0.5		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		
1.0 Sample	0.0	To be estimated	5		0.5		

Result: The given unknown sample contains ----µg protein/mL.

Experiment No. 4: Estimation of Protein by Bradford's Method.

Aim: To estimate the protein using Bradford's method.

Principle: The assay is based on the ability of protein to bind coomassie brilliant blue G250 and form a complex whose *extinction coefficient* is much greater than that of the free dye.

Reagents Required:

1. Dye Concentrate: Dissolve 100 mg of coomassie brilliant blue G250 in 50 mL of 95 % ethanol. Add 100 mL of concentrated orthophosphoric acid. Add distilled water to a final volume of 200 mL. store refrigerated in amber bottles; the solution is stable at least 6 months.

- Mix 1 volume of concentrated dye solution with 4 volumes of distilled water for use. Filter with Whatman No. 1 paper.

2. Protein Standard: 100 µg/mL in PBS.

Apparatus and Glass wares required: Test tubes, Pipettes, Colorimeter, *etc*.,

Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard in to the series of labeled test tubes. Also, Pipette out 1 mL of the given sample in another test tube.
2. Make up the volume to 1 mL in all the test tubes with PBS. A tube with 1 mL of distilled water serves as the blank.
3. Now add 5 mL of diluted dye solution to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
4. Mix the contents of the tubes by vortexing / shaking the tubes and allow the colour to develop for at least 5 min but not more than 30 min. The red dye turns blue when it binds protein. Now record the absorbance at 595 nm against blank.
5. Then plot the standard curve by taking concentration of protein along X-axis and absorbance at 595 nm along Y-axis.
6. Then from this standard curve calculate the concentration of protein in the given sample.

Observations and Calculations

Volume of standard BSA (mL)	Volume of distilled water (mL)	Concentration of Protein (µg)	Volume of Biuret reagent (mL)	Allow to develop colour from 5 30 min	A_{595}
0.0	1.0	00	5		0.00
0.2	0.8	1	5		
0.4	0.6	2	5		
0.6	0.4	3	5		
0.8	0.2	4	5		
1.0	0.0	5	5		
1.0 Sample	0.0	To be estimated	5		

Result: The given unknown sample contains ----µg protein/mL.

Experiment No. 5: Estimation of Sugar by Titrimetric (HAGEDORN-JENSON) Method.

Aim: To estimate reducing sugars by Hagedorn and Jensen method.

Principle: The potassium ferricyanide is reduced to potassium ferrocynide when heated with alkaline solution by reducing sugars. The ferrocynide formed is precipitated as potassium zinc salt. The remaining ferricyanide is determined from the amount of iodine liberated.

The principle reactions are

$$2K_3[Fe(CN)_6] + 2KI \rightarrow 2K_4[Fe(CN)_6] + I_2$$

$$2K_4[Fe(CN)_6] + ZnSO_4 \rightarrow K_2Zn[Fe(CN)_6] + 3K_2SO_4$$

Reagents Required

1. **Iodide sulfate chloride solution:** Dissolve 25 g of $ZnSO_4$ and 12.5 g of $NaCl_2$ in 500 mL of water. To this, add 12.5 g of KI on the day of the experiment.
2. **3% acetic acid.**
3. **Potassium ferricyanide solution:** Dissolve 0.825 g of $K_3[Fe(CN)_6]$ and5.3 g of anhydrous sodium carbonate in 500 mL water. Store it in a dark bottle.
4. **Preparation of standard sugar solution:** Dissolve 100 mg of standard sugar in 100 mL of water. Take 10 mL of stock and make it up to 100 mL.
5. **0.1N $Na_2S_2O_3$solution:** 12.4 g of sodium thiosulfate in 500 mL of distilled water. Take 5 mL of stock and make it up to 100 mL for 0.005 N $Na_2S_2O_3$solution.

6. **Starch indicator:** 1 g of starch in 100 mL of H_2O and 5 g of NaCl.

Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard in to the series of labeled test tubes.
2. Pipette out 1 mL of the given sample in another test tube.
3. Make up the volume to 2 mL in all the test tubes. A tube with 2 mL of distilled water serves as the blank.
4. Now add 2 mL of potassium ferricyanide reagent to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and incubate in a boiling water bath for 15 min.
6. Then add 3 mL of Iodine sulfate chloride solution, mix well and add 2 mL of 3% acetic acid.
7. Now add few drops of starch solution as indicator and totrate against sodium thiosulphate. Titrate until the blue color disappears. Note the readings.
8. Then plot the standard curve by taking concentration of glucose along X-axis and difference of burette readings along Y-axis.
9. From this standard curve calculate the concentration of glucose in the given sample.

Observations and Calculations

Volume of standard glucose (mL)	Volume of distilled water (mL)	Concentration of creatinine (µg)	Potassium ferricyanide reagent (ML)	Boil in boiling water bath for 15 Min & Coo	Iodine sulfate chloride solution (mL)	3% acetic acid (mL)	Volume of sodium thiosulphate used (mL)	Difference in burette reading
0.0	2.0	00	2		3	2		
0.2	1.8	200	2		3	2		
0.4	1.6	400	2		3	2		
0.6	1.4	600	2		3	2		
0.8	1.2	800	2		3	2		
1.0	1.0	100	2		3	2		
1.0 Sample	1.0	To be estimated	2		3	2		

Result: The given sample contains _____ µg of glucose/mL.

Experiment No. 6: Extraction of Starch from Potato.

Aim: To isolate the starch from the given potato sample.

Principle: Starch is an important polysaccharide found in plant sources. The microscopic appearance of starch is in the form of granules. It is typical for the individual starch granules. They differ in size depending on the source from which they were isolated. Starch is insoluble in water and rapidly settles at the bottom and can be collected by decanting the supernatant.

Materials Required: Potato, Muslin Cloth, Watch Glass, Mortar and Pestle, Test Tube, Iodine solution *etc*.,

Procedure:

1. Peal a raw potato and cut into small pieces, and record the initial weight.
2. Grind them in a motor and pestle with sufficient water.
3. Collect the potato homogenate into a beaker and add enough water.
4. Then filter the homogenate through a muslin cloth to remove the particles.
5. Allow the filtrate to settle. Starch rapidly settles at the bottom. Decant the starch free supernatant carefully.
6. Wash 3-4 times and decant the supernatant. Collect the compact mass of starch and allow it to dry.
7. Record the final weight of isolated starch and calculate the yield.

Iodine Test: Take a small quantity of test solution with a drop of 1N HCl and then add two drops of iodine solution. Formation of blue colour indicates the presence of starch.

Result: The given sample contains _____ g of starch/100 g potato.

Experiment No. 7: Isolation of DNA from Plant Source.

Aim: To isolate DNA from plant tissue.

Principle: Extraction of DNA is accomplished by the rupturing of cell wall, cell and nuclear membrane followed by deproteinization and precipitation of the nucleic acid using ethanol.

Materials Required:

1. Extraction buffer: 100mM Tris-Cl buffer containing 0.15 M NaCl, 0.1MEDTA, 2.5% SDS pH 7.8.
2. Chloroform and isoamyl alcohol mixture 24:1.
3. 3M sodium acetate
4. Tris-EDTA buffer: 10 mM Tris-Cl pH 7.5, 1mM EDTA.
5. 70% ethanol, Sample, Glass wares, centrifuge, water bath *etc*.,

Procedure:

1. Grind 0.5g of the sample (mulberry leaf) material in 0.5 – 0.75 mL of extraction buffer in a mortar and pestle.
2. Transfer the homogenized sample in to a micro centrifuge tube and incubate at 65°C for 30 min.
3. After incubation centrifuge at 3000 rpm for 10 min at room temperature.
4. Collect the supernatant in to an another micro centrifuge tube. To this, add an equal amount of chloroform – isoamyl alcohol mixture and shake the contents.
5. Centrifuge the contents at 8000 rpm for 10 min at room temperature.
6. After centrifugation, carefully pipette out the upper clear aqueous phase from the coagulated protein emulsion at the interface in to a fresh tube.
7. To this, add $1/20^{th}$ volume of 3 M sodium acetate and double volume chilled ethanol and swirl gently to precipitate DNA. White fibrous DNA appears in the tube. For further precipitation incubate the tube at -20°C for 30 – 60 min.
8. Centrifuge the contents for 10 min at 8000 rpm, discard the supernatant and air dry the DNA pellet. Wash the DNA with 70 % ethanol twice.
9. Suspend the pellet in 100 µl TE buffer and store at -20° for future use.

Result: DNA is isolated from plant source, which is observed as white fibrous material.

Flow chart:

0.5 g Plant Tissue

0.5 – 0.75 mL of extraction buffer

Homogenize and incubate at 65 °C for 30 min

Centrifuge the homogenate at 3000 rpm for 10 min

Transfer the supernatant in to a fresh tube

Add equal volume of Chloroform-isoamyl alcohol mixture, mix and centrifuge at 8000 rpm for 10 min

Collect the upper clear supernatant in to fresh tube and add 1/20th volume of sodium acetate and double volume of chilled ethanol and swirl gently

Fibrous White DNA appears

Keep the tube at -20°C for 30-60 min for further precipitation

Centrifuge 8000 rpm for 5 min; discard the supernatant and air dry the pellet

Wash in 70% ethanol (1-2 times) and suspend in 100 µl TE buffer and preserve for future use

Experiment No. 8: Isolation of DNA from Animal Source.

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Aim: To isolate DNA from chicken liver.

Principle: Extraction of DNA is accomplished by the rupturing of cell wall, cell and nuclear membrane followed by deproteinization and precipitation of the nucleic acid using ethanol.

Materials Required:

1. **Extraction buffer:** 100mM Tris-Cl buffer containing 0.15 M NaCl, 0.1MEDTA, 2.5% SDS pH 7.8.
2. **Chloroform and isoamyl alcohol mixture 24:1.**
3. **3M sodium acetate**
4. **Tris-EDTA buffer:** 10 mM Tris-Cl pH 7.5, 1mM EDTA.
5. Absolute alcohol, 70% ethanol, Sample, Glass wares, centrifuge, water bath *etc*.,

Procedure:

1. Grind 0.5g of the silkworm midgut (or any tissue) in 0.5 – 0.75 mL of extraction buffer in a mortar and pestle.
2. Transfer the homogenized sample in to a micro centrifuge tube and incubate at 65 °C for 30 min.
3. After incubation centrifuge at 3000 rpm for 10 min at room temperature.
4. Collect the supernatant in to another micro centrifuge tube. To this, add an equal amount of chloroform – isoamyl alcohol mixture and shake the contents.
5. Centrifuge the contents at 8000 rpm for 10 min at room temperature.
6. After centrifugation, carefully pipette out the upper clear aqueous phase from the coagulated protein emulsion at the interface in to a fresh tube.
7. To this, add 1/20th volume of 3 M sodium acetate and double volume chilled absolute ethanol and swirl gently to precipitate DNA. White fibrous DNA appears in the tube. For further precipitation incubate the tube at -20°C for 30 – 60 min.
8. Centrifuge the contents for 10 min at 8000 rpm, discard the supernatant and air dry the DNA pellet. Wash the DNA with 70 % ethanol twice.
9. Suspend the pellet in 100 µl TE buffer and store at -20° for future use.

Result: DNA is isolated from plant source, which is observed as white fibrous material.

Flow chart:

0.5 g Chicken Liver

0.5 – 0.75 mL of extraction buffer

Homogenize and incubate at 65°C for 30 min

Centrifuge the homogenate at 3000 rpm for 10 min

Transfer the supernatant in to a fresh tube

Add equal volume of Chloroform-isoamyl alcohol mixture, mix and centrifuge at 8000 rpm for 10 min

Collect the upper clear supernatant in to fresh tube and add 1/20th volume of sodium acetate and double volume of chilled absolute ethanol and swirl gently

Fibrous White DNA appears

Keep the tube at -20°C for 30-60 min for further precipitation

Centrifuge 8000 rpm for 5 min; discard the supernatant and air dry the pellet

Wash in 70% ethanol (1-2 times) and suspend in 100 µl TE buffer and preserve for future use

Experiment No. 9: Estimation of DNA by Diphenylamine Method.

Aim: To estimate the concentration of DNA by diphenylamine reaction.

Principle: This is a general reaction given by deoxypentoses. The 2-deoxyribose of DNA, in the presence of acid, is converted to ω-hydroxilevulinic aldehyde, which reacts with diphenylamine to form a blue coloured complex, which can be read at 595 nm.

Requirements:

1. Standard DNA solution- Dissolve calf thymus DNA/ any available standard DNA (200µg/mL) in 1N perchloric acid/buffered saline.
2. Diphenylamine solution- Dissolve 1g of diphenylamine in 100 mL of glacial acetic acid and 2.5 mL of concentrated H_2SO_4. This solution must be prepared fresh
3. Buffered Saline- 0.5 mol/liter NaCl; 0.015 mol/liter sodium citrate, pH 7.

Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard in to the series of labeled test tubes.
2. Pipette out 1 mL of the given sample in another test tube.
3. Make up the volume to 1 mL in all the test tubes. A tube with 1 mL of distilled water serves as the blank.
4. Now add 2 mL of DPA reagent to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and incubate on a boiling water bath for 10 min.
6. Then cool the contents and record the absorbance at 595 nm against blank.
7. Then plot the standard curve by taking concentration of DNA along X-axis and absorbance at 595 nm along Y-axis.
8. Then from this standard curve calculate the concentration of DNA in the given sample.

Observations and Calculations

Volume of standard (200 µg/mL) DNA (mL)	Volume of distilled water (mL)	Concentration of DNA (µg)	Volume of DPA reagent (mL)	Incubate in boiling water bath for 10 Min & Cool	A595
0.0	1.0	00	2		0.00
0.2	0.8	40	2		
0.4	0.6	80	2		
0.6	0.4	120	2		
0.8	0.2	160	2		
1.0	0.0	200	2		
1.0 Unknown	0.0	To be Estimated	2		

Result: The given unknown sample contains ----µg DNA/mL.

Experiment No. 10: Isolation of RNA.

Aim: To isolate RNA from yeast.

Principle: Total yeast RNA is obtained by extracting a whole cell homogenate with phenol. The concentrated solution of phenol disrupts hydrogen bonding in the macromolecules, causing denaturation of protein. The turbid suspension is centrifuged and two phases appear; the lower phenol phase contains DNA, and the upper aqueous phase contains carbohydrates and RNA. Denaturated protein, which is present in both the phases, is removed by centrifugation. The RNA is then precipitated with alcohol.

Materials Required:

1. Dried Yeast.
2. Phenol solution: 900g/liter.
3. Potassium acetate: 200g/liter, pH 5.
4. Absolute ethanol.
5. Diethyl ether.
6. Glass wares, centrifuge, water bath *etc*.,

Procedure:

1. Suspend 0.15 g of dried yeast in 0.6 mL of warm (37ºC) water and incubate in an water bath for 15 min at 37ºC.
2. Then add 0.8 mL of concentrated phenol solution and stir the suspension mechanically for 30 min at room temperature, then centrifuge at 3000g for 15 min in cold (5ºC) to break the emulsion.
3. Carefully collect the upper aqueous layer with a Pasteur pipette and centrifuge at 10000 g for 5 min in cooling centrifuge to sediment denatured protein.
4. After centrifugation collect supernatant into a fresh tube and add $1/10^{th}$ volume of potassium acetate and two volumes of ethanol. Cool the solution in ice and leave to stand for one hr.
5. Collect the precipitate by centrifuging at 2000 g for 5 min at 5ºC.
6. Wash the RNA with ethanol: water (3:1), ethanol and finally ether; air dry and preserve for future use.

Result: RNA is isolated from yeast, which is observed as white fibrous material.

Flow chart

0.15 g yeast granules

0.6 mL of warm water and incubate at 37 ºC for 15 min

Add 0.8 mL of concentrated phenol solution and stir the suspension mechanically for 30 min at R T

Centrifuge at 3000g for 15 min in cold (5 ºC) to break the emulsion.

Carefully collect the upper aqueous layer with a Pasteur pipette and centrifuge at 10000 g for 5 min at 5 ºC

Collect supernatant into a fresh tube and add $1/10^{th}$ volume of potassium acetate and two volumes of ethanol.

Collect the precipitate by centrifuging at 2000 g for 5 min at 5 ºC.

Wash the RNA with ethanol: water (3:1), ethanol and finally ether; air dry and preserve for future use.

Experiment No. 11: Estimation of RNA by Orcinol Reaction.

Aim: To estimate the concentration of RNA by Orcinol reaction.

Principle: This is a general reaction for pentoses and depends on the formation of furfural when the pentose is heated with concentrated hydrochloric acid. Orcinol reacts with the furfural in the presence of ferric chloride as a catalyst to give a green colour, which can be measured at 665 nm.

Requirements:

1. Standard RNA solution- 200µg/mL in 1 N perchloric acid/buffered saline.
2. Orcinol Reagent- Dissolve 0.1g of ferric chloride in 100 mL of concentrated HCl and add 3.5 mL of 6% w/v Orcinol in alcohol.
3. Buffered Saline- 0.5 mol/liter NaCl; 0.015 mol/litre sodium citrate, pH 7.

Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard in to the series of labeled test tubes.
2. Pipette out 1 mL of the given sample in another test tube.
3. Make up the volume to 1 mL in all the test tubes. A tube with 1 mL of distilled water serves as the blank.
4. Now add 2 mL of Orcinol reagent to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and heat on a boiling water bath for 20 min.
6. Then cool the contents and record the absorbance at 665 nm against blank.

7. Then plot the standard curve by taking concentration of RNA along X-axis and absorbance at 665 nm along Y-axis.
8. Then from this standard curve calculate the concentration of RNA in the given sample.

Observations and Calculations

Volume of standard (200 µg/mL) RNA	Volume of distilled water (mL)	Concentration of RNA (µg)	Volume of Orcinol reagent (mL)	Incubate in boiling water bath for 20 Min & Cool	A_{665}
0.0	1.0	00	2		0.00
0.2	0.8	40	2		
0.4	0.6	80	2		
0.6	0.4	120	2		
0.8	0.2	160	2		
1.0	0.0	200	2		
1.0 Unknown	0.0	To be estimated	2		

Result: The given unknown sample contains ----µg RNA/mL.

Experiment No. 12: Qualitative Analysis of Urine for Abnormal Constituents.

Substances which are not present in easily detectable amounts in urine of normal healthy individuals but are present in the urine under certain diseased conditions are said to be *Abnormal* constituents of urine.

1. Glucose:

Benedict's Test:

Principle: Copper sulphate of Benedict's qualitative solution is reduced by reducing substances on boiling to form the coloured precipitate of cuprous oxide.

Test for Glucose:

Procedure	Observation	Inference
To about 5 mL of Benedict's reagent, add 0.5 mL of urine and boil for 2 min.	Blue color appears	Sugar Absent
	Light green precipitate appears	0.1-0.5 % 0f reducing sugar present
	Green precipitate appears	0.5 to 1.0 % of reducing sugar present
	Yellow precipitate appears	1-2 % reducing sugar present
	Brick red precipitate appears	Above 2 % reducing sugar present

Normal urine also contains a trace of glucose and glucuronates, but their amount is too small to cause reduction in Benedict's test. In *Diabetes mellitus* and in renal glycosuria, glucose is found in urine. This gives a Benedict's test positive.

2. Albumin:

a. Sulphosalicylic acid test:

Principle: Albumin, the protein, is denatured by sulphosalicylic acid a coagulation.

Procedure	Observation	Inference
Add a few drops of Sulphosalicylic acid to 2 mL of urine	Turbidity appears	Indicates the presence of Albumin

b. Heat coagulation test:
Principle: The albumin is coagulated after being heated.

Procedure	Observation	Inference
Fill 3/4th of the test tube by urine. Heat the upper 1/3rd of the test tube by a small flame.	Turbidity appears on the heated portion of the tube	Indicates the presence of Albumin

c. Heller's Nitric acid test:

Principle: Nitric acid causes precipitation of protein.

Procedure	Observation	Inference
To 3 mL of nitric acid in a tube add 3 mL of urine by the wall of the tube in such a way that the two liquids do not mix	White ring appears at the junction of the two fluids	Indicates the presence of Albumin

3. Ketone bodies:

Rothera's Test:

Principle: Acetoacetic acid forms a complex with nitroprusside in alkaline solution developing a permanganate colour.

Procedure	Observation	Inference
Saturate 5 mL of urine with Ammonium sulphate by shaking vigorously. Then add 2 drops of freshly prepared 5% solution of sodium nitroprusside and 1 mL of ammonium hydroxide. Allow it to stand in a rack for a while	A permanganate colour develops just above the layer of undissolved ammonium crystals	Indicates the presence of Ketone bodies

Experiment No. 13: Estimation of Creatinine in Urine.

Aim: To estimate creatinine in urine.

Principle: Creatinine develops an orange red colour when treated with picric acid in the presence of strong alkali. This colour is due to the formation of creatinine picrate and measured at 460 nm.

Normal adults excrete creatinine from 1-1.8 mg/day. Under heavy meat diet, wasting disease, prolonged starvation, fever *etc*., it increases.

Requirements:

1. Creatinine standard solution: Dissolve 100 mg creatinine in 100 mL of 0.1 N HCl. Working standard solution may be prepared by appropriate dilution of the stock solution.
2. Saturated picric acid solution (about 1%).
3. Sodium hydroxide solution – 0.75 N.
4. Hydrochloric acid solution – 0.1N.
5. Sodium tungstate: 10%
6. Sulfuric acid - 2/3 N

Preparation of protein free filtrate: To 1 mL urine/blood sample, add 8 mL distilled water, 0.5 mL of 2/3 N sulfuric acid and 0.5 mL of 10% sodium tungstate solution in a stoppered

centrifuge tube and mix the contents. Then centrifuge at 3000 rpm for 10 min and collect the supernatant.

Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard in to the series of labeled test tubes.
2. Pipette out 1 mL of the given sample in another test tube.
3. Make up the volume to 1 mL in all the test tubes. A tube with 1 mL of distilled water serves as the blank.
4. Now add 1.5 mL of saturated picric acid solution and 1.5 mL of 0.75 N NaOH. Mix the contents of the tubes and incubate at room temperature for 15 min.
5. Then read the absorbance at 460 nm against blank.
6. Then plot the standard curve by taking concentration of creatinine along X-axis and absorbance at 460 nm along Y-axis.
7. From this standard curve calculate the concentration of creatinine in the given sample.

Observations and Calculations

Volume of standard creatinine (mL)	Volume of distilled water (mL)	Concentration of creatinine (µg)	Volume of picric acid (mL)	Volume of NaOH (mL)	Incubate at Room Temperature for 10 Min	A460
0.0	1.0	00	1.5	1.5		0.00
0.2	0.8	200	1.5	1.5		
0.4	0.6	400	1.5	1.5		
0.6	0.4	600	1.5	1.5		
0.8	0.2	800	1.5	1.5		
1.0	0.0	1000	1.5	1.5		
1.0 UK	0.0	To be estimated	1.5	1.5		

Result: The given unknown sample contains ----µg /mL.

Experiment No. 14: Estimation of Total Titratable Acidity in Urine.

Aim: To estimate the titratable acidity in urine.

Principle: The titratable acidity of urine is expressed in terms of the mL of standard alkali necessary to bring the urine from its original pH to phenolphthalein end point pH 8.5 or 9 by the use of the phenolphthalein indicator.

The total acid excreted per day under normal conditions by normal adults varies from 200 to 500 mL of 0.1N NaOH. Under meat diet, active gastric digestion, renal diseases, fasting and severe diabetic acidosis values may go up to 1500 mL. The acidity of the urine decreased under vegetable diet.

Requirements:

1. 0.1 N NaOH solution.
2. 1% Phenolphthalein solution.
3. Potassium oxalate.

Procedure:

1. Take 25 mL of urine in a conical flask.
2. Add 5 g of freshly powdered potassium oxalate and 0.5 mL of phenolphthalein solution to the flask containing urine sample.
3. Shake vigorously and titrate immediately with 0.1 N NaOH solution from a burette till a permanent pink colour appears.
4. Repeat the experiment thrice and take the mean of the three readings for calculation.

Calculation:

$$\frac{25}{a} = \frac{b}{x}$$

Therefore

$$x = \frac{a \times b}{25}$$

Where, a = mL of 0.1N NaOH solution used

b = volume of urine excreted in 24 hours *i.e.*, 2000 mL per day under normal conditions.

x = Total acidity of the 24 hour urine specimen.

The result is expressed as mL of 0.1 N NaOH.

Report: The given urine sample required _____ mL of 0.1 N NaOH.

Experiment No. 15: Estimation of Sugar by Folin-Wu Method.

Aim: To estimate the glucose level in the given blood sample.

Principle: Glucose reduces the cupric ions present in the alkaline copper reagent to cuprous ions or the cupric sulfate is converted into cuprous oxide, which reduces the phosphomolybdic acid to phosphomolybdous acid, which is blue when optical density is measured at 420 nm.

Reagents Required:

1. Alkaline copper-reagent: Dissolve 40 gs of anhydrous sodium carbonate (Na_2CO_3) in about 400 mL of water and transfer it into a 1-liter flask. Dissolve 7.5 gs of tartaric acid in this solution and 4.5 gs of $CuSO_4$, which is dissolved in 100 mL water. Mix the solution and make it up to 1 liter.
2. Phosphomolybdic acid reagent: Add 5 gs of sodium tungstate to 35 g of phosphomolybdic acid. Add 20 mL 10% NaOH and 50 mL distilled water. Boil vigorously for 20-30 min so as to remove the NH_3 present in molybdic acid, cool and dilute to 350 mL with water. Add 125 mL of orthophosphoric acid and make up the volume up to 500 mL.
3. Standard glucose solution: Dissolve 100 mg of glucose in 100 mL distilled water and take 10 mL from this stock and make it up to 100 mL.

Preparation of protein free filtrate: To 1 mL blood sample, add 8 mL distilled water, 0.5 mL of 2/3 N sulfuric acid and 0.5 mL of 10% sodium tungstate solution in a stoppered centrifuge tube and mix the contents. Then centrifuge at 3000 rpm for 10 min and collect the supernatant as sample.

Procedure:

1. Pipette out 0.0, 0.4, 0.8, 1.2, 1.6 and 2 mL of working standard in to the series of labeled test tubes.
2. Pipette out 2 mL of the given sample (protein free filtrate) in another test tube.

3. Make up the volume to 2 mL in all the test tubes. A tube with 2 mL of distilled water serves as the blank.
4. Now add 2 mL of copper sulfate solution and incubate in a boiling water bath for 8 min and cool it.
5. Then add 2 mL of phosphomolybdic acid to all the test tubes. Dilute the solution up to 25 mL mark on the Folin-Wu tube. Mix the contents and read the absorbance at 420 nm against blank.
6. Then plot the standard curve by taking concentration of glucose along X-axis and absorbance at 420 nm along Y-axis.
7. From the standard curve calculate the concentration of glucose in the given sample.

Observations and Calculations

Volume of standard glucose (mL)	Volume of distilled water (mL)	Concentration of glucose (µg)	Volume of copper sulfate solution (mL)	Incubate In boiling water bath for 8 min and cool to R T	Volume of phosphomolybdic acid (mL)	Dilute the solution up to 25 mL mark on the Folin-Wu tube	A420
0.0	2.0	00	1.5		2		0.00
0.4	1.6	40	1.5		2		
0.8	1.2	80	1.5		2		
1.2	0.8	120	1.5		2		
1.6	0.4	160	1.5		2		
2.0	0.0	200	1.5		2		
2.0 Sample	0.0	To be estimated	1.5		2		

Result: The given unknown sample contains ----µg /mL.

Experiment No. 16: Agarose Gel Electrophoresis.

Aim: To determine the molecular weight of the DNA sample.

Principle: Agarose forms a gel by hydrogen bonding and the gel pore size depends on the agarose concentration. The DNA molecules are separated by electrophoresis on the basis of their size, shape and the magnitude of net charge on the molecules.

Requirements:

1. Tris-Acetate buffer (TAE) stock solution (5X): Prepare by mixing Tris 24.2 g, glacial acetic acid 5.71 mL and 10.0 mL 0.5 M EDTA in 1000 mL of distilled water. Set the pH 8.0. Dilute 5 times before use to obtain the working solution.
2. 1 % Agarose: Dissolve 1 g agarose in 100 mL of 1 X TAE buffer by boiling and maintain it at 50° C till it is to be used.
3. Gel Loading Buffer: Chromous biotech green view gel loading buffer.
4. Standard DNA marker. Take 20 µl of DNA ladder and mix with 10 µl of gel loading buffer.
5. Adhesive tape, gloves *etc*.,

Procedure:

12. Take a clean dry gel casting plate and make a gel mould using an adhesive tape along the sides of the plate to prevent running off of the material to be poured on the plate.
13. Pour required amount of 1 % agarose solution kept at 50 °C on to the casting plate. Immediately place the comb about 1 cm from one end of the plate ensuring that teeth of the comb do not touch the glass plate. Wait till a firm layer of gel is formed.
14. Pour 1 X TAE buffer in to the tank until the gel is completely submerged. Connect the electrodes to the power supply.
15. Load the DNA samples as well as marker in to the separate wells with the help of micro syringe.
16. Turn on the power supply and run at 100 V. Monitor the progress of fast running tracking dye during electrophoresis.
17. Turn off the power supply when the tracking dye has reached near the opposite edge of the gel.
18. Transfer the gel from casting plate onto a UV-transilluminator and record the result. Then determine the molecular weight of the given DNA sample using the Vilber Laurmat Bioprofil image analysis system software.

Result: The given unknown sample contains ------ bp.

Experiment No. 17: Estimation of Haemoglobin.

Aim: To determine the concentration of haemoglobin in the given blood by Sahli's method.

Principle: Haemoglobin is converted in to acid haematis with 0.1 N HCl which will be a reddish orange coloured samle, upon diluting with distilled water it turns yellow which can be compared to the permanent standard in Sahli's haemometer. The concentration of Hb is directly read on the graduations of the tube when the colour intensity exactly matches to that of the permanent.

Requirements:

Blood sample. 0.1N HCl, Blood lancet, alcohol, Sahli's haemometer, water.

Procedure:

1. Fill the graduated tube till the bottom graduation (mark 2) with 0.1N HCl.
2. Clean thoroughly the finger tip with alcohol and take a drop of blood with a blood lancet, suck 20µl of blood in to the capillary pipette precisely up to the mark.
3. Wipe the pipette point and blow the blood in to the measuring graduated tube. Mix it well with the help of micro pestle provided to get dark brown colour which will clear after one minute.
4. Add distilled water by means of water pipette and stir it with micro pestle until the colour of the solution matches exactly to the permanent standard colour when observed against the white background.
5. Read the concentration of haemoglobin directly on the graduations and express in terms of g/dl.

Report: The concentration of haemoglobin in the given unknown blood sample is ___ g/dl.

Experiment No. 18: RBC Counting.

Aim: To estimate the number of RBCs in one cubic millimeter of blood.

Introduction: Red blood cells or erythrocytes occur only in vertebrates. Their main function is to transport respiratory gases. In the early stages of development, they are nucleated but when mature, they lose nucleus hence it is biconcaval disc shaped. They are extremely elastic

and deform readily in passing through smallest blood vessel. A plasma membrane surrounds the erythrocytes. This is an enzyme containing functional membrane of the RBCs. In normal healthy human being, the number of RBCs per cubic ml is 4.5 to 5 millions. This is counted by haemocytometer.

Principle: To count the erythrocytes, a dilution (isotonic) solution is used to prevent haemolysis. RBCs are counted using Neubauer's haemocytometer, which consists of 400 boxes. Four corner boxes and one corner area are used for counting. Number of RBCs is calculated using following formula.

$$\text{Number of RBCs} = \frac{\text{No. of cells counted x Dilution factor}}{\text{Depth factor X Area Counted}}$$

Requirements:

EDTA, diluting fluid, RBC pipette, counting chamber, Microscope.

Procedure: Draw blood in the RBC pipette up to 0.5 mark. Wipe the tip with clean tissue paper. Draw dilution fluid up to 101 mark of the same pipette. Shake well for few minutes. Charge the chamber and count the number of RBCs using 40 X objective of the microscope.

Report: Number of cells counted from the blood sample is 430 million cells / mm^3.

Experiment No. 19: WBC Counting.

Aim: To estimate the number of WBCs.

Reagents required:

1. EDTA
2. Diluting fluid: 1.5 mL of gacial acetic acid + 1 mL of giemsa stain or crystal violet + 97.5 mL of distilled water.

Procedure: Draw blood up to mark 0.5 mark in the WBC pipette. Wipe the tip with clean tissue paper and draw diluting fluid up to 11 mark. Shake well gently avoiding air bubbles. Place cover slip on the counting chamber. Transfer the mixture in the WBC pipette into the counting chamber. Allow the cells to settle for two minutes. Place the counting chamber on the stage of microscope. Using 10x objective, count the WBCs in the four large corner squares. Total number of WBCs is calculated using the formula.

$$\text{Number of WBCs} = \frac{\text{No. of cells counted x Dilution factor}}{\text{Depth factor X Area Counted}}$$

Result: Number of WBCs counted in the given sample is _____ / mm3.

Experiment No. 20: Differential Counting.

Aim: To count different types of WBC cells from the given blood sample.

Introduction: In human, usual blood smear which is stained with different dyes shows leucocytes which vary in concentration according to their affinity to acidic or basic dyes. Due to this differential staining, different types of WBCs could be identified by staining with giemsa stain. Leucocytes are distinguished based on size and shape of the nuclei, colour and presence or absence of granules.

Procedure:

1. Take a drop of blood on clean glass slide and spread it using another glass slide to get a thin smear. A properly made smear must be about one cell thick throughout the slide.
2. Dry the blood smear and stain it with 8-9 drops of giemsa stain. After two minutes add double the amount of buffer water and mix by sucking the mixture in and out of the pipette. Leave it for 2-10 minutes and wash excess stain in running water.
3. Dry the smear and observe under a microscope.
4. Count the cells by preparing a block with hundred squares. Fill each square with 'N' for Neutrophills, 'L' for Lymphocytes, 'B' for Basophills, 'E' for Eosinophills and 'M' for Monocytes as and when they are observed. Count hundred cells in this manner and calculate the percentage of each type.

Result: per cent age of each type of leucocytes

Monocytes (M)=___%

Lymphocyte (L) = ___ %

Basophills (B) = ___%

Eosinophills (E) = ___%

Neutrophils (N) = ___ %

Experiment No. 21: Estimation of Albumin and Globulin Ratio in Blood.

Aim: To estimate the albumin and globulin ratio in blood.

Introduction: The concentration of proteins in the serum is determined by biuret reaction during colormetric method. The intensity of colour developed is proportional to the number of peptide bonds and hence to the concentration of proteins. In the differential estimation of albumin and globulins, the property of globulins by virtue of their molecular weight to get precipitated by 28% sodium sulphate solution is used. The serum is treated with sodium sulphate where globulins are precipitated. The precipitate is filtered. The filtrate contains only albumins and is treated with biuret reagent and the intensity of colour developed gives the measurement of albumin. Besides the above two solutions, a standard containing a known amount of protein and a blank with distilled water are also treated with biuret reagent. Read O D at 540 nm. The difference between total proteins and albumin gives the content of globulins. To calculate the A/G ratio, divide the amount of albumin to that f globulin, if the albumin content is more or vice versa.

Reagents required

Biuret reagent: Dissolve 3 g of copper sulphate ($CuSO_4.5H_2O$) and 9 g of sodium potassium tartarate in 500 ml of 0.2 mol/liter sodium hydroxide; add 5 g of potassium iodide and make up to 1 liter with 0.2 mol/liter sodium hydroxide.

Procedure: Sample Preparation: Pipette 0.2 mL of serum in to a test tube. Add 5.8 mL of sodium sulphate. Mix by inversion and allow to stand for 10 min. filter through a Whatman 44 paper or centrifuge it. The clear filtrate or supernatant is used for albumin estimation.

Estimation of Albumin and Total protein:

1. Take four clean and dry test tubes. Label first tube as blank and add 3 mL of distilled water to it.
2. To the second tube, add 3 mL of standard protein solution (BSA 5000 µg/mL).
3. To the third tube, add 0.1 mL serum (for total protein estimation) and 2.9 mL of NaCl.
4. To the fourth tube, add 3 mL of biuret reagent. Mix and allow the tubes to stand for 10 min. read the O D at 540 nm.

Report: The amount of total protein present in the sample is ____ mg/mL.

The amount of albumin is 1.7625.

The amout of globulin is 2.6475.

Observation and Calculations:

Total Protein = $\frac{\text{O D of (total protein – blank) x concentration of standard x 100 x 1}}{\text{O D of (Standard – blank)}}$

Total Albumin = $\frac{\text{O D of (total albumin – blank) x concentration of standard x 100 x 1}}{\text{O D of (Standard – blank)}}$

Globulin = Total protein – Albumin

A/G Ratio = Albumin : Globulin

Experiment No. 21: Estimation of Total Cholesterol in Serum.

Aim: To estimate total cholesterol in serum by Zak's method.

Reagents required:

Preparation of Stock Solution: 100 mg of cholesterol is dissolved in 100mL of glacial acetic acid. This is kept in cool dark place and it can be preserved for about a month.

Working Standard Solution: 4 ml of stock solution is taken in 100 mL volumetric flask and the solution is made up to the mark using $FeCl_3$-CH_3COOH solution.

Sample preparation for total Cholesterol:- 0.1 mL of serum is taken, to this 10 mL of $FeCl_3$-CH_3COOH solution is added. Now this is centrifuged at 2000rpm for 5 min. Take 5mL of supernatant for analysis.

Procedure:

1. Pipette out 0-5 mL of working standard solution in a series of 6 tubes. In the 7^{th} tube, take 5 mL of total cholesterol sample.
2. Now make up the volume up to 5 mL using 0.05% $FeCl_3$-CH_3COOH solution. Add 0.05 mL of 0.9 % NaCl solution followed by 3 mL of Concentrated H_2SO_4 to all the test tubes, mix well and incubate at room temperature for 30 min.
3. Record the absorbance at 570 nm.

4. Then plot the standard curve by taking concentration of protein along X-axis and absorbance at 570 nm along Y-axis.

Observation and Calculations:

Sl. No.	Volume of Standard Cholesterol (mL)	Concentration of Cholesterol (µg)	Volume of $FeCl_3$-CH_3COOH (mL)	Volume of NaCl (mL)	Volume of Concentrated H_2SO_4 (mL)	Incubation At R T For 30’	A_{570}
1	0	0	5	0.05	3		0.00
2	1	40	4	0.05	3		
3	2	80	3	0.05	3		
4	3	120	2	0.05	3		
5	4	160	1	0.05	3		
6	5	200	0	0.05	3		
7	Unknown 5 ml	To be estimated	0	0.05	3		

Report: The amount of total cholesterol is found to be _____ µg/ml.

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