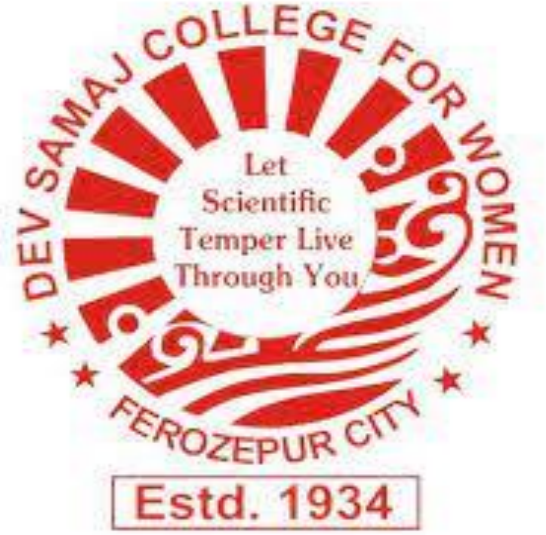
ZOOLOGY

LAB MANUAL

B.Sc. I



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EXPERIMENT NO. 1

AIM: Studying the application of the different microscope- with the help of the different permanent slides & temporary mount

Theory: microscopes are employed as a basic tool to observe microorganisms and to study the structural details of the cells. Nowadays, highly sophisticated microscopes such as phase contrast, dark field and electron microscopes are available but simple and compound microscopes remain as a basic tool in routine microbiology research work. A simple microscope is just a hand lens and magnifying glass whose magnification power is relatively lower. In order to achieve higher magnification power, combination of lenses is used as objective lens and ocular lens in components are grouped into two categories:

I. Structural components

II. Optical components

I. Structural components: three structural components of microscope are head, arm and base. Head contains the optical parts in the upper part of the microscope. Base supports it and contains eliminator (condenser). The arm is fitted with the coarse and fine adjustment knobs and help in easy handling of microscope.

II. Optical components: there are 2 types of optical structures:

a. Eye piece/ocular: it is a cylinder containing 2 or more lenses. Its function is to bring the image into focus for the eye. Typical magnification values for the ocular lens is 2x, 10x and 50x.

b. Objective lens: objective lens is usually a cylinder containing lens attached to the circular disk with the movable head of the microscope. Magnification of the objective lens ranges from 10x, 40x to 100x. Three to four objective lenses are attached to the piece.

c. Stage: stage is the flat platform used to place the slide. An eliminator is provided under the stage to provide the sufficient light which passes through the holes on the stage for focussing the contents of the slide.

d. Condenser: it is used to collect the light from the light source.

Working:

The objective lens is placed close to the object to be viewed and ocular lens is placed closed to the eyes. The primary enlargement of the object is produced by objective lens. The image produced thus is transmitted to the ocular where the final enlargement occurs. Therefore, the magnifying capacities of the compound microscope are the product of magnification of the objective and the ocular lens. For e.g.; using an objective lens of 40x and ocular lens of 10x will produce a magnification of 400x. The primary image produced by the objective lens is real and inverted image.

Light Microscope General Operating Procedure:

The following step-by-step operating procedure should be carefully observed when first using the microscope. Go through these steps now and again when carrying out the procedures for parts B, C, and D. This instrument is designed for fast-simplified use and correct operating technique should soon become automatic.

1. Plug in and turn on the in-base illuminator.

2. Raise the nosepiece using the coarse adjustment knob. This provides greater access to the stage when the slide is in position.

3. Rotate the nosepiece so that the 10X objective is in operating position. You should feel a definite position stop for the objective. Turn the nosepiece clockwise and counter-clockwise until you are familiar with this position stop.

4. Open the iris diaphragm approximately half way.

5. Place the slide in the stage slide holder securely. Be sure the slide has the *specimen side up*. Roughly center the specimen over the light coming from the condenser.

6. Move the microscope condenser by means of the condenser rack and pinion knob until the top of the condenser is approximately the thickness of a piece of paper beneath the slide.

7. Lower the objective using the coarse control knob until it reaches a stop. *Do not* force the knob. The stop should be obvious if you are moving the controls gently. Again, become familiar with *the feel* of the position stop.

8. View through the eyepieces and without disturbing the coarse adjustment setting, slowly rotate the fine adjustment knob in the appropriate direction until the specimen detail is in sharpest possible focus.

9. Adjust the interpupillary distance between the two eyepieces by sliding the eyepiece plates in or out.

10. If your eyes are very different the left eyepiece tube is adjustable to compensate for refraction differences of the eyes. The correct procedure is to bring the specimen into sharpest possible focus with a fine adjustment knob using the right eyepiece only, while closing your left eye. To focus for the left eye, view the specimen with the left eye only and turn the knurled collar until the specimen is in sharp focus. *Do not* adjust the fine adjustment knob during this procedure.

11. Once the specimen detail is in sharp focus using the 10X objective, it is then possible to rotate the nosepiece to other objectives without changing the position of the coarse adjustment knob. Very little refocusing with the fine adjustment is required since the Nikon Alphaphot-2 microscope objectives are *parfocal*. The term parfocal means that if an object is in focus with one objective lens, it will be in focus with all of the lenses.

Make certain that the portion of the specimen you wish to view is exactly centered in the field of the low-power objective. This is necessary because the microscopic field of view is smaller under high magnification than under low magnification.

Remember that the iris diaphragm setting must be changed whenever a different objective is used. As magnification increases the condenser iris diaphragm is opened as required. With these microscopes you are effectively increasing the light reaching the higher power objective. Adjustment of the condenser iris diaphragm results in proper contrast for viewing specimens under varying magnifications.

12. Try the highest magnification, the l00X-oil immersion objective. To focus with the oil immersion lens proceed in the following manner:

a) Revolve the nosepiece so that there is no objective directly over the specimen. This allows enough space so that a drop of immersion oil may be placed on the specimen.

b) Place *one drop* of immersion oil in the center of the circle of light formed on the specimen slide.

c) Turn the nosepiece until the 100X objective snaps into place. The objective should be in the oil but must not touch the slide.

d) Rotate the fine adjustment knob to obtain a sharp focus of the specimen. Remember to make the adjustments noted in step 10.

Problems:

Certain mechanical difficulties, real or apparent, may be encountered while operating your microscope.

A common problem is the failure of the fine adjustment to turn in the direction required for sharp focusing. This indicates that it has been screwed to the limits of its threads, either upward or downward, as the case may be. Screw it back to about one-half the thread distance, use the coarse adjustment to raise or lower the objective sufficiently to bring the specimen into view, and then refocus with the fine adjustment.

If the coarse adjustment fails to lower the objective sufficiently to bring the specimen into view, the fine adjustment has been screwed up too far and should be screwed down to about half its thread distance.

Proper Care of the Microscope:

The compound microscope used in microbiology is a precision instrument. Its mechanical parts, such as the calibrated mechanical stage and the adjustment knobs, are easily damaged and all lenses, particularly the oil immersion objective, are delicate and expensive. Thus, the instrument must be handled with care. The following rules, cautions, and maintenance should be observed:

1. Use both hands when carrying the microscope; one firmly grasping the arm of the microscope, the other beneath the base. Avoid sudden jars.

2. To keep the microscope and lens systems clean:

- Never touch the lenses. If the lenses become dirty, wipe them gently with lens paper.

- Never leave a slide on the microscope when it is not in use.

- Always use lens paper to remove oil from the oil-immersion objective after its use. *Do not* wipe the lower power objectives with the same piece of lens paper used to clean the oil-immersion objective. If by accident oil should get on either of the lower power objectives, wipe it off immediately with clean lens paper.

- Keep the stage of the microscope clean and dry.

3. To avoid breaking the microscope:

• Never force the adjustments. All adjustments should work freely and easily.

• Never allow an objective lens to jam into or even to touch the slide or cover slip.

• Never focus downward with the coarse adjustment while you are looking through the microscope. Always incline your head to the side with eyes parallel to the slide, so that downward movement can be arrested before the objective touches the slide.

• Never exchange the objectives or eyepieces of different microscopes and never under any circumstance remove the front lenses from the objectives.

• Never attempt to carry two microscopes at one time.

4. Storage of the microscope:

- ***Make sure the immersion oil has been removed from the lens.***
- Put the low power (10X) objective into position and turn the stage all the way down.
- Be sure the slide holder does not extend beyond the *left* edge of the microscope.
- Wrap the electrical cord around the cord hanger.
- Your TA will place the microscope in the cabinet.

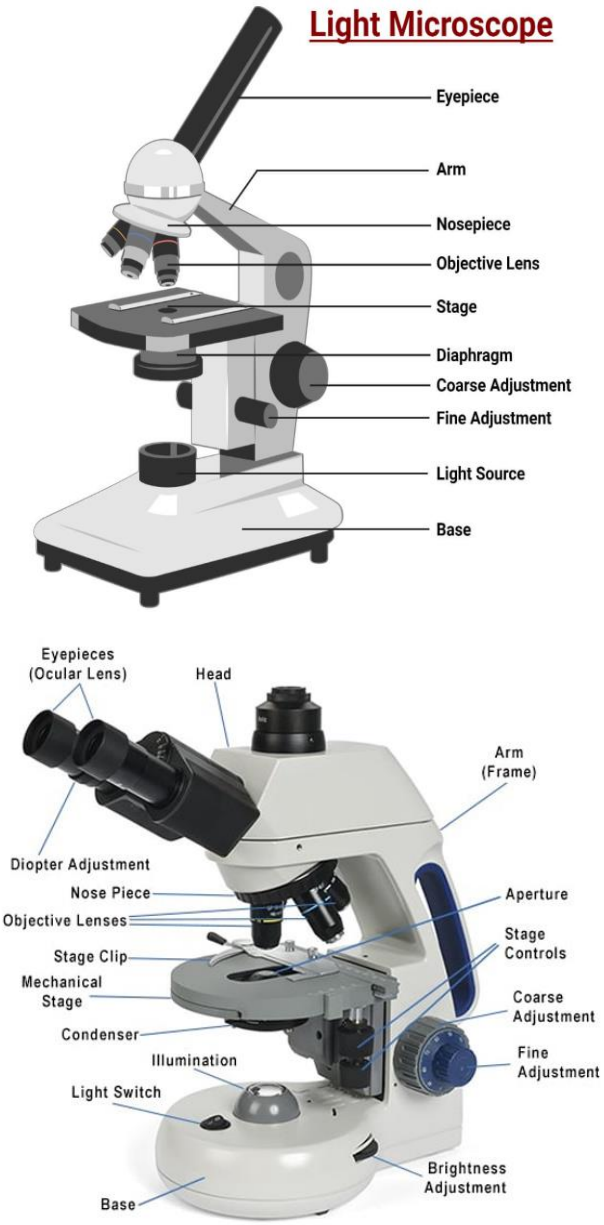

Fig 1: Light and Compound Microscope

EXPERIMENT NO. 2

Performing the procedure of the electrophoresis and demonstrate to the students and provide technical details to the students.

Theory: Gel electrophoresis is a laboratory method used to separate mixtures of DNA, RNA, or proteins according to molecular size. In gel electrophoresis, the molecules to be separated are pushed by an electrical field through a gel that contains small pores.

Preparing TAE and TBE Buffer for electrophoresis systems

These buffers are used because they both have a basic pH which gives the phosphate group of the DNA a net negative charge allowing migration of the DNA toward the positive anode.

Buffer TAE or TBE TAE is best used when DNA is to be recovered from the gel and for electrophoresis of large (>20kb) fragments. TBE is best used for smaller (<1kb) fragments due to high ionic strength and high buffering capacity. Also it reacts with agarose making smaller pores and tighter matrix.

TAE-Tris acetate w/EDTA (40mM Tris base, 40mM acetic acid, 1mM EDTA)

50x Stock solution, pH ~8.5

242g Tris base

57.1ml glacial acetic acid

18.61g Na2EDTA-2H2O (MW 372.24)

Distilled/RO water to 1 liter final volume

TBE-Tris borate with EDTA (89mM Tris base, 89mM boric acid, 2mM EDTA)

10x Stock solution

108g Tris base

55g boric acid

7.44g Na2EDTA-2H2O (MW 372.24) (Or 40ml of 0.5M EDTA, pH 8.0)

Distilled/RO water to 1 liter final volume

Making a GEL

Please note an increased % in agarose gives better separation of small fragments and bands that are close in size.

FOR MAKING A 1% GEL

1. Dissolve 0.4g of agarose in 40ml of electrophoresis buffer (TBE or TAE) by heating in a microwave.

! CAUTION HOT USE GLOVES WHEN REMOVING!

2. After heating and fully dissolving the agarose add 4uL of SYBR SAFE directly into the liquid via pipette, mix by gentle swirling.
3. Use this mixture to cast a gel
4. Place the gel tray into the gel box so that the gasket (Ends with rubber strip) forms a seal against the walls of the gel box make sure to press the gel tray all the way down so that the gel box and gel tray are level.
5. After the gel mix has cooled to 60C (Higher temps will damage and warp the gel box) pour the mix into the gel tray. Upon pouring the gel mix immediately insert the gel comb with the desired number of teeth/wells.
6. Allow the gel to solidify completely. Then lift the gel tray out of the gel box turn it 90^{O} and replace it into the gel box with the comb closest to the cathode.
7. Pour running buffer into the gel box to fill the chamber and completely submerge the gel (300ml).
8. Carefully remove the comb using a light tapping motion to avoid damage to the wells.

Loading samples

Please note the maximum volume for a gel of this size using a B1A-10 comb is 13.5uL

For other combs use the calculation below to determine well volume and x 0.75 of this value for loading volume. Please note well height will remain **6mm** unless volume of gel cast is increased from 40ml.

(Well height (mm)-1.5) x (Tooth width x Comb thickness)

1. Pipette 10uL of your sample into a clean tube.
2. Add 2uL of loading dye(Bromophenol blue) (Blue dye) for visual tracking.
3. Mix by low vortex and spin on mini-fuge to ensure all sample is at the bottom of the well.
4. Plan out your gel before pipetting and create a reference chart/diagram to ensure correct samples are put into the correct wells. Also that you have a negative and positive control in addition to a hyperladder on one or both ends of the gel (First and last well).
5. Carefully pipette all of sample into the correct well on your gel using 10xL pipette tips (Or any compatible extended length tips)
6. Add 10uL of the hyperladder for reference/comparison in at least one or if possible both ends of your gel (First and last wells)

Connecting and starting power supply

1. Carefully place the lid on the top of the gel box, ensure it is completely pressed down and level.

! MAKE SURE RED WIRES CONNECT TO RED PORT ON BOX AND BLACK WIRES TO BLACK PORT!

2. Connect the wires to the correct color terminals on the power supply.
3. Plug in the power supply
4. Set voltage to 150V
5. Ensure bubbles start to form on both sides of the gel box where the buffer reservoirs are.
6. After 45 mins the gel should be complete check that sampleshave migrated by looking through the lid.
7. Turn off power supply.
8. Disconnect power supply from outlet.

Visualizing the gel

1. Ensure power supply is turned off.
2. Remove lid from gel box.
3. Wait approx. 10mins for gel to cool.
4. Carefully remove gel.
5. Place gel onto a blue light transilluminator (Optimal due to maxima excitation wavelength).
6. Place orange filter over to protect eyes.
7. Turn on transilluminator, bands should be visible in addition to a clear hyperladder
8. Take picture via gel doc system on other device (Cell phone) as soon as possible to avoid degradation of fluorescence.
9. Annotate gel picture to transcribe your reference chart for sample in each well including negative and positive control (See below).

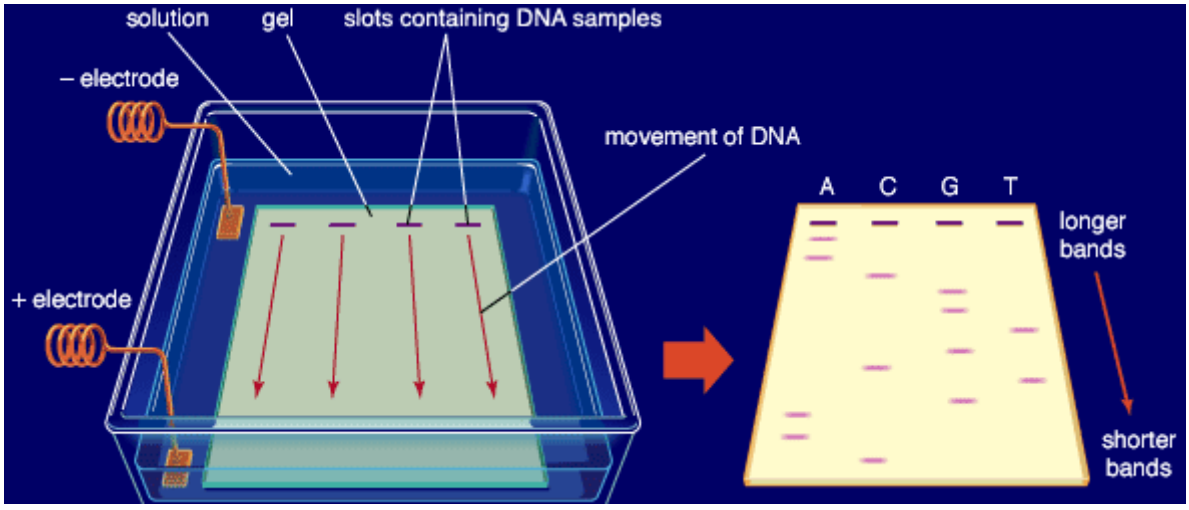

Fig 2: Horizontal Gel Electrophoresis

EXPERIMENT NO. 3

A) To find out the quality of soil of given ecosystem and its effect on flora & founa of the system

Aim

To study soil samples from two different sites and analyse their properties such as texture, moisture content, water-retaining capacity and pH. Also, the study aims to correlate the plants found in such soil.

Necessary Materials & Apparatus

For this experiment, soil collected from the **roadside** and **garden** are to be used. Apart from the soil samples, other required materials are:

- Tile.
- Beaker.
- Funnel.
- Burner.
- Dropper.
- Crucibles
- Petri dish.
- Glass rods.
- Test tubes.
- Wire gauze.
- Filter Paper.
- Distilled water.
- Mortar and Pestle.
- pH paper booklet.
- Measuring cylinder.
- Universal pH indicator solution.
- Tin Box with a perforated bottom.
- Weighing scale or Electronic balance.

Procedure

The following are the steps taken to prepare the soil samples for experiments to analyse various properties.

To study the pH of the Soil Samples

- Take the collected roadside soil and garden soil into two different beakers containing water.
- Mix the test tubes with the soil solution slowly
- Now into a clean and dried two test-tube, arrange a funnel spread covered with a filter paper.
- Now gently pour the soil solutions into the test tubes separately.
- Let the water to completely filter off from the filter paper.
- Take the collected filtrates (soil) into the two different test tubes for testing the pH values.
- With the help of a dropper, add a few drops of universal indicator solution to both the test tubes.
- Observe the changes.

Observation

When the universal pH indicator is added to the test tube containing the soil solution, the colour changes. These colour changes can be tracked using the pH colour chart. Roadside soil has a pH level of 7 while garden soil has a pH level of 6. Most crops grow between pH levels of 6.0 and 7.0.

To study the texture of Soil Samples

- Collect 50 gm of any soil sample in a beaker.
- Take a clean and moisture-free measuring cylinder and the collected soil sample into it.
- Now pour little water into the same measuring cylinder and shake well.
- Keep the apparatus undisturbed for a few minuted and wait for the particles to settle down.
- After a while, observe the changes in the measuring cylinder.
- The soil particles in the measuring cylinder will start to settle down in layers.
- Record the thickness of these layers.

Observation

Using a soil textural triangle, draw the corresponding percentage of the soil components (silt, clay & sand). The resultant lines which, intersect indicate the type of soil.

To study the Moisture Content of Soil Samples

- Collect two different soil sample in two different crucibles.
- Weight the soil samples using a weighing balance.
- Make a note of the reading.

- Place the two crucibles over the bunsen burner and heat it until it becomes dry.
- Now again weigh the soil samples and record the weight of the dry soil samples.
- The samples are now ready to be used to determine the moisture content of the soil.
- Calculate the two different readings to know the moisture content of soil samples.

Observation

The sample where the initial and final weight is the larger indicates a higher moisture content. Lower values mean the moisture content is quite low.

To study the Water Holding Capacity of Soil Samples

- Collect a garden soil sample in a beaker.
- To a clean and dried mortar pestle add the collected soil sample.
- Now slowly grind the soil sample into a fine powder using a pestle.
- Place a filter paper at the bottom of the tin box.
- Weigh the entire contents of the tin box.
- Now, add the powered soil into the tin box.
- Use the glass rod to press and tap the box, so that the soil is uniformly layered.
- Now, the weight of the tin box is measured and to be recorded.
- Next, take two glass rods and place them parallel to each other. Ensure that the distance between the two is not long.
- Position the tin on the two glass rods in such a way that the bottom is in contact with the water.
- The complete setup should be left undisturbed until the water seeps through the upper surface of the soil.
- Now, remove the tin and allow all the water to flow out from the bottom.
- Wait until no more water percolates from the tin.
- Now wipe the bottom dry and use the weighing machine to note down the weight.
- Calculate the two different readings to know the water holding capacity of the given soil samples.

Observation

The water holding capacity of the soil is determined by the quantity of water held by the soil sample versus the dry weight of the soil sample.

B) To find out the quality of water of given ecosystem and its effect on flora & founa of the system

1.0 PURPOSE

To lay down the procedure for the analysis of water.

2.0 SCOPE

Applicable to all sampling points of the water system.

3.0 RESPONSIBILITY

Microbiologist

4.0 ACCOUNTABILITY

Head of Department

5.0 PROCEDURE

Collect the sample as per Standard Operating Procedure for water sampling and analysis for chemical and microbiological parameters as per their specifications.

5.1 Chemical Analysis

Prepare the solutions/ reagents for chemical analysis.

5.1.1 Description

Examine the water physically such as color, odor.

5.1.2 Hardness

Take 100 ml sample add 2 ml of ammonia buffer pH 10.0, 50 mg of mordant black 11 mixture and add of 0.01 M disodium edetate until, a pure blue color is produced. Measures the volume of disodium edetate used and calculate the hardness by the following formula.

Hardness as mg/L = ml of EDTA used x 1000 mg/L Sample volume

5.1.3 Total Suspended Solids (TSS)

Take the gouch crucible clean and dry in oven for one hour at 105°C, Cool the gouch crucible in desiccator and take the empty weight of gouch crucible and then filter the 30 ml water sample from the gouch crucible with the help of vacuum pump and calculate the TSS with the help of the formula.

TSS = W2-W1 × 1000 (mg/L)/ml of solution taken

W1 : Weight of Gouch crucible before filtration

W2 : Weight of Gouch crucible After filtration

5.1.4 Total dissolved solids (TDS)

Measure the conductivity at 25 °C with a calibrated conductivity meter and convert the value in TDS by the following formula.

TDS in mg/L= conductivity in mS X 0.667 (Geographical factor of area)

5.1.5 Acidity

Take 10 ml sample freshly boiled and cooled sample, add 0.05 ml of methyl red solution and mix, the resulting solution is not red. Interpretation of result: If the solution is in red color the sample is Acidic

5.1.6 Alkalinity

Take 10 ml sample freshly boiled and cooled sample, add 0.1 ml of bromothymol blue solution and mix. Interpretation of result: If the solution is in blue color the sample is Alkaline.

5.1.7 Ammonium

Take 20 ml sample add 1 ml of alkaline potassium mercuri-iodide solution and allow standing for 5 minutes. When vertically viewed the solution is not more intensely colored than a solution prepared at the same time by adding 1 ml of alkaline potassium mercuri-iodide solution to a solution containing 2.5 ml of dilute ammonium chloride solution and 7.5 ml of the liquid being examined.

5.1.8 Calcium & Magnesium

Take 100 ml sample add 2 ml of ammonia buffer pH 10.0, 50 mg of mordant black 11 mixture and 0.5 ml of 0.01 M disodium edetate, a pure blue color is produced.

5.1.9 Heavy Metals

In a glass-evaporating dish evaporate 150 ml of sample to 15 ml on a water bath. Standard solution Into a small Nessler Cylinder, pipette 10.0 ml of lead standard solution (1ppm Pb). Test Solution Pipette 12 ml into a small nessler cylinder. Procedure To the cylinder containing the standard solution add 2.0 ml of the test solution and mix. To each cylinder add 2 ml of acetate buffer pH 3.5, mix, add 1.2 ml of thioacetamide reagent, allow to stand for 2 minutes and view downwards over a white surface, the colour produced with the test solution is not more intense than that produced with the standard solution.

5.1.10 Chloride

Take 10 ml sample add 1 ml of 2 M nitric acid and 0.2 ml of 0.1 M silver nitrate, the appearance of the solution does not change for at least 15 minutes.

5.1.11 Nitrate

Take 5 ml sample in a test tube immersed in ice add 0.4 ml of a 10% w/v solution of Potassium chloride, 0.1 ml of diphenylamine solution and dropwise with shaking 5 ml of sulphuric acid. Transfer the tube to a water bath at 50°C to allow standing for 15 minutes. Any blue colour in the solution is not more intense than that in a solution prepared at the same time and in the same manner using a mixture of 5.5 ml of nitrate free water and 0.5 ml of nitrate standard solution (2 ppm NO3).

5.1.12 Sulphate

Take 10 ml sample add 0.1 ml of 2 M Hydrochloric acid and 0.1 ml of barium chloride solution. The appearance of the solution does not change for at least 1 hour.

5.1.13 Oxidisable substances

Take 100 ml sample add 10 ml of 1 M sulphuric acid and 0.1 ml of 0.02 M potassium permanganate and boil for 5 minutes, the solution should remain faintly pink.

5.1.14 Residue on evaporation

Evaporate 100 ml sample to dryness into hot plate and dry to a constant weight at 105°C. The residue weighs not more than 1 mg (0.001%).

Residue on evaporation: W2-W1 × 100 (mg/L)/ml of solution taken

W1 : Weight of Evaporating dish

W2 : Weight of Evaporating dish + Residue

5.1.15 Total Organic Carbon

Analyse the sample for TOC in a calibrated TOC Analyser as per SOP. 5.1.15.1 Alert and Action limit for Total Organic Carbon of water system

S.No.	Type of Water	Alert Limit (ppb)	Action Limit (ppb)
1	Purified water	300.0	500
2	Water for injection	250.0	500
3	Pure Steam	250.0	500

5.1.15.2 If the TOC results are above alert and action limit, follow the SOP.

5.1.16 Conductivity

Take the 100 ml sample in a suitable container, and stir the test sample by maintaining the temperature 25°C ± 1°C, measuring the conductivity with the help of a calibrated conductivity meter. Temperature and the respective Conductivity.

Temperature (°C)	Conductivity µS cm^{-1}
0	0.6
5	0.8
10	0.9
15	1.0
20	1.1
25	1.3
30	1.4
35	1.5
40	1.7
45	1.8
50	1.9
55	2.1
60	2.2
65	2.4
70	2.5
75	2.7
80	2.7
85	2.7
90	2.7
95	2.9
100	3.1

5.1.17 pH

Take 100 ml of sample and add 0.3 ml of saturated KCL solution. Mix the solution well and then measure the pH with the help of a Calibrated pH meter.

NOTE : If results are observed out of limit in chemical analysis of water, follow the SOP.

5.2 Microbiological Analysis

Analyse the water samples for Microbiological analysis as per specifications.

5.2.1 Pour Plate Method

Dispense one ml of sample into two Petri dishes. Approximately add 15-20 ml of R2A / Plate count Agar into each Petri dishes. Cool the media approximately 45°C (feel on the dorsal side of the hand, it should be bearable). Cover the Petri dish, mix the sample with the agar by tilting or rotating the dishes and allow the contents to solidify at room temperature. Invert the Petri dishes and incubate at 30-35°C for 5 days. After incubation, examine the plates for growth, count the number of colonies and express the average for the two plates in terms of the number of colony forming units per ml. Related: Incubation Conditions for Common Media used for Fungus and Bacteria

5.2.2 Membrane Filtration Technique

The procedure gives the use of a single disposable/ autoclaveable filtration funnels and filter holder, using MILLIFLEX system. Preparation of the Filtration apparatus Operate the Milliflex as per its SOP. Use sample size as specified in the specification for filtration through the 0.45 m filter. After completion of filtration of the sample, rinse the filter with 100 ml sterile water remove the filter using sterilized forceps and transfer it immediately to the previously prepared petri-dish with appropriate medium (R2A agar/Plate count agar). Place the membrane filter carefully so that the air should not be trapped inside the filter, as this will prevent the nutrient medium from reaching the entire membrane surface. Replace the lid. Incubate the plates in an upright position (in case of filter) at 30-35°C for 5 days. Count the number of colonies on the membrane and express the results as per specification.

5.2.3 Bacterial Endotoxin Test

Refer the SOP for bacterial endotoxin test.

5.2.4 Pathogens

The sample shall be tested for the following four specific pathogens.

(A) Salmonella species

(B) Escherichia coli

(C) Pseudomonas aeruginosa

(D) Staphylococcus aureus

Filter 100 ml of water sample through the 0.45 membrane filter fixed on Milliflex system. After filtration removes the filter aseptically and put it in 100 ml Soybean Casein Digest Medium and incubate at 30-35°C for 24-48 hours. From Soybean Casein Digest Medium, inoculate sterile 10 ml volumes of the following enrichment broths using 1 ml of inoculated broth

1. Selenite Cystine Broth for Salmonella species.
2. Tetrathinate Broth for Salmonella species.
3. MacConkey's Broth for Escherichia coli
4. Cetrimide Broth for Pseudomonas aeruginosa
5. Giolitti Cantoni Broth for Staphylococcus aureus (use sterile liquid paraffin for anaerobic conditions). Incubate the tubes for 24-48 hours at 30-35°C.

(A) Test for Salmonella species:

If growth is present in Selenite Cystine Broth and Tetrathionate Broth, inoculate the following selective media plates and incubate at 30-35° C for 24-48 hours for presumptive identification of the pathogen.

Medium	Description of Colony
Xylose-Lysine Deoxycholate agar medium	Red with or without Black Centre
Bismuth Sulphite agar medium	Black or Green colonies
Brilliant Green agar	Small, transparent, colorless or pink to white Opaque (frequently surrounded by pink to red zone)

Confirmatory Test

From the selective media plates pick the suspected colonies and go for confirmatory tests with the following biochemical/media and by gram reaction. Individually transfer the suspected colony by first streaking the slope of slant, of Triple Sugar-Iron Agar with inoculating loop and then stabbing with inoculating straight wire well in the butt. Incubate at 30-35° C for 24-48 hours After incubation, examine the tube of Triple Sugar Iron Agar Medium for the presence of microbial growth and for the following Physical characteristics.

(a) Slant Surface: Alkaline reaction (red color)

(b) Butt: Acid reaction (yellow color) and/or gas bubble (with or without concomitant blackening).

If the butt, slant of Triple Sugar Iron Agar shows growth and physical characteristics conforming to the above descriptions the presence of Salmonella species is indicated.

(B) Test for Escherichia coli

If the inoculated MacConkey's broth tube shows acid and gas formation, inoculate the following selective media plates and incubate at 30-35°C for 24-48 hours for presumptive identification of the pathogen.

Medium	Description of Colony
MacConkey's Agar	Brick red may have surrounding zone of precipitated bile.
Eosin Methylene Blue Agar	Metallic sheen with dark grey colonies

Confirmatory Test

From the selective media plates pick the suspected colonies and go for confirmatory tests into the following biochemicals/media and by gram reaction.

Add 0.1 ml of the contents of the tube showing acid and gas to tubes containing 10 ml of peptone water From peptone, water tube perform Indole test as follow
Add 0.5 ml of Kovac's reagent to peptone water tube, allow to stand for one minute, if a red color is produced in the reagent layer indole is present

The presence of acid and gas in MacConkey's broth, in peptone water and indole, indicates the presence of Escherichia coli. Presence of Escherichia coli shall be confirmed by Gram staining (Gram-ve rods) and by streaking a loopful of the MacConkey's broth, with acid and gas production on plates of MacConkey Agar, and Levine Eosin Methylene Blue Agar. Incubate the plates at 30-35°C for 24-48 hours. If after incubation, plates show colonies of following characteristics presence of Escherichia coli is confirmed. MacConkey's Agar: Brick red colonies with or without surrounding zone of precipitates. Levine Eosin Methylene blue Agar: Colonies with characteristic of metallic sheen under reflected light and blue-black appearance under transmitted light

Test for *Pseudomonas aeruginosa*

If the inoculated Cetrimide broth tube shows growth with greenish/bluish pigmentation, inoculate the following selective media plates and incubate at 30-35°C for 24-28 hours for presumptive identification of the pathogens.

Medium	Description of Colony
Cetrimide Agar	Greenish colonies, which exhibit a greenish fluorescence under ultra violet light.
Pseudomonas Agar (For Pyocyanin)	Colourless to yellowish, yellowish under ultra violet light.
Pseudomonas Agar (For Fluorescein)	Colourless to yellowish, yellowish under ultra violet light.

Confirmatory Test

From the selective media plates pick the suspected colonies and go for confirmatory tests Streak suspected colony on Pseudomonas Agar for Fluorescenin (PAF) Detection and Psedomonas Agar for Pyocyanin (PAP) Detection using inoculating loop. Incubate the plates in inverted condition at 30-35°C for 24-28 hours. Simultaneously inoculate the suspected colony in 100 ml of Soyabean casein digest medium and incubate at 41° to 43°C for 18 to 24 hours. After incubation, examine the plates and tube of Soybean casein digest medium for the presence of microbial colonies of Gram-Negative rods exhibiting following characteristics. Pseudomonas Agar for fluorescenin detection: Colorless to yellowish fluorescence under the ultra violet light. Pseudomonas Agar for Pyocyanin Detection: Greenish colonies, which exhibit a blue fluorescence ultraviolet light. Soybean casein digest medium: Growth occurs. If colonies are found confirming to above descriptions, Oxidase test shall be performed to confirm identification as follow: With the aid of an inoculating loop, transfer suspected colonies to strip or discs of filter paper impregnated with N, N-dimethyl-p-phenylenediamine dihydrochloride. If a Pink-Purple colour is produced within five to ten seconds, the presence of Pseudomonas aeruginosa is confirmed.

(D) Test for Staphylococcus aureus

If growth is present in Giolitti Cantoni (G.C) broth, usually characterized by black settled growth at the bottom of the broth under anaerobic conditions, inoculate the following selective media plates and incubate at 30-35°C for 24-28 hours for presumptive identification of pathogen.

Medium	Description of Colony
Mannitol Salt Agar Medium	Yellow colonies with yellow zones
Vogel Johnson Agar Medium	Black surrounded by yellow zone
Baird Parker Agar Medium	Black, shiny, surrounded by clear zones of 2-5 mm

Confirmatory Test

From the selective media plates pick the suspected colonies and go for confirmatory tests If colonies are found confirming to the above descriptions identification shall be performed by a coagulase test as follow. With the aid of an inoculating loop, individually transfer suspected colonies to separate tubes containing 0.5 ml of mammalian plasma (preferably rabbit or horse). Incubate in a water-bath / incubator at 370C for 3 to 24 hours, in parallel with positive control using known strain of Staphylococcus aureus and negative control using Plasma alone. Examine after 3 hours and at suitable intervals thereafter for the presence of coagulation. If coagulation in any degree is observed, the presence of Staphylococcus aureus is indicated. And perform the gram staining for the presence of gram Positive cocci.

5.2.5 Coli forms

Filter 100 ml of test sample and transfer the filter to M-Endo agar and incubate at 35°C for 22-24 hrs count colonies that are pink to dark red with a green metallic surface sheen, the sheen may vary from pinpoint to complete coverage of colony. Report the as number of Coliforms colonies per 100 ml.

5.2.6 After completion of testing prepare a test report.

5.2.7 If the counts obtained are above the limits specified below investigate the results and take necessary actions as per SOP.

5.3 Alert and Action limit for TAMC of water system

S.No	Type of water	Alert limit	Action limit
1	Raw water	300	500
2	Soft water	200	500
3	Potable water	150	500
4	Drinking water	100	500
5	Purified water	50	100
6	Water for injection (100 ml)	3	10
7	Pure steam (100 ml)	3	10

If the Microbial results are above alert and action limit, follow the SOP for Out of Specification.