Title: Procedure for Analysis of Water			
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1.0 PURPOSE

To lay down the procedure for the analysis of water.

2.0 SCOPE

Applicable to all sampling points of water system as per the Annexure-II.

3.0 RESPONSIBILITY

Microbiologist

4.0 PROCEDURE

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

4.1 Chemical Analysis

Prepare the solutions/ reagents as per Annexure-I for chemical analysis.

4.1.1 Description

Examine the water physically such as Colour, Odour.

4.1.2 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 colour is produced. Measures the volume of disodium edetate used and calculate the hardness by the following formula.

$$\text{Hardness as mg/L} = \frac{\text{ml of EDTA used}}{\text{Sample volume}} \times 1000 \text{ mg/L}$$

4.1.3 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.

$$TSS = \frac{W_2 - W_1}{} \times 1000 \text{ (mg/L)}.$$

ml of solution taken

W_1 : Weight of Gouch crucible before filtration

W_2 : Weight of Gouch crucible After filtration

4.1.4 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 µS X 0.667 (Geographical factor of area)

4.1.5 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 colour the sample is Acidic

4.1.6 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 colour the sample is Alkaline.

4.1.7 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 coloured 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.

4.1.8 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 colour is produced.

4.1.9 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.

4.1.10 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.

4.1.11 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 drop wise 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 4.5 ml of nitrate free water and 0.5 ml of nitrate standard solution (2 ppm NO_3).

4.1.12 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.

4.1.13 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.

4.1.14 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: $\frac{W_2-W_1}{\text{ml of solution taken}} \times 100$ (mg/L)

W_1 : Weight of Evaporating dish

W_2 : Weight of Evaporating dish + Residue

4.1.15 Total Organic Carbon

Analyse the sample for TOC in a calibrated TOC Analyser as per SOP.

4.1.15.1 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	290.0	500
2	Water for injection	240.0	500
3	Pure Steam	240.0	500

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

4.1.16 Conductivity

Take the 100 ml sample in a suitable container, and stir the test sample by maintaining the temperature 25°C ± 1°C, measure the conductivity with the help of 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

4.1.17 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 Calibrated pH meter.

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

4.2 Microbiological Analysis

Analyse the water samples for Microbiological analysis as per specifications.

4.2.1 Pour Plate Method

Dispense one ml of sample into two petridishes. Approximately add 15-20 ml of R_2A / Plate count Agar into each petridishes. Cool the media approximately 45°C (feel on the dorsal side of the hand, it

should be bearable). Cover the petridish, mix the sample with the agar by tilting or rotating the dishes and allow the contents to solidify at room temperature. Invert the petridishes 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.

4.2.2 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 µ filter.

After completion of filtration of sample, rinse the filter with 100 ml sterile water remove the filter using sterilised forceps and transfer it immediately to the previously prepared petri-dish with appropriate medium (R_2A agar/Plate count agar).

Place the membrane carefully so that the air should not be trapped inside the filter, as this will prevent nutrient medium from reaching the entire membrane surface. Replace the lid. Incubate the plates in 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.

4.2.3 Bacterial Endotoxin Test Refer the SOP.

4.2.4 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 remove the filter aseptically and put it in 100 ml Soyabean 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, colourless 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 colour)

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

If the butt, slant of Triple Sugar Iron Agar shows growth and physical characteristics confirming 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 bio-chemicals/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 colour 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 shows 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.

(C) 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 Soyabean casein digest medium for the presence of microbial colonies of Gram-Negative rods exhibiting following characteristics. Pseudomonas Agar for fluorescenin detection: Colourless to yellowish fluorescence under ultra violet light. Pseudomonas Agar for Pyocyanin Detection: Greenish colonies, which exhibit a blue fluorescence under ultra violet light. Soyabean 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 37^0C 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.

4.2.5 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.

4.2.6 After completion of testing prepare a test report according to Annexure-II.

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

4.3 Alert and Action limit for TAMC of water system

S.No.	Type of water	Alert limit	Action limit
1.	Raw water	143	500
2.	Soft water	134	500
3.	Potable water	167	500
4.	Drinking water	108	500
5.	Purified water	50	100
6.	Water for injection	3	10
7.	Pure steam	3	10

NOTE : If the Microbial results are above alert and action limit, follow the SOP.

5.0 ABBREVIATIONS AND DEFINITIONS

SOP	Standard Operating Procedure
QCM	Quality Control Microbiology
QAD	Quality assurance Department
Rev.	Revision
No.	Number
M	Molarity
mg	Milli gram
TSS	Total Suspended Solids
TDS	Total Dissolved Solids
ppm	Parts per million
TOC	Total Organic Carbon
%	Percent
°C	Degree Centigrade
PAF	Pseudomonas agar for Fluorescenin
PAP	Pseudomonas agar for Pyocyanin
G.C	Giolitti Cantoni broth
TAMC	Total Aerobic Microbial Count
mL	Milli litre

Action limit: Established criteria, requiring immediate follow-up and corrective action if exceeded

Alert limit : Established criteria giving early warning of potential drift from normal condition which are not necessarily grounds for definitive corrective action but which require follow-up

investigation.

6.0 REFERENCE DOCUMENTS

SOP	Water sampling procedure
SOP	Procedure for Operation, Calibration & System suitability TOC analyzer
SOP	Operation of Miliflex Water filtration unit.
SOP	Bacterial endotoxin (LAL) test
SOP	Handling of out of trend results in environmental monitoring and water analysis.

7.0 ANNEXURE

Annexure I : Form 1-Preparation of Standard solutions/ reagents for water.

Annexure II : Form 2- Test data sheet for water

8.0 REVISION LOG

Revision Number	Effective Date	Reason for Revision