برنامج المسار الوظيفي للعاملين بقطاع مياه الشرب والصرف الصحي

دليل المتدرب

البرنامج التدريبي كيميائي مياه

Bacteriological Analysis of Water- الدرجة الثالثة

تم اعداد المادة بواسطة الشركة القابضة لمياه الشرب والصرف الصحي
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Definitions

- **Water Microbiology**: Study of small living microorganisms in water.
- **Drinking Water**: Regarding to World Health Organization (WHO) should be “Suitable for human consumption and for all usual domestic purposes”.
- **Drinking Water (from microbiological perspective):** Should be free of any microorganism that may cause health risk (pathogen).
- **Pathogen** : Is an organism with the potential to cause disease

Agenda

This material will cover:

- History of Water Microbiology.
- Waterborne Diseases
- Why water microbiology is important?
- Microbial Indicators.
- Methods of Analysis.

History of Water Microbiology

In 1852, Dr. John Snow (1813 – 1858), British physician, traced pump incident in London.

- He noticed that cholera cases were isolated from region that served by specific company, while other areas did not affected.
- After removal of the pump, no other cholera cases were found.
- He concluded that “water can cause human health disease”.

In 1880, Louis Pasteur (1822-1895), French chemist and microbiologist

- He defined the "germ theory" of disease, stating that “infectious disease wasn't spontaneous but a result of the presence and/or growth of harmful microorganisms”.

In 1877, Robert Koch (1843-1910), German physician

- He isolated Bacillus anthracis as the causative agent Anthrax.

In 1883, Koch published an article entitled:

"About Detection Methods for Microorganisms in Water"

- In that historic paper he marked the introduction of the application of microbial indicators.
- He described for the first time the methodology for bacteria measurement in water.

In 1884, Koch identified ***Vibrio cholerae*** as the causative agent of cholera after his research with French team in governmental hospital in Alexandria, Egypt.

- He is considered one of the founders of microbiology science.

In 1850, Dr. Snow used Chlorine as water disinfection agent in England.

In 1918, The US Department of Treasury called all world water companies to disinfect water with chlorine to kill harmful microorganisms.

Waterborne Diseases

Examples:

- Bacterial:
  - Cholera by *Vibrio Choloerae*
  - Typhoid by *Salmonella typhi*
- Viral:
  - Poliomyelitis by Poliovirus
  - Acute gastroenteritis by Rotavirus

Why Water microbiology is important?

- It helps us to:
- Identify harmful microorganisms in water.
- Realize the diseases cause.
- Measure effectiveness of water treatment.
- Monitor water safety.
- Confirm that water will not cause human disease.

Microbial Indicators

Definitions

- Microbial Indicators are:
- Microorganism or group of microorganisms that its presence in water indicate the possibility of pathogens contamination,
- Its absence indicates no pathogenic contamination. No single indicator organism can universally be used for all purposes of water quality surveillance.
- The challenge is to select the appropriate indicator, or combination of indicators, for each particular purpose of water quality assessment.

Why indicators and not direct detection of pathogens؟

We use indicators because:

- Pathogens are in small numbers.
- Takes long times to detect (72 hours for Typhoid).
- Cannot predict likelihood of infectious dose from average concentration.
- Many pathogens present, and each one require different method

Microbial Indicators:

Indicator Characters are:

- Present when there is a risk of contamination by pathogens
- Should not multiply in environmental conditions under which pathogens cannot multiply
- Should correlate with the degree of faecal contamination.
- Easy to enumerate and identify by simple methods
- Specific for Faecal or sewage pollution.
- At least as resistant as pathogens to conditions in natural water environments, and water treatment processes.
- Should have stable characteristics and give consistent reactions in these analyses
- Present in the same or higher numbers than pathogens.
- Non-pathogenic.

Microbial Indicators Examples:

Water Treatment and Quality:

- Heterotrophic Plate Count.
- Total Coliform

Fecal Pollution:

- Thermotolerant Coliforms
- E.coli
- Enterococci
- C. perfringens
- Coliphage

Total Coliforms:

- Coliforms are gram-negative non-sporing rod-shaped bacteria, oxidase-negative, capable of aerobic and facultative anaerobic growth in the presence of bile salts or other surface-active agents with similar growth-inhibiting properties.
- They are able to ferment lactose with the production of acid within 48 hours at 35–37°C.
- Fermentation by these organisms begins with the cleavage of lactose into galactose and glucose by the enzyme ß-galactosidase
- Coliforms comprise Escherichia coli (E.coli), other thermotolerant coliforms such as Citrobacter and regrowth bacteria such as Klebsiella, and other related enterobacteria.

Escherichia coli and thermo-tolerant coliforms:

- Escherichia coli (E. coli) is the faecal indicator of choice used in WHO Guidelines for Drinking-water Quality (WHO, 2004) and several countries are including this organism in their regulations as the primary indicator of faecal pollution.
- Current data suggest that E. coli is almost exclusively derived from the feces of warm-blooded animals.
- Its presence in drinking-water is interpreted as an indication of recent or substantial post-treatment faecal contamination or inadequate treatment.

- Thermotolerant coliforms, including E. coli, can ferment lactose (or mannitol) at 44.5± 0.2°C with the production of acid within 24 hr.
- Thermotolerant coliforms include E. coli and also some types of Citrobacter, Klebsiella and Enterobacter

Fecal Streptococci and Enterococci:

- **Faecal streptococci** are species of Gram-positive cocci belonging to two genera, *Enterococcus* and *Streptococcus*.
- The relevant species are linked by common biochemical and antigenic properties and are found in the faeces of humans and other animals.
- Many will grow in 6.5% sodium chloride and at 45°C.
- **Enterococci**: All faecal streptococci that grow at pH 9.6, 10° C and 45°C and in 6.5% NaCl. Nearly all are members of the genus Enterococcus, and also fulfill the following criteria: resistance to 60°C for 30 min and ability to reduce 0.1% methylene blue.
- The enterococci are a subset of faecal streptococci that grow under the conditions outlined above.

Heterotrophic Bacteria

- Heterotrophs are broadly defined as microorganisms that require organic carbon for growth. They include bacteria, yeasts and moulds.
- A variety of simple culture-based tests that are intended to recover a wide range of microorganisms from water are collectively referred to as “heterotrophic plate count” or “HPC test” procedures. Exposure to general HPC microbiota is far greater through foodstuffs than through drinking-water.
- Levels of exposure regarded as acceptable from foods are much greater than those regarded as acceptable from drinking-water.
- Limited data are available with which to characterize exposure to specific microorganisms through these two routes.
- Exposure to HPC microbiota also occurs through air and other environmental sources.

Methods of Analysis

1. Estimation of Heterotrophic Bacteria by Pour Plate Method

1. Scope:

The heterotrophic plate count (HPC), formerly known as the standard plate count is a procedure for estimating the number of live cultivable heterotrophic bacteria in water and measuring changes during water treatment and distribution or in swimming pools.

Colonies may arise from pairs, chains, clusters, or single cells, all of which are included in the term “colony-forming units” (CFU).

The final count also depends on interaction among the developing colonies.

Estimation of live cultivable heterotrophic bacteria by pour plate method in water samples in 48 h at 35.0 °C and/or 7 days at 22.0°C on the basis of culturing in non-selective low nutrient agar medium.

This procedure can be applied for different types of water.

2. Principle:

Volume of water to be tested is mixed with R2A medium in sterile petri dishes, incubated at 35 °C for 48h and/or 22 °C for 7 days. The advantage of R2A medium is due to low nutrient formulation that stimulates the growth of stressed and chlorine-tolerant bacteria. The final count also depends on interaction among the developing colonies.

The test has little value as an indicator of pathogen presence but can be useful in operational monitoring as a treatment and disinfectant indicator.

In addition, HPC measurement can be used as indicator of stagnation, tuberculation, residual disinfectant concentration, assessing the cleanliness and integrity of distribution systems, presence of biofilms and availability of nutrients for bacterial growth.

It should be noted that heterotrophic plate count results are not an indicator of water safety and, as such, should not be used as an indicator of potential adverse human health effects. It should also

be noted that the results obtained using heterotrophic plate count test are not an accurate assessment of total heterotrophic concentrations but, instead, are indications of culturable organisms present.

Pour plate method is a simple technique that can accommodate volumes of sample or diluted sample ranging from 0.1 to 2.0 ml. The colonies produced are relatively small and compact, showing fewer tendencies to encroach on each other than those produced by surface growth. On the other hand, submerged colonies often are slower growing and are difficult to transfer.

3. Definition:

Heterotrophs are those microorganisms that use organic compounds for most or all of their carbon requirements.

Heterotrophic plate count (HPC): is a microbial method that uses colony formation on culture media to approximate the levels of heterotrophic flora.

Colony-Forming Units (CFU): Colony that may arise from pairs, chains, clusters, or single cells.

Note: Various synonyms are frequently used instead of “heterotrophic count”. These include colony count, viable count, standard plate count and culturable micro-organisms etc…….

4. Environmental Conditions:

All sample analysis steps will be carried out under Laminar Air Flow (LAF) to ensure aseptic environmental conditions.

5. Interference:

Significant heat shock to bacteria from the transient exposure of the sample to 45 to 46 °C agar may occur, to minimize, use a thermostatically controlled water bath for tempering the agar.

6. Equipment:

- Water bath: 45°C to 46°C.
- Pre-sterilized plastic Petri dishes about 90 x 15 mm, with tight-fitting lids.
- Automatic pipettes and associated sterile tips capable of delivering 1 ml.
- Automatic pipettes and associated sterile tips capable of delivering 100 µl.
- Buffered Dilution water screw cap bottles.
- Incubator: 35°C ± 0.5°C.
- Incubator: 22°C ± 1°C.
- Colonies counter aid.
- Indelible ink marker for labeling plates.

7. Chemicals and Reagents:

R2A medium.

Phosphate buffered dilution water.

8. Precautions:

Follow the normal safety procedures required in a microbiology laboratory.

Mouth-pipetting is prohibited.

Do not re-sterilize plating medium, re-melting is allowed for only one time.

Decontaminate all the used plates and materials at the end of the analyses.

Do not prepare dilutions or pour plates in direct sunlight.

Avoid spilling medium on outside of dish lid when pouring plates.

Do not depend on the sense of touch to indicate proper medium temperature when pouring agar.

Avoid prolonged exposure to unreasonably high temperatures during melting agar.

Use sterile tips for initial and subsequent transfers from each different dilution.

Replace the tip with a sterile one, if it becomes contaminated before transfers completed.

Avoid contamination when removing sterile tips from the container by not dragging tip across exposed ends of tip in the tip container or across lips and necks of dilution bottles.

Do not insert tips more than 2 to 3 cm below the surface of sample or dilution when removing the sample volume.

Discard melted agar that contains precipitate.

9. Procedure:

9.1 Responsibility:

Microbiologists are responsible for the implementation of the test procedures inside the microbiology laboratory according to analysis work flow.

Laboratory technician is responsible for preparation of the media and reagents used in this procedure under supervision of the microbiologist(s).

10. Sample Handling:

Sample Holding Time and Limitations: samples should be analyzed within 8 h of collection (maximum transit time 6 h, maximum processing time 2 h).

When analysis cannot begin within 8 h, maintain sample at temperature below 4.0 °C but do not freeze. Maximum elapsed time between collection and analysis must not exceed 24h.

11. Instrument Calibration:

Calibrate and verify balances daily using reference weights.

Calibrate pH meter prior to use, using at least two standards points.

Check temperature of incubators and refrigerators daily to ensure operation within stated limits of method.

Check sterilization procedure and efficiency to ensure media sterility used in this SOP.

12. Media and solutions preparation:

Prepare and handle media and reagents appropriately, carry out suitable performance test to ensure homogeneity, sterility, and suitability of each prepared batch.

12.1 Phosphate buffered dilution water

Stock phosphate buffer solution: Dissolve 34.0 g KH_2PO_4 in a 500 ml reagent-grade distilled water, adjust the pH of the solution to 7.2 with 1 N NaOH and bring volume to 1000 ml with reagent-grade distilled water. Sterilize by filtration or autoclave for 15 minutes at 121°C, store in the refrigerator until using (If evidence of mold or other contamination appears in the stock, the solution should be discarded, and a fresh solution should be prepared).

$MgCl_2$ solution: Dissolve 38 g anhydrous $MgCl_2$ (or 81.1 g $MgCl_2.6H_2O$) in one liter of reagent-grade distilled water. Sterilize by filtration or autoclave for 15 minutes at 121°C, store in the refrigerator until using (If evidence of mold or other contamination appears in the stock, the solution should be discarded, and a fresh solution should be prepared).

Working solution: Add 1.25 ml phosphate buffer stock and 5 ml $MgCl_2$ stock for each liter of reagent-grade distilled water prepared, mix well(Final pH 7.0 ± 0.2), and dispense in appropriate amounts for dilutions in screw-cap dilution bottles or culture tubes, and/or into larger containers for use as rinse water. Autoclave at 121°C for 15 minutes.

12.2 R2A medium:

Prepare and sterilize medium according to manufacture instructions.

12.3 Melting Agar:

Melt sterile solid agar medium in boiling water bath.

Maintain melted medium in a water bath between 44° C and 46°C until used, preferably for no longer than 3 h.

13. Sample Dilution and preparations:

Label the bottom of plates with the sample identification number, analyst initials, and sample volume to be analyzed.

Prepare buffered dilution water directed in section 10.4.1.

Select the dilution(s) so that the total number of colonies on a plate will be between 30 and 300. For example, where a heterotrophic plate counts high as 3000 is suspected, prepare plates with 10^{-2} dilution.

Shake the sample container vigorously by rapidly making about 25 complete up-and-down (or back-and-forth) movements. Optionally, use a mechanical shaker to shake samples or dilutions for 15 s.

Hold micropipette inside neck of dilution bottle to transfer 10 ml of sample to 90 ml of sterile dilution water bottle, cap, and mix by vortex, 1ml of this dilution is considered 10^{-1} of the original sample.

Repeat the previous step to prepare further dilutions.

14. Selection of Sample Size:

Pipette 1ml, and 0.1 ml directly from tested water sample. In examining, known, turbid water, do not measure 0.1 ml inoculum of original sample, but prepare an appropriate dilution.

15. Plating:

Limit the number of samples to be plated in any one series so that no more than 20 minutes elapse between dilution of the first sample and pouring of the last plate in the series.

Shake the sample container vigorously.

Lift cover of petri dish just high enough to insert micropipette.

Pipet selected sample volume by holding micropipette at an angle of about 45°.

Remove micropipette without touching it to dish.

Pour at least 10 to 12 ml liquefied medium maintained at 44 to 46°C into each dish by gently lifting cover just high enough to pour.

Mix melted medium carefully with test portions in petri dish.

Rotate the dish gently containing the mixture first in one direction and then in the opposite direction.

Let plates solidify on a level surface.

16. Incubation:

Invert and incubate plates at 35°C for 48hr and/or 22°C for 7 days.

17. Counting:

Count colonies using dark-field colony counter.

If counting must be delayed temporarily, store plates at 5 to 10°C for no more than 24 h.

Quebec counter

Count all colonies on selected plates promptly after incubation.

Count plates that have 30 to 300 colonies.

If there is no plate with 30 to 300 colonies, and one or more plates have more than 300 colonies, use the plate(s) having a count nearest 300 colonies.

When the total number of colonies is less than 30, disregard the rule above and record result observed.

Count as individual colonies similar-appearing colonies growing in close proximity but not touching, provided that the distance between them is at least equal to the diameter of the smallest colony.

Well isolated colonies

Count impinging colonies that differ in appearance, such as morphology or color, as individual colonies.

If spreading colonies (spreaders) are encountered on the plate(s) selected, count colonies on representative portions only when colonies are well distributed in spreader-free areas and the area covered by the spreader(s) does not exceed one-half the plate area.

Count each of the following types of spreading colonies as one: a chain of colonies that appears to be caused by disintegration of a bacterial clump as agar and sample were mixed; a spreader that develops as a film of growth between the agar and bottom of Petri dish; and a colony that forms in a film of water at the edge or over the agar surface.

Spreading colonies

18. Calculations:

Compute bacterial count per milliliter by the following equation:

$$\text{CFU/mL} = \frac{\text{colonies counted}}{\text{actual volume of sample in dish, mL}}$$

19. Quality Control:

19.1 Analytical Quality Control Batch:

Prepare duplicate plates for each volume of sample or dilution examined; concentration value for that volume will be obtained by getting the arithmetic mean for both plates.

Plate(s) with non-selective agar medium, labeled as "**Environmental Control**", will be opened along the samples analysis run time to ensure adequate environmental conditions.

Procedural Blank: Inoculate 1 ml of sterile phosphate buffered dilution water, using separate sterile micropipette tip, after inoculation of samples series; incubate under the same conditions as a sample. Absence of growth indicates absence of cross contamination.

19.2 Non Conformance and Corrective Action:

If the media performance and sterility assessment are performed concurrently with the use of the media in testing and the media quality is deemed unsatisfactory, the specific use of the media in the test(s) should be investigated to determine the potential impact on the test results and the necessity for repeating the test(s).

Testing must be repeated when unsatisfactory media and/or reagents are used for critical aspects of testing.

If a procedural blank test gives unsatisfactory results, the samples results should be investigated to determine the potential impact of contamination on the test result, if test results deemed unsatisfactory the test must be repeated.

If, one or more of, environmental control plate(s) gives unsatisfactory results, the samples results should be investigated to determine the potential impact on the test result, if test results deemed unsatisfactory the test must be repeated.

For all non-conformance findings, trace all media, reagents, equipment operations, and tools used in analysis to find out root cause of non-conformance.

Take appropriate corrective action, if cause was temporary e.g. if unsterile batch of media used accidently in the analysis; discard all batch of prepared media, or preventive actions if cause was permanent e.g. re-maintenance of autoclave if actual temperature was less than adjusted value, to eliminate reoccurrence of non-conformance in the future.

For Invalid results, request a new sample from the same location, explaining the reason for re-sampling need.

20. Method Performance Data:

A limited intra-laboratory study was conducted. Four participated microbiological analysts tested two water matrices (ground water and drinking water), both spiked with natural flora culture which previously analyzed five times by each analyst to determine spike volume and level of inoculation.

Results of this intra-laboratory study, table 1, may not be typical of results for samples other than those studied, hence secondary validation (cross validation) in form of long-term study shall be carried out depending on routine samples results.

Table 1

Criteria	Drinking Water @ 22.0 °C	Drinking Water @ 35.0 °C	Ground Water @ 35.0 °C
Precision/ repeatability	0.02	0.084	0.112
Precision/Reproducibility	0.16	0.22	0.12
Bias %	±0.051	±1.04	±0.74
Uncertainty %	10	15	9

Detection limit was originally established for any culture-based technique as one colony.

Upper working limit was previously established for this method as 300 colonies per plate.

Range of reliable count was previously established for this method between 30-300 colonies.

Reporting:

Report results as the number of colony forming units (CFU) per ml.

If sample was delayed more than permissible time, write “Delayed” in report comment.

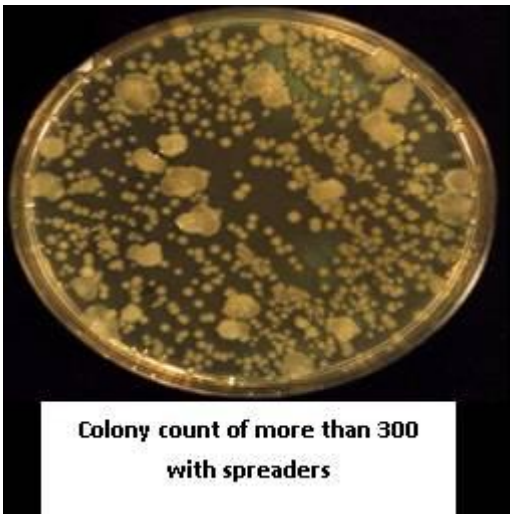
If plates from all dilutions of any sample have no colonies, report the count as less than one (< 1) divided by the corresponding largest sample volume used. For example, if no colonies develop from the 0.01-ml sample volume, report the count as less than 100 (< 100) CFU/ml

When colonies on duplicate plates and/or consecutive dilutions are counted and results are averaged before being reported, round off counts to two significant figures only when converting to colony-forming units.

Raise the second digit to the next higher number when the third digit from the left is 5, 6, 7, 8, or 9; use zeros for each successive digit toward the right from the second digit. For example, report a count of 142 as 140 and a count of 155 as 160, but report a count of 35 as 35.

If the number of colonies per plate far exceeds 300, do not reports result as ‘‘too numerous to count’’ (TNTC) otherwise use one of the following rules:

When fewer than 10 colonies/cm^2 are present, count colonies in 19 squares (of the colony counter) having representative colony distribution. Multiply sum of the number of colonies by 3 to compute estimated colonies per plate when the plate area is 57 cm^2.



When more than 10 colonies/cm^2 are present, count four representative squares, take average count per square centimeter, and multiply by 57 to compute estimated colonies per plate when the plate area is 57 cm^2.

When bacterial counts on crowded plates are greater than 100 colonies/cm^2, report result as greater than (>) 5700 divided by the smallest sample volume plated for plastic plates.

If plates have excessive spreader growth, report as ‘‘spreaders’’ (Spr)

When plates are uncountable because of missed dilution, accidental dropping, and contamination, or the control plates indicate that the medium or other material or lab ware was contaminated, report as ‘‘laboratory accident’’ (LA).

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2. Estimation of Heterotrophic Bacteria by Spread Plate Method

1. Scope:

- The spread plate method causes no heat shock and all colonies are on the agar surface where they can be distinguished readily from particles and bubbles.
- Colonies can be transferred quickly, and colony morphology easily can be discerned and compared to published descriptions.
- This is a detailed procedure for estimation of live culturable heterotrophic bacteria by spread plate method in water samples in 48 h at 35.0 °C on the basis of culturing on non-selective low nutrient agar medium.
- This procedure can be applied for all types of water, in microbiology laboratory it will be applied for the analysis of raw water.

2. Principle:

- Volume of water to be tested is spread over R2A medium in sterile Petri dishes, and incubated at 35 oC for 48h. The advantage of R2A medium is due to low nutrient formulation that stimulates the growth of stressed and chlorine-tolerant. The final count also depends on interaction among the developing colonies.
- The test has little value as an indicator of pathogen presence but can be useful in operational monitoring as a treatment and disinfectant indicator. In addition, HPC measurement can be used as indicator of stagnation, tuberculation, residual disinfectant concentration, assessing the cleanliness and

integrity of distribution systems, presence of biofilms and availability of nutrients for bacterial growth.

- It should be noted that heterotrophic plate count results are not an indicator of water safety and, as such, should not be used as an indicator of potential adverse human health effects. It should also be noted that the results obtained using heterotrophic plate count test are not an accurate assessment of total heterotrophic concentrations but, instead, are indications of culturable organisms present.
- Spread plate method has the advantage of using solidified agar, eliminating the possibility of heat shock. The resultant colonies can be easily transferred, and their colony morphology can be distinguished. However, this method is limited by the small volume of sample or diluted sample that can be absorbed by the agar: 0.1 to 0.5 ml, depending on the degree to which the pre-poured plates have been dried.

3. Definitions:

- Heterotrophs are those microorganisms that use organic compounds for most or all of their carbon requirements.
- Heterotrophic plate count (HPC): is a microbial method that uses colony formation on culture media to approximate the levels of heterotrophic flora.
- Colony-Forming Units (CFU): Colonies may arise from pairs, chains, clusters, or single cells.
- Note: Various synonyms are frequently used instead of “heterotrophic count”. These include colony count, viable count, standard plate count and culturable micro-organisms etc.

4. Environmental Conditions:

- All sample analysis steps will be carried out under Laminar Air Flow (LAF) to ensure aseptic environmental conditions.

5. Interference:

- Spreading colonies, such as a film of growth between the agar and bottom of petri dish or a film of water at the edge or over the agar surface, are mainly develop because of an accumulation of moisture at the point from which the spreader

originates. If those spreading colonies cover more than half the plate, it will interfere with obtaining reliable plate count.

6. Equipment:

- Pre-sterilized plastic Petri dishes about 90 x 15 mm, with tight-fitting lids.
- Automatic pipettes and associated sterile tips capable of delivering 100 µl.
- Automatic pipettes and associated sterile tips capable of delivering 1 ml
- Pre-sterilized plastic spreader.
- Buffered dilution screw-capped bottles.
- Incubator: 35°C ± 0.5°C.
- Colonies counter aid.
- Indelible ink marker for labeling plates.

7. Chemicals and Reagents:

- R2A medium.
- Phosphate buffered dilution water.

8. Precautions:

8.1 General:

- Follow the normal safety procedures required in a microbiology laboratory.
- Mouth-pipetting is prohibited.
- Do not re-sterilize plating medium, re-melting is allowed for only one time..
- Decontaminate all the used plates and materials at the end of the analyses.
- Do not prepare dilutions or pour plates in direct sunlight.
- Avoid prolonged exposure to unreasonably high temperatures during melting agar.
- Use sterile tips for initial and subsequent transfers from each different dilution.
- Replace the tip with a sterile one, if it becomes contaminated before transfers completed.

- Avoid contamination when removing sterile tips from the container by not dragging tip across exposed ends of tip in the tip container or across lips and necks of dilution bottles.
- Do not insert tips more than 2 to 3 cm below the surface of sample or dilution when removing the sample volume.
- Discard melted agar that contains precipitate.
- Pre-dry the dish of excess moisture before use to prevent water film formation.
- Allow the inoculum to soak into the agar to prevent colony spreading over agar layer.

9. Procedure:

9. 1 Responsibility:

- Microbiologists are responsible for the implementation of the test procedures inside the microbiology laboratory.
- Laboratory technician is responsible for preparation of the media and reagents used in this procedure under supervision of the microbiologist.

9. 2 Sample Handling:

- Sample Holding Time and Limitations: Samples should be analyzed within 8 h of collection (maximum transit time 6 h, maximum processing time 2 h).
- When analysis cannot begin within 8 h, maintain sample at temperature below 4.0°C but do not freeze. Maximum elapsed time between collection and analysis must not exceed 24h.

9. 3 Instrument Calibration:

- Calibrate and verify balances daily using reference weights.
- Calibrate pH meter prior to use, using at least two standards points.
- Check temperature of incubators and refrigerators daily to ensure operation within stated limits of method.
- Check sterilization procedure and efficiency to ensure media sterility used in this SOP.

9. 4 Media and solutions preparation:

- Prepare and handle media and reagents appropriately, carry out suitable performance test to ensure homogeneity, sterility, and suitability of each prepared batch.

9.4.1 Phosphate buffered dilution water:

- **Stock phosphate buffer solution**: Dissolve 34.0 g KH_2PO_4 in a 500 ml reagent-grade distilled water, adjust the pH of the solution to 7.2 with 1 N NaOH and bring volume to 1000 ml with reagent-grade distilled water. Sterilize by filtration or autoclave for 15 minutes at 121°C, store in the refrigerator until using (If evidence of mold or other contamination appears in the stock, the solution should be discarded, and a fresh solution should be prepared).
- **$MgCl_2$ solution**: Dissolve 38 g anhydrous $MgCl_2$ (or 81.1 g $MgCl_2C6H_2O$) in one liter of reagent-grade distilled water. Sterilize by filtration or autoclave for 15 minutes at 121°C, store in the refrigerator until using (If evidence of mold or other contamination appears in the stock, the solution should be discarded, and a fresh solution should be prepared).
- **Working solution**: Add 1.25 ml phosphate buffer stock and 5 ml $MgCl_2$ stock for each liter of reagent-grade distilled water prepared, mix well(Final pH 7.0 ± 0.2), and dispense in appropriate amounts for dilutions in screw-cap dilution bottles or culture tubes, and/or into larger containers for use as rinse water. Autoclave at 121°C for 15 minutes.

9.4.2 R2A medium:

- Prepare and sterilize medium according to manufacture instructions.
- If plates are previously prepared and stored in the refrigerator, allow them to warm at room temperature. The crystals that form on agar plates after refrigeration will disappear as the plates warm up.
- Use plates within two weeks after preparation.

9. 5 Preparation of Plates:

- Pour 15 ml of R2A medium into sterile 90 × 15 petri dishes.
- Let agar solidify.
- Pre-dry plates inverted, with lids on, so that there is a 2- to 3-g water loss over night.
- For pre-drying and using plates the same day, pour 25 ml agar into petri dish and air dry in temperature at 50 °C) with the lid off for 78 minutes- 114 minutes to obtain the desired 2- to 3-g weight loss.
- Prepare sample dilutions as directed in 10.6.

9. 6 Sample Dilution and preparations :

- Label the bottom of plates with the sample identification number, analyst initials, and sample volume to be analyzed.
- Prepare buffered dilution water as directed in section 10.4.1.
- Select the dilution(s) so that the total number of colonies on a plate will be between 30 and 300. For example, where a heterotrophic plate counts high as 3000 is suspected, prepare plates with 10^{-2} dilution.
- Use decimal dilutions in preparing sample volumes of less than 0.1 ml.
- Shake the sample container vigorously by rapidly making about 25 complete up-and-down (or back-and-forth) movements. Optionally, use a mechanical shaker to shake samples or dilutions for 15 s.
- Hold micropipette inside neck of sample bottle to transfer 100 µl of sample to 900 µl of sterile dilution water tube, cap, and mix by vortex.
- 100 µl of this dilution is considered 10^{-1}.
- Repeat the previous step to prepare further dilutions.

9. 7 Selection of Sample Size:

- For raw water, Pipette (500 µl and 100 µl) from 10^{-1} dilution.

9. 8 Inoculation:

- Allow the dish to equilibrate to room temperature when a prepared petri dish is used.
- Pipet selected volume onto surface of pre-dried agar plates.
- Distribute inoculum over surface of the medium, using a sterile spreader, by manually rotating.
- Let inoculum to be absorbed completely onto the medium before incubation.

9. 9 Incubation :

- Invert and incubate plates at 35°C for 48 hr.

9. 10 Counting

- Count colonies using dark-field colony counter.
- If counting must be delayed temporarily, store plates at 5 to 10°C for no more than 24 h.
- Count all colonies on selected plates promptly after incubation.
- Count plates that have 30 to 300 colonies.
- If there is no plate with 30 to 300 colonies, and one or more plates have more than 300 colonies, use the plate(s) having a count nearest 300 colonies.
- When the total number of colonies is less than 30, disregard the rule above and record result observed.
- Count as individual colonies similar-appearing colonies growing in close proximity but not touching, provided that the distance between them is at least equal to the diameter of the smallest colony.
- Count impinging colonies that differ in appearance, such as morphology or color, as individual colonies.
- If spreading colonies (spreaders) are encountered on the plate(s) selected, count colonies on representative portions only when colonies are well distributed in spreader-free areas and the area covered by the spreader(s) does not exceed one-half the plate area.
- Count each of the following types of spreading colonies as one: a chain of colonies that appears to be caused by disintegration of a bacterial clump as agar and sample were mixed; a

spreader that develops as a film of growth between the agar and bottom of Petri dish; and a colony that forms in a film of water at the edge or over the agar surface

9. 11 Calculations:

- Compute bacterial count per milliliter by the following equation:

$$\text{CFU/mL} = \frac{\text{colonies counted}}{\text{actual volume of sample in dish, mL}}$$

10. Quality Control:

10.1 Analytical Quality Control Batch:

- Prepare **duplicate plates** for each volume of sample or dilution examined; concentration value for that volume will be obtained by getting the arithmetic mean for both plates.
- Plate(s) with non-selective agar medium, labeled as "**Environmental Control**", will be opened along the samples analysis run time to ensure adequate environmental conditions.
- **Procedural Blank**: Inoculate 1 ml of sterile phosphate buffered dilution water by pour plate technique, using separate sterile micropipette tip, after inoculation of samples series; incubate under the same conditions as a sample. Absence of growth indicates absence of cross-contamination.
- **Buffered dilution water controls**: After inoculation of samples series; Inoculate 1 ml of sterile phosphate buffered dilution water, using separate sterile micropipette tip on TSA plates, and incubate at 35°C for 24 hours. Absence of growth indicates sterility of the dilution water.

10.2 Non Conformance and Corrective Action:

- If the media performance and sterility assessment are performed concurrently with the use of the media in testing and the media quality is deemed unsatisfactory, the specific use of the media in the test(s) should be investigated to determine the potential impact on the test results and the necessity for repeating the test(s).
- Testing must be repeated when unsatisfactory media and/or reagents are used for critical aspects of testing.

- If a procedural blank test gives unsatisfactory results, the samples results should be investigated to determine the potential impact of contamination on the test result, if test results deemed unsatisfactory the test must be repeated.
- If dilution water gives positive result, the samples results should be investigated to determine the potential impact of dilution water on the test result, if test results deemed unsatisfactory the test must be repeated.
- If, one or more of, environmental control plate(s) gives unsatisfactory results, the samples results should be investigated to determine the potential impact on the test result, if test results deemed unsatisfactory the test must be repeated.
- For all non-conformance findings, trace all media, reagents, equipment operations, and tools used in analysis to find out root cause of non-conformance.
- Take appropriate corrective action, if cause was temporary e.g. if unsterile batch of media used accidently in the analysis; discard all batch of prepared media, or preventive actions if cause was permanent e.g. re-maintenance of autoclave if actual temperature was less than adjusted value, to eliminate reoccurrence of non-conformance in the future.
- For Invalid results, request a new sample from the same location, explaining the reason for re-sampling need.

11. Method Performance Data:

- A limited intra-laboratory study was conducted. Four participated microbiological analysts tested two water matrices (ground water and drinking water), both spiked with natural flora culture which previously analyzed five times by each analyst to determine spike volume and level of inoculation.
- Results of this intra-laboratory study, table 1, may not be typical of results for samples other than those studied, hence secondary validation (cross validation) in form of long-term study shall be carried out depending on routine samples results.

Table 1

Criteria	Raw Water @ 35.0 °C
Precision/ repeatability	0.104
Precision/Reproducibility	0.34
Bias %	+0.045
Uncertainty %	28

- Detection limit was originally established for any culture-based technique as one colony.
- Upper working limit was previously established for this method as 300 colonies per plate.
- Range of reliable count was previously established for this method between 30-300 colonies.

12. Reporting:

- Report results as the number of colony forming units (CFU) per ml.
- If sample was delayed more than permissible time, write “Delayed” in report comment.
- If plates from all dilutions of any sample have no colonies, report the count as less than one (< 1) divided by the corresponding largest sample volume used. For example, if no colonies develop from the 0.01-ml sample volume, report the count as less than 100 (< 100) CFU/ml
- When colonies on duplicate plates and/or consecutive dilutions are counted and results are averaged before being reported, round off counts to two significant figures only when converting to colony-forming units.
- Raise the second digit to the next higher number when the third digit from the left is 5, 6, 7, 8, or 9; use zeros for each successive digit toward the right from the second digit. For example, report a count of 142 as 140 and a count of 155 as 160, but report a count of 35 as 35.
- If the number of colonies per plate far exceeds 300, do not report result as ‘‘too numerous to count’’ (TNTC) otherwise use one of the following rules:

- When fewer than 10 colonies/cm^2 are present, count colonies in 19 squares (of the colony counter) having representative colony distribution. Multiply sum of the number of colonies by 3 to compute estimated colonies per plate when the plate area is 57 cm^2.
- When more than 10 colonies/cm^2 are present, count four representative squares, take average count per square centimeter, and multiply by 57 to compute estimated colonies per plate when the plate area is 57 cm^2.
- When bacterial counts on crowded plates are greater than 100 colonies/cm^2, report result as greater than (>) 5700 divided by the smallest sample volume plated for plastic plates.
- If plates have excessive spreader growth, report as ‘‘spreaders’’ (Spr)
- When plates are uncountable because of missed dilution, accidental dropping, and contamination, or the control plates indicate that the medium or other material or lab ware was contaminated, report as ‘‘laboratory accident’’ (LA).

13. References:

- Eugene W. Rice, Rodger B. Baired, Andrew D. Eaton, Lenore S. Clesceri eds.2012 Standard Methods for the Examination of Water and Wastewater. Method 9215C#, 22nd edition (on-line edition). American Public Health Association, American Water Works Association, and Water Environment Federation
- World Health Organization. 2011, Guidelines for Drinking-Water Quality, Fourth Edition. Geneva,
- U.S. Environmental Protection Agency.2005.Manual of The Certification of laboratories Analyzing Drinking Water, Criteria and Procedures Quality Assurance. EPA 815-R-05-054. Office of Ground Water and Drinking Water. Cincinnati, Ohio.
- Health Canada. 2006. Guidelines for Canadian Drinking water: Guideline Technical Document. Heterotrophic Plate Count. Ottawa. Ontario.

3. Estimation of Total Coliform by Multiple Tube Fermentation Technique

1. Scope:

- Estimation of Total Coliforms (TC) by Multiple-Tube Fermentation technique (MTF), also called Most Probable Number (MPN) procedure, in 96 hours, or less, in water samples on the basis of the production of gas and acid from fermentation of lactose.
- This procedure can be applied for different types of water, in microbiology laboratory it will be applied for the analysis of surface raw water.

2. Principle:

- Volumes/dilutions of water sample to be tested are added to tubes containing lauryl Tryptose broth as presumptive media (5 tubes per dilution) with inverted vials, or pH indicator. The selectivity of media is due to sodium lauryl sulfate that acting as inhibitor of bacteria other than coliforms. After incubation, the tubes are examined for growth, gas, and/or acidic reaction (shades of yellow color) if inner vial is omitted, growth with acidity (yellow color) is signifies a positive presumptive reaction.
- An additional confirmatory test by brilliant green bile broth 2% is required to confirm the result. The selectivity is due to presence of both oxgall (bile) and brilliant green dye acting as inhibitor of gram positive and selected gram negative bacteria. Organisms, primarily coliform, which are resistant to those inhibitor and ferment lactose with gas formation, indicated by Durham tube, can replicate in this medium.

- Total coliform bacteria (excluding E. coli) occur in both sewage and natural waters. Some of these bacteria are excreted in the faeces of humans and animals, but many coliforms are heterotrophic and able to multiply in water and soil environments.
- Total coliforms include organisms that can survive and grow in water distribution system, particularly in the presence of biofilms. They were, traditionally, regarded as belonging to the genera Escherichia, Citrobacter, Klebsiella and Enterobacter, but the group is more heterogeneous and includes a wider range of genera, such as Serratia and Hafnia. Hence, they are not useful as an index of faecal pathogens, but they can be used as an indicator of treatment effectiveness and to assess the cleanliness and integrity of distribution systems and the potential presence of biofilms. However, there are better indicators for these purposes.
- The presence of Total Coliform after disinfection indicates inadequate treatment, and presence in distribution system or stored water supplies can reveal regrowth and possible biofilm formation or contamination through ingress of foreign material, including soil or plants.
- The MTF procedure, in comparison with the membrane filter (MF) procedure, is more difficult to perform, takes longer to produce results, and lacks precision; the precision depends on the number of tubes used. The most satisfactory information will be obtained when the largest sample inoculum examined shows acid and/or gas in some or all of the tubes and the smallest sample inoculum shows no acid and/or gas in any or a majority of the tubes. However, the MTF procedure is still of value when conditions render the MF technique unusable e.g. with turbid, colored, or grossly contaminated water
- Results are reported in terms of the Most Probable Number (MPN/100ml) of organisms present. It can be estimated by the formula given below (section 11) or from MPN tables (Appendix 2) using the number of positive tubes in the multiple dilutions. This number, based on certain probability formulas, is an estimate of the mean density of total coliforms in the sample.

3. Definitions:

- Total coliform bacteria in this SOP are those facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 hours at 35°C.

4. Environmental Conditions:

- All sample analysis steps will be carried on disinfected working bench inside cultivation room to ensure controlled environmental conditions.

5. Interference:

- Since the MPN indexes are based on assumption of a Poisson distribution (random distribution) that's for if the sample is not adequately shaken before portions are removed or if bacterial cells clump, the MPN value will be an underestimate of actual bacterial density.
- High densities of non-coliform bacteria and the inhibitory nature of some MTF media, e.g. Lauryl Tryptose broth, may prevent gas formation. It's recommended to treat all tubes with turbidity, indicating growth, regardless of gas production, as presumptive total coliform-positive tubes in case if tubes form the next dilution gives true positive reaction.

6. Equipment:

- 16 × 100 mm tubes, borosilicate glass, with heat-resistant plastic caps.
- 16 × 150 mm tubes, borosilicate glass, with heat-resistant plastic caps.
- Durham tubes, borosilicate glass.
- Test tubes racks to hold sterile culture tubes.
- Automatic pipettes and associated sterile long tips capable of delivering 1 ml.
- Dilution screwed cap bottles.
- Incubator: 35°C ± 0.5°C.
- Sterile disposable applicator stick.
- Sterile plastic petri dishes 90 mm.
- Sterile plastic petri dishes 50 mm.

- Indelible ink marker for labeling plates.

7. Chemicals and Reagents:

- Lauryl Tryptose broth.
- Brilliant Green broth.
- Phosphate buffered dilution water.
- Nutrient agar.
- Gram Stain Reagent set.
- M-Endo LES agar.
- Bromcresol purple.

8. Precautions:

- Follow the normal safety procedures required in microbiology laboratory.
- Mouth-pipetting is prohibited.
- Use a separate sterile tip for initial and subsequent transfers from each tube, and different dilution.
- Replace the tip with a sterile one if becomes contaminated before transfers are completed.
- Do not prepare dilutions in the direct of sunlight.
- Do not insert tips more than 2 to 3 cm below the surface of sample or dilution when removing the sample volume.
- Avoid contamination when removing sterile tips from the container by not dragging tip across lips and necks of dilution bottles.
- Avoid picking up any membrane or scum on the needle while transferring by inclining the fermentation tube.
- Insert end of loop or needle into the liquid in the tube to a depth of approximately 0.5 cm.
- Decontaminate all the used tubes, plates, and materials at the end of the analyses.

9. Procedure:

9. 1 Responsibility :

- Microbiologists are responsible for the implementation of the test procedures inside the microbiology laboratory.
- Laboratory technician is responsible for preparation of the media and reagents used in this procedure under supervision of the microbiologist.

9. 2 Sample Handling:

- Analyze samples on day of receipt whenever possible. The time between sample collection and the placement of samples in the incubator must not exceed 30 hours.
- If arrival is too late for processing on same day, refrigerate overnight as long as holding time conditions still be met i.e. **preservation temperature <8.0°C and time did not exceeded 30 h.**

9. 3 Instrument Calibration:

- Calibrate and verify balances daily using reference weights.
- Calibrate pH meter prior to use, using at least two standards points.
- Check temperature of incubators and refrigerators daily to ensure operation within stated limits of method.
- Check sterilization procedure and efficiency to ensure media sterility used in this SOP.

9. 4 Presumptive Phase:

9.4.1 Selection of sample size:

- Inoculate a series of tubes with at least three decimal dilutions (see 10.4.3) of water sample (multiples and submultiples of 10 ml), based on the probable total coliform density.

9.4.2 Media Preparation:

- Prepare and handle media and reagents appropriately, and carry out suitable performance test to ensure homogeneity, sterility, and suitability for each prepared batch.
- **Phosphate buffered dilution water**:
- **Stock phosphate buffer solution**: Dissolve 34.0 g KH_2PO_4 in a 500 ml reagent-grade distilled water, adjust the pH of the solution to 7.2 with 1 N NaOH and bring volume to 1000 ml with reagent-grade distilled water. Sterilize by autoclave for 15 minutes at 121°C, store in the refrigerator until using (If evidence of mold or other contamination appears in the stock, discard the solution, and prepare a fresh solution).
- **$MgCl_2$ solution**: Dissolve 38 g anhydrous $MgCl_2$ (or 81.1 g $MgCl_2.6H_2O$) in one liter of reagent-grade distilled water. Sterilize by autoclave for 15 minutes at 121°C, store in the refrigerator until using (If evidence of mold or other contamination appears in the stock, discard the solution, and prepare a fresh solution).
- **Working solution**: Add 1.25 ml phosphate buffer stock and 5 ml $MgCl_2$ stock for each liter of reagent-grade distilled water prepared, mix well(Final pH 7.0 ± 0.2), and dispense in appropriate amounts for dilutions in screw-cap dilution bottles or culture tubes, and/or into larger containers for use as rinse water. Autoclave at 121°C for 15 minutes.
- **Lauryl Tryptose broth:**
- Prepare and sterilize medium according to manufacturer instructions.
- Dispense 10 ml medium, before sterilization, in fermentation tubes with an inverted Durham tube.
- Alternatively, and only in case of unavailability of Durham tubes, add 0.01 g/L bromcresol purple to medium to determine acid production.
- Close tubes with heat-resistant plastic caps.
- Ensure, after sterilization, that media cover inverted tube with at least one-half to two-thirds, and completely filled with media i.e. no air space inside inverted Durham tubes.
- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes).

9.4.3 Samples Dilutions:

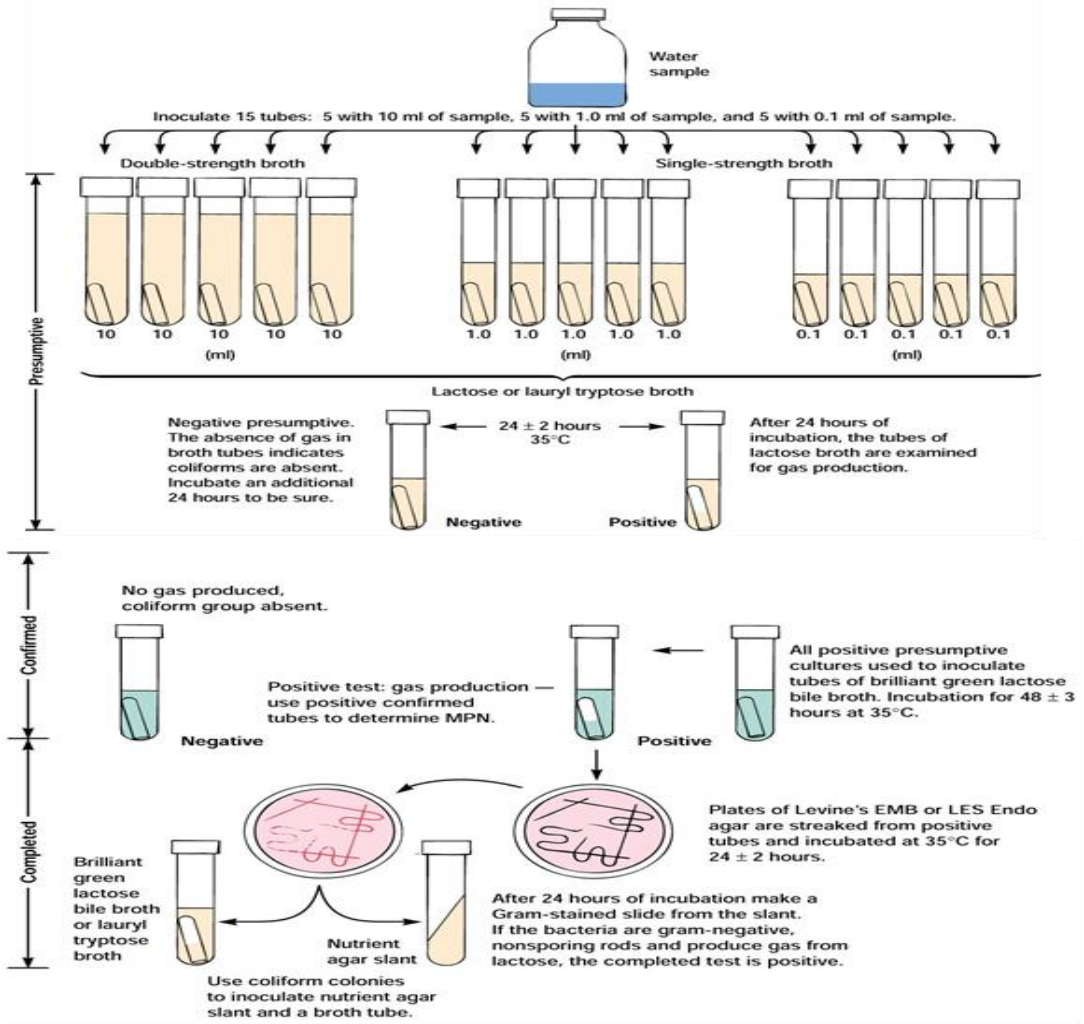
- Mix the sample by vigorously shakes the bottle.
- Use a sterile tip to transfer 10 ml of sample to 90 ml, or 1 ml of sample to 9 ml of sterile dilution water bottle, cap, and mix. 1 ml of this dilution is considered 10-1 of the original sample.
- Repeat the previous step to prepare further dilutions.

9.4.4 Inoculation:

- Use lauryl tryptose broth for presumptive phase.
- Arrange fermentation tubes in rows.
- Prepare four sets of serial dilutions (1.0, 0.1, 0.01, 0.001 ml) using five tubes per dilution.
- Shake sample or dilutions vigorously.
- Inoculate each tube in the set with sample or dilution volume.
- Mix test portions in the medium by gentle agitation.

9.4.5 Incubation:

- Incubate inoculated tubes at 35 ± 0.5°C.
- After 24 ± 2 h swirl each tube gently and examine it for growth, gas, and acidic reaction (shades of yellow color). If inner vial is omitted, growth with acidity (yellow color) is signifies a positive presumptive reaction
- If no gas or acidic reaction is evident, reincubate and reexamine at the end of 48 ± 3 h.
- Record presence or absence of growth, gas, and acid production.



9.4.6 Interpretation:

- Production of an acidic reaction and/or gas in the tubes within 48 ±3 h constitutes a positive presumptive reaction.
- The absence of acidic reaction and/or gas formation at the end of 48 ±3 h of incubation constitutes a negative test.

9. 5 Confirmed Phase:

9.5.1 Media Preparation:

- **Brilliant green broth:**
- Prepare and sterilize medium according to manufacturer instructions.
- Dispense 10 ml medium, before sterilization, in fermentation tubes with an inverted Durham tube.
- Close tubes with heat-resistant plastic caps.
- Ensure, after sterilization, that media cover inverted tube with at least one-half to two-thirds, and completely filled with media i.e. no air space inside inverted Durham tubes.

- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes) after preparation.

9.5.2 Inoculation:

- Submit tubes with a positive presumptive reaction to the confirmed phase within 24 ± 2 h of incubation to the confirmed phase.
- If additional presumptive tubes showed active fermentation or acidic reaction at the end of 48 ± 3 h incubation period, submit to the confirmed phase.
- Arrange brilliant green tubes rows in a similar manner to positive presumptive tubes.
- Gently rotate positive presumptive tubes to resuspend the organisms.
- Use a sterile loop 3.0 mm in diameter to transfer three loopfuls from positive presumptive tube to a brilliant green tube confirmation tube.
- Repeat for all other positive presumptive tubes.

9.5.3 Incubation:

- Incubate the inoculated tubes at 35 ± 0.5°C for 48 ± 3 h

9.5.4 Interpretation:

- Formation of gas in any amount, in Durham tube, of the brilliant green tubes, at any time, within 48 ± 3 h constitutes a positive confirmed result.
- Calculate the MPN value (see section 11.0) of the number of positive brilliant green lactose bile tubes from MPN index (see A.1).

9. 6 Completed Phase:

9.6.1 Application:

- This phase is aiming to verify the presence of coliform bacteria and to provide quality control data for non-potable water sample analysis.
- Use the completed test on 10% of positive confirmed tubes on seasonal basis. Analysis of samples for thermotolerant (fecal)

coliforms at elevated temperature (44.5±0.2°C) can be considered as a completed test.

- Alternatively, the completed test for positive total coliforms may be performed as follows:

9.6.2 Media Preparation:

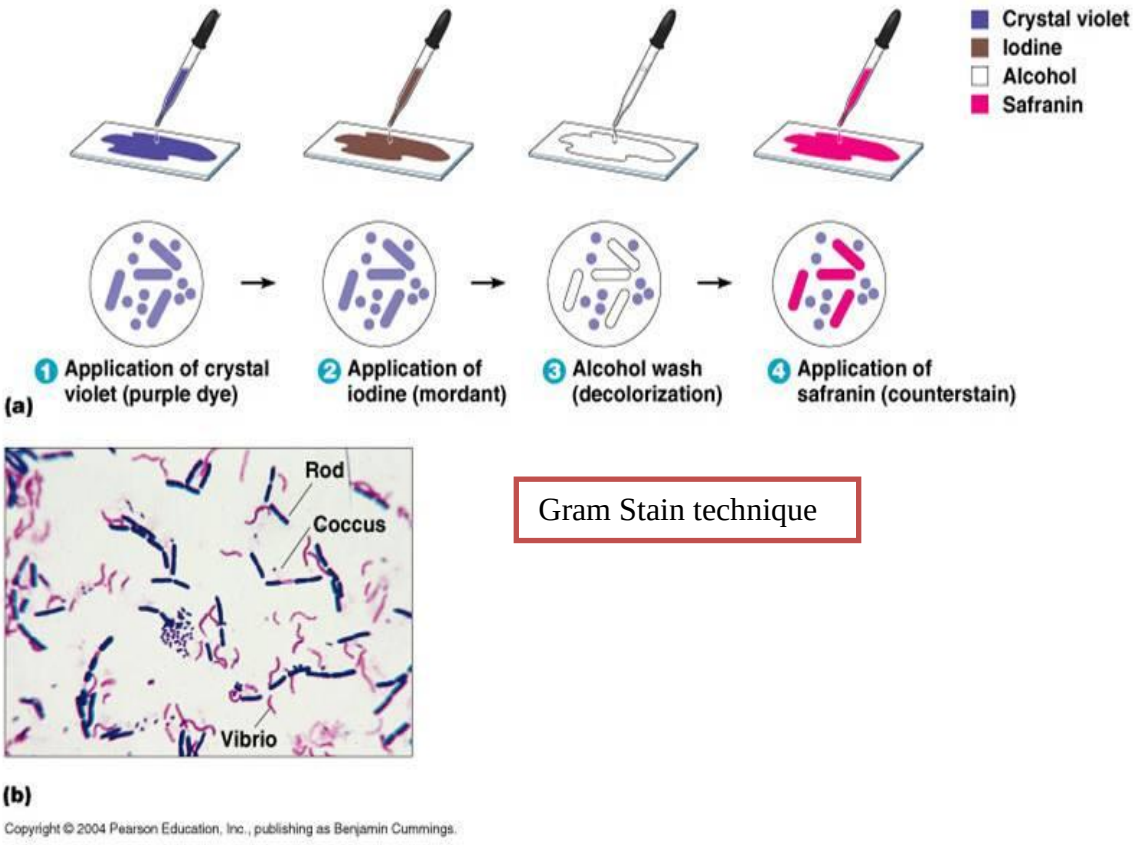
- Use LES Endo agar plates and Nutrient agar slants for completed phase.
- **m-Endo LES agar**:
- Prepare and sterilize medium according to manufacturer instructions.
- Dispense in 5 to 7 ml quantities into lower section of 50 mm Petri dishes, if dishes of any other size are used, adjust quantity to give an equivalent depth of 4 to 5 mm.
- If plates are previously prepared and stored in the refrigerator, allow them to warm at room temperature. The crystals that form on agar plates after refrigeration will disappear as the plates warm up. Use plates within two weeks after preparation.
- **Nutrient agar:**
- Prepare and sterilize medium according to manufacturer instructions.
- Dispense 10 ml in screw-capped tubes before sterilization.
- Place tubes in an inclined position immediately after sterilization, so that the agar will solidify with a sloped surface.
- Tighten screw caps after cooling and store in a protected, cool storage area.
- If plates or tubes are made ahead of time and stored in the refrigerator, remove them and allow warming at room temperature before use.

9.6.3 Procedure:

- Use a sterile 3-mm-diam loop or an inoculating needle slightly curved at the tip
- Tap and incline the fermentation tube to avoid picking up any membrane or scum on the needle
- Insert the end of the loop or needle into the liquid in the tube to a depth of approximately 0.5 cm

- Streak a plate for isolation with the curved section of the needle in contact with the agar to avoid a scratched or torn surface
- Change loop between second and third quadrants to improve colony isolation.
- Incubate plates (inverted) at 35 ± 0.5°C for 24 ± 2 h.
- Pick from each plate one or more typical (pink to dark red with a green metallic surface sheen) well-isolated coliform colonies. If no typical colonies are present, pick two or more colonies considered most likely to consist of organisms of the coliform group.
- Transfer growth from each isolate, by barely touching the surface of colony to minimize the danger of transferring mixed culture, to a single-strength lauryl tryptose broth fermentation tube and onto a nutrient agar slant.
- Incubate nutrient agar slants at 35 ± 0.5°C for 24 ± 2 h.
- Incubate secondary broth tubes at 35 ± 0.5°C for 24 ± 2 h; if gas is not produced within 24 ± 2 h reincubate and examine again at 48 ± 3 h.
- Examine Gram stained preparations from nutrient agar slant cultures corresponding to the secondary tubes that showed gas; include gram positive and gram negative cultures as controls.
- **Gram Stain technique :**
- Prepare separate light emulsions of the test bacterial growth using drops of distilled water on the slide.
- Prepare separate light emulsions of the positive and negative control cultures on the same slide using drops of distilled water on the slide.
- Fix the slides by air drying.
- Stain for 1 min with ammonium oxalate-crystal violet solution, rinse slides in tap water and drain off excess.
- Apply Lugol’s solution for 1 min, rinse stained slides in tap water.
- Decolorize for approximately 15 to 30 s with acetone alcohol by holding slide between the fingers and letting acetone alcohol flow across the stained smear until the solvent flows colorlessly from the slide. Do not over-decolorize to not remove crystal violet from gram positive bacteria.
- Counterstain with safranin for 15 s, rinse with tap water.

- Blot dry with absorbent paper or air dry, and examine microscopically.
- Gram-positive organisms are blue (violet); gram-negative organisms are red.
- Results are only acceptable when controls given proper reactions.



Gram Stain technique

9.6.4 Interpretation:

- Formation of gas in the secondary tube of lauryl tryptose broth within 48 ± 3 h and demonstration of gram-negative, nonspore-forming, rod-shaped bacteria from the agar culture constitute a positive result for completed test, demonstrating the presence of a member of the coliform group.

10. Calculations:

- The MPN values, for variety of positive and negative tubes combinations, are given in appendix A.1.
- The samples volumes indicated in indexes A.1 illustrates MPN values for combinations of positive results when five 10 ml, five 1 ml, and five 0.1 ml sample portion volumes are tested.

- If the sample portion volumes used are those found in the tables, report the value corresponding to the number of positive and negative results in the series as the MPN/100ml.
- When the series of decimal dilutions is different from that in the table, select the MPN value from index A.1 for the combination of positive results and calculate according to the following formula :

$$\text{MPN / 100 ml} = (\text{Table MPN/ 100 ml}) \times 10/V$$

Where:

V = volume of sample portion at the lowest selected dilution.

- When more than three dilutions are used in a decimal series of dilutions, select the three most appropriate dilutions and refer to index A.1.
- Selected examples(A though C) illustrating correct selection are listed below, more examples are available in Standard Methods (see 16.0):

Example	Volumes *ml*					Combination of positive	MPN index / 100 ml
	10	1.0	0.1	0.01	0.001		
A	5	**5**	**1**	**0**	0	5-1-0	330
B	**4**	**5**	**1**	0	0	4-5-1	48
C	**4**	**3**	**0**	1	1	4-3-2	39

- Example A: First, remove the highest dilution (smallest sample volume) if it has all negative tubes and at least one remaining dilution has a negative tube. Next, remove the lowest dilution (largest sample volume) if it has all positive tube and at least one remaining dilution has a positive tube. According to these guidelines, the three dilutions in Example A are selected by removal of the highest (0.001-ml) and the lowest (10-ml) dilutions.
- Example B: If the lowest dilution does not have all positive tubes, and several of the highest dilutions have all negative tubes, then remove the highest negative dilutions.

- Example C: If no dilution has all positive tubes, select the lowest two dilutions, corresponding to 10 and 1 ml sample. For the third dilution, add the number of positive tubes in the remaining dilutions (0.1, 0.01, and 0.001 ml sample), to yield a final combination of 4-3-2
- If MPN value for combinations not appearing in the table, or for other combinations of tubes or dilutions, estimate the result as follows:
- First, select the lowest dilution that does not have all positive results.
- Second, select the highest dilution with at least one positive result.
- Select all dilutions between them.
- Use the selected dilutions in the following formula:

$$MPN / 100 ml (approx.) = 100 \times P / (N \times T)^{½}$$

Where:

P: number of positive results.

N: Volume of sample in all negative portions combined.

T: Total volume of sample in the selected dilutions.

- Examples :
- From (5/5, 10/10, 4/10, 1/10, 0/5) Use only (-, -, 4/10, 1/10. -), 4/10 @ 0.1 ml sample/tube and 1/10 @ 0.01 ml sample/tube. calculations will be:

MPN / 100 ml (approx.) = 100 × 5 / (0.69 ×1.1) ½ = 500/0.87 = 570/ 100 ml

- From (5/5, 10/10, 10/10, 0/10, 0/5) Use only (-, -, 10/10, 0/10. -) 10/10 @ 0.1 ml sample/tube and 0/10 @ 0.01 ml sample/tube.

calculations will be:

MPN / 100 ml (approx.) = 100 × 10 / (0.1×1.1) ½ = 1000/0.332 = 3000/ 100 ml.

11. Quality Control:

11.1 Analytical Quality Control:

- Apply the presumptive-confirmed phase of the multiple-tube procedure to all samples, if samples were analyzed for fecal coliform using MTF technique there will be no need to apply completed phase, otherwise proceed completed test (Section 10.6) to not less than 10% of all coliform-positive samples on a seasonal basis.
- Plates with non-selective agar medium, labeled as "**Environmental Control**", will be opened along the samples analysis run time to ensure adequate environmental conditions.
- Procedural Blank: Inoculate, under the same conditions as sample, one additional tube for each dilution with sterile phosphate buffered dilution water after inoculation of series of 10 samples. Absence of growth indicates absence of cross contamination.

11.2 Non Conformance and Corrective Action:

- If the media performance and sterility assessment are performed concurrently with the use of the media in testing and the media quality is deemed unsatisfactory, the specific use of the media in the test(s) should be investigated to determine the potential impact on the test results and the necessity for repeating the test(s).
- Testing must be repeated when unsatisfactory media and/or reagents are used for critical aspects of testing.
- If procedural blank test gives unsatisfactory results, the samples results should be investigated to determine the potential impact of contamination on the test result, if test results deemed unsatisfactory the test must be repeated.
- If dilution water gives positive result, the samples results should be investigated to determine the potential impact of dilution water on the test result, if test results deemed unsatisfactory the test must be repeated.
- If one or more of environmental control plates gives unsatisfactory results, the samples results should be investigated to determine the potential impact on the test result, if test results deemed unsatisfactory the test must be repeated.

- For all non conformance findings, trace all media, reagents, equipment operations, and tools used in analysis to find out root cause of non conformance.
- Take appropriate corrective action, if cause was temporary e.g. if unsterile batch of media used accidently in the analysis; discard all batch of prepared media, or preventive actions if cause was permanent e.g. re-maintenance of autoclave if actual temperature was less than adjusted value, to eliminate reoccurrence of non conformance in the future.

12. Method Performance Data:

- A limited intralaboratory study was conducted. Four participated microbiological analysts tested surface raw water matrix.
- Results of this intralaboratory study, table 1, may not be typical of results for samples other than those studied, hence secondary validation (cross validation) in form of long-term study shall be carried out depending on routine samples results.
- Table 1

Criteria	Surface Raw
Precision/ repeatability	0.461
Precision/Reproducibility	0.81
Bias %	±0.03

- Detection limit was originally established as 1.8 MPN/100 ml.
- Upper working limit was previously established for this method as 1600 MPN/100ml.
- Range of reliable estimation was previously established for this method between 1.8-1600 MPN/ 100ml.

13. Reporting:

- Report coliform concentration as the Most Probable Number (MPN)/100 ml.
- If sample was delayed more than permissible time, write “Delayed” in report comment.

14. References:

- Eugene W. Rice, Rodger B. Baired, Andrew D. Eaton, Lenore S. Clesceri eds.2012 Standard Methods for the Examination of Water and Wastewater. Method 9221B#, 22nd edition (on-line edition). American Public Health Association, American Water Works Association, and Water Environment Federation
- World Health Organization. 2011, Guidelines for Drinking-Water Quality, Fourth Edition. Geneva
- Guidelines for Canadian Drinking water: Guideline Technical Document. 2006. Total Coliform. Health Canada. Ottawa. Ontario.

A.1 MPN index and 95% confidence limits for various combinations of positive results when five tubes are used per dilution (10 ml, 1.0 ml, 0.1 ml).

Combination of Positives	MPN Index/100 mL	Confidence Limits		Combination of Positives	MPN Index/100 mL	Confidence Limits	
		Low	High			Low	High
0-0-0	<1.8	—	6.8	4-0-3	25	9.8	70
0-0-1	1.8	0.090	6.8	4-1-0	17	6.0	40
0-1-0	1.8	0.090	6.9	4-1-1	21	6.8	42
0-1-1	3.6	0.70	10	4-1-2	26	9.8	70
0-2-0	3.7	0.70	10	4-1-3	31	10	70
0-2-1	5.5	1.8	15	4-2-0	22	6.8	50
0-3-0	5.6	1.8	15	4-2-1	26	9.8	70
1-0-0	2.0	0.10	10	4-2-2	32	10	70
1-0-1	4.0	0.70	10	4-2-3	38	14	100
1-0-2	6.0	1.8	15	4-3-0	27	9.9	70
1-1-0	4.0	0.71	12	4-3-1	33	10	70
1-1-1	6.1	1.8	15	4-3-2	39	14	100
1-1-2	8.1	3.4	22	4-4-0	34	14	100
1-2-0	6.1	1.8	15	4-4-1	40	14	100
1-2-1	8.2	3.4	22	4-4-2	47	15	120
1-3-0	8.3	3.4	22	4-5-0	41	14	100
1-3-1	10	3.5	22	4-5-1	48	15	120
1-4-0	10	3.5	22	5-0-0	23	6.8	70
2-0-0	4.5	0.79	15	5-0-1	31	10	70
2-0-1	6.8	1.8	15	5-0-2	43	14	100
2-0-2	9.1	3.4	22	5-0-3	58	22	150
2-1-0	6.8	1.8	17	5-1-0	33	10	100
2-1-1	9.2	3.4	22	5-1-1	46	14	120
2-1-2	12	4.1	26	5-1-2	63	22	150
2-2-0	9.3	3.4	22	5-1-3	84	34	220
2-2-1	12	4.1	26	5-2-0	49	15	150
2-2-2	14	5.9	36	5-2-1	70	22	170
2-3-0	12	4.1	26	5-2-2	94	34	230
2-3-1	14	5.9	36	5-2-3	120	36	250
2-4-0	15	5.9	36	5-2-4	150	58	400
3-0-0	7.8	2.1	22	5-3-0	79	22	220
3-0-1	11	3.5	23	5-3-1	110	34	250
3-0-2	13	5.6	35	5-3-2	140	52	400
3-1-0	11	3.5	26	5-3-3	170	70	400
3-1-1	14	5.6	36	5-3-4	210	70	400
3-1-2	17	6.0	36	5-4-0	130	36	400
3-2-0	14	5.7	36	5-4-1	170	58	400
3-2-1	17	6.8	40	5-4-2	220	70	440
3-2-2	20	6.8	40	5-4-3	280	100	710
3-3-0	17	6.8	40	5-4-4	350	100	710
3-3-1	21	6.8	40	5-4-5	430	150	1100
3-3-2	24	9.8	70	5-5-0	240	70	710
3-4-0	21	6.8	40	5-5-1	350	100	1100
3-4-1	24	9.8	70	5-5-2	540	150	1700
3-5-0	25	9.8	70	5-5-3	920	220	2600
4-0-0	13	4.1	35	5-5-4	1600	400	4600
4-0-1	17	5.9	36	5-5-5	>1600	700	—
4-0-2	21	6.8	40				

* Results to two significant figures.

4. Estimation of Fecal Coliform by Multiple Tube Fermentation Technique

1. Scope:

- This is a detailed procedure for estimation of Fecal Coliforms (FC) by Multiple-Tube Fermentation technique (MTF), also called Most Probable Number (MPN) procedure, in 72 hours, or less, in water samples on the basis of the production of gas and acid from fermentation of lactose.
- This procedure can be applied for different types of water, in microbiology laboratory it will be applied for the analysis of surface raw water.

2. Principle:

- Volumes/dilutions of water sample to be tested are added to tubes containing lauryl Tryptose broth as presumptive media (5 tubes per dilution) with inverted vials, or pH indicator. The selectivity of media is due to sodium lauryl sulfate that acting as inhibitor of bacteria other than coliforms. After incubation, the tubes are examined for, gas, and/or acidic reaction (shades of yellow color). If inner vial is omitted, growth with acidity (yellow color) is signifies a positive presumptive reaction.
- An additional confirmatory test by EC broth is required to confirm the result. The selectivity is due to presence of bile salt that acting as inhibitor of gram positive bacteria, particularly bacilli and fecal streptococci. Incubation at elevated incubation temperature (44.5±0.2°C) inhibits other coliform bacteria, which is not thermo-tolerant, from growing up.
- Total coliform bacteria that are able to ferment lactose at 44.5°C are known as thermo-tolerant coliforms. Thermo-tolerant coliforms were traditionally called fecal coliforms, but they also have been documented in organically rich waters or tropical climates in the absence of recent fecal contamination. So, testing for E. coli, a specific indicator of fecal contamination, is recommended.
- In most waters, the predominant genus is ***Escherichia***, but some types of ***Citrobacter***, ***Klebsiella*** and ***Enterobacter*** are also thermo-tolerant. They are usually found in sewage and water recently subjected to fecal pollution.

- Populations of thermo-tolerant coliforms are composed predominantly of *E. coli*; as a result, this group is regarded as a less reliable but acceptable index of faecal pollution. The presence of thermo-tolerant coliform (fecal coliform) provides evidence of recent fecal pollution
- The MTF procedure, in comparison with the membrane filter (MF) procedure, is more difficult to perform, takes longer to produce results, and lacks precision; the precision depends on the number of tubes used. The most satisfactory information will be obtained when the largest sample inoculum examined shows acid and/or gas in some or all of the tubes and the smallest sample inoculum shows no acid and/or gas in any or a majority of the tubes. However, the MTF procedure is still of value when conditions render the MF technique unusable e.g. with turbid, colored, or grossly contaminated water
- Results are reported in terms of the Most Probable Number (MPN/100ml) of organisms present. It can be estimated by the formula given below (section 11) or from MPN tables (Appendices) using the number of positive tubes in the multiple dilutions. This number, based on certain probability formulas, is an estimate of the mean density of total coliforms in the sample.

3. Definitions:

- Fecal coliform bacteria in this SOP are those facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 24 hours at 44.5°C.

4. Environmental Conditions:

- All sample analysis steps will be carried on disinfected working bench inside cultivation room to ensure controlled environmental conditions.

5. Interference:

- Since the MPN indexes are based on assumption of a Poisson distribution (random distribution) that's for if the sample is not adequately shaken before portions are removed or if bacterial

cells clump, the MPN value will be an underestimate of actual bacterial density.

- High densities of non-coliform bacteria and the inhibitory nature of some MTF media, e.g. Lauryl Tryptose broth, may prevent gas formation. It's recommended to treat all tubes with turbidity, indicating growth, regardless of gas production, as presumptive fecal coliform-positive tubes in case if tubes form the next dilution gives true positive reaction.

6. Equipment:

- 16 × 100 mm tubes, borosilicate glass, heat-resistant plastic caps.
- 16 × 150 mm tubes, borosilicate glass, with heat-resistant plastic caps.
- Durham tubes, borosilicate glass.
- Test tubes racks to hold sterile culture tubes.
- Automatic pipettes and associated sterile long tips capable of delivering 1 ml and up to10 ml.
- Dilution screwed cap bottles.
- Incubator: 35°C ± 0.5°C.
- Incubator: 44.5°C ± 0.2°C.
- Sterile disposable applicator stick.
- Sterile plastic petri dishes 90 mm.
- Sterile plastic petri dishes 50 mm.
- Indelible ink marker for labeling plates.

7. Chemicals and Reagents:

- Lauryl Tryptose broth.
- EC broth.
- Phosphate buffered dilution water.
- Bromcresol purple.

8. Precautions:

- Follow the normal safety procedures required in microbiology laboratory.
- Mouth-pipetting is prohibited.

- Use a separate sterile tip for initial and subsequent transfers from each tube, and different dilution.
- Replace the tip with a sterile one if becomes contaminated before transfers are completed.
- Do not prepare dilutions in the direct of sunlight.
- Do not insert tips more than 2.5 cm below the surface of sample or dilution when removing the sample volume.
- Avoid contamination when removing sterile tips from the container by not dragging tip across lips and necks of dilution bottles.
- Avoid picking up any membrane or scum on the needle while transferring by inclining the fermentation tube.
- Insert end of loop or needle into the liquid in the tube to a depth of approximately 0.5 cm.
- Decontaminate all the used tubes, plates, and materials at the end of the analyses.

9. Procedure:

9. 1 Responsibility:

- Microbiologists are responsible for the implementation of the test procedures inside the microbiology laboratory.
- Laboratory technician is responsible for preparation of the media and reagents used in this procedure under supervision of the microbiologist(s),.
- Microbiologists and technician are well trained in the implementation and performance of these procedures.

9. 2 Sample Handling:

- Analyze samples on day of receipt whenever possible. The time between sample collection and the placement of samples in the incubator must not exceed 30 hours.
- If arrival is too late for processing on same day, refrigerate overnight as long as holding time conditions still be met i.e. preservation temperature <8.0°C and time did not exceeded 30 hr.

9. 3 Instrument Calibration:

- Calibrate and verify balances daily using reference weights.

- Calibrate pH meter prior to use, using at least two standards points.
- Check temperature of incubators and refrigerators daily to ensure operation within stated limits of method.
- Check sterilization procedure and efficiency to ensure media sterility used in this SOP.

9. 4 Presumptive Phase:

9.4.1 Selection of sample size:

- Inoculate a series of tubes with at least three decimal dilutions (see 10.4.3) of the water (multiples and submultiples of 10 ml), based on the probable fecal coliform density.

9.4.2 Media Preparation:

- Prepare and handle media and reagents appropriately, and carry out suitable performance test to ensure homogeneity, sterility, and suitability for each prepared batch.
  - **Phosphate buffered dilution water**:
- **Stock phosphate buffer solution**: Dissolve 34.0 g KH_2PO_4 in a 500 ml reagent-grade distilled water, adjust the pH of the solution to 7.2 with 1 N NaOH and bring volume to 1000 ml with reagent-grade distilled water. Sterilize by autoclave for 15 minutes at 121°C, store in the refrigerator until using (If evidence of mold or other contamination appears in the stock, discard the solution, and prepare a fresh solution).
- **$MgCl_2$ solution**: Dissolve 38 g anhydrous $MgCl_2$ (or 81.1 g $MgCl_2.6H_2O$) in one liter of reagent-grade distilled water. Sterilize by autoclave for 15 minutes at 121°C, store in the refrigerator until using (If evidence of mold or other contamination appears in the stock, discard the solution, and prepare a fresh solution).
- **Working solution**: Add 1.25 ml phosphate buffer stock and 5 ml $MgCl_2$ stock for each liter of reagent-grade distilled water prepared, mix well(Final pH 7.0 ± 0.2), and dispense in appropriate amounts for dilutions in screw-cap dilution bottles or culture tubes, and/or into larger containers for use as rinse water. Autoclave at 121°C for 15 minutes.
  - **Lauryl Tryptose broth:**

- Prepare and sterilize medium according to manufacturer instructions.
- Dispense 10 ml medium, before sterilization, in fermentation tubes with an inverted Durham tube.
- Alternatively, and only in case of unavailability of Durham tubes, add 0.01 g/L bromo-cresol purple to medium to determine acid production.
- Close tubes with heat-resistant plastic caps.
- Ensure, after sterilization, that media cover inverted tube with at least one-half to two-thirds, and completely filled with media i.e. no air space inside inverted Durham tubes.
- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes) after preparation.

9.4.3 Samples Dilutions:

- Mix the sample by vigorously shakes the bottle.
- Use a sterile tip to transfer 10 ml of sample to 90 ml, or 1 ml to 9 ml, of sterile dilution water bottle, cap, and mix. 1 ml of this dilution is considered 10^{-1} of the original sample.
- Repeat the previous step to prepare further dilutions.

9.4.4 Inoculation:

- Use lauryl tryptose broth for presumptive phase.
- Arrange fermentation tubes in rows.
- Prepare four sets of serial dilutions (1, 0.1, 0.01, 0.001 ml) using five tubes per dilution.
- Shake sample or dilutions vigorously.
- Inoculate each tube in the set with sample or dilution volume.
- Mix test portions in the medium by gentle agitation.

9.4.5 Incubation:

- Incubate inoculated tubes at 35 ± 0.5°C.
- After 24 ± 2 h swirl each tube gently and examine it for growth, gas, and acidic reaction (shades of yellow color) If inner vial is omitted, growth with acidity (yellow color) is signifies a positive presumptive reaction.

- If no gas or acidic reaction is evident, re-incubate and reexamine at the end of 48 ± 3 h.
- Record presence or absence of growth, gas, and acid production.
- If the inner vial is omitted, growth with acidity signifies a positive presumptive reaction.

9.4.6 Interpretation:

- Production of an acidic reaction and/or gas in the tubes within 48 ±3 h constitutes a positive presumptive reaction.
- The absence of acidic reaction and/or gas formation at the end of 48 ±3 h of incubation constitutes a negative test.

9. 5 Confirmed Phase:

9.5.1 Media Preparation:

- **EC broth.**

- Prepare and sterilize medium according to manufacturer instructions.
- Dispense 10 ml medium, before sterilization, in fermentation tubes with an inverted Durham tube.
- Close tubes with heat-resistant plastic caps.
- Ensure, after sterilization, that media cover inverted tube with at least one-half to two-thirds, and completely filled with media i.e. no air space inside inverted Durham tubes.
- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes) after preparation.

9.5.2 Inoculation:

- Submit tubes with a positive presumptive reaction to the confirmed phase within 24 ± 2 h of incubation to the confirmed phase.
- If additional presumptive tubes showed active fermentation or acidic reaction at the end of 48 ± 3 h incubation period, submit to the confirmed phase.
- Arrange EC tubes rows in a manner similar to the positive presumptive tubes.
- Gently shake or rotate positive presumptive tubes to re-suspend the organisms.

- Use a sterile loop 3.0 mm in diameter to transfer three loopfuls from positive presumptive tube to EC tube confirmation tube.
- Repeat for all other positive presumptive tubes.

9.5.3 Incubation:

- Incubate the inoculated tubes at 44.5 ± 0.2°C for 24 ± 2 h.

9.5.4 Interpretation:

- Formation of gas in any amount, in Durham tube, of the EC tubes, at any time, within 24 ± 2 h constitutes a positive confirmed result.
- Calculate the MPN value (see section 11.0) of the number of positive EC broth tubes from MPN index (see A.1).

10. Calculations:

- The MPN values, for variety of positive and negative tubes combinations, are given in appendix A.1.
- The samples volumes indicated in indexes A.1 illustrates MPN values for combinations of positive results when five 10 ml, five 1 ml, and five 0.1 ml sample portion volumes are tested.
- If the sample portion volumes used are those found in the tables, report the value corresponding to the number of positive and negative results in the series as the MPN/100ml.
- When the series of decimal dilutions is different from that in the table, select the MPN value from index A.1 for the combination of positive results and calculate according to the following formula :

$$\text{MPN / 100 ml} = (\text{Table MPN/ 100 ml}) \times 10/V$$

Where:

V = volume of sample portion at the lowest selected dilution.

- When more than three dilutions are used in a decimal series of dilutions, select the three most appropriate dilutions and refer to index A.1.
- Selected examples(A though C) illustrating correct selection are listed below, more examples are available in Standard Methods (see 16.0):

Example	Volumes ml					Combination of positive	MPN index / 100 ml
	10	1.0	0.1	0.01	0.001		
A	5	5	1	0	0	5-1-0	330
B	4	5	1	0	0	4-5-1	48
C	4	3	0	1	1	4-3-2	39

- Example A: First, remove the highest dilution (smallest sample volume) if it has all negative tubes and at least one remaining dilution has a negative tube. Next, remove the lowest dilution (largest sample volume) if it has all positive tube and at least one remaining dilution has a positive tube. According to these guidelines, the three dilutions in Example A are selected by removal of the highest (0.001 ml) and the lowest (10 ml) dilutions.
- Example B: If the lowest dilution does not have all positive tubes, and several of the highest dilutions have all negative tubes, then remove the highest negative dilutions.
- Example C: If no dilution has all positive tubes, select the lowest two dilutions, corresponding to 10 and 1 ml sample. For the third dilution, add the number of positive tubes in the remaining dilutions (0.1, 0.01, and 0.001 ml sample), to yield a final combination of 4-3-2If MPN value for combinations not appearing in the table, or for other combinations of tubes or dilutions, estimate the result as follows:
- First, select the lowest dilution that does not have all positive results.
- Second, select the highest dilution with at least one positive result.
- Select all dilutions between them.
- Use the selected dilutions in the following formula:

$$\text{MPN / 100 ml (approx.)} = 100 \times P / (N \times T)\ ½$$

Where:

P: number of positive results.

N: Volume of sample in all negative portions combined.

T: Total volume of sample in the selected dilutions.

- Examples :
- From (5/5, 10/10, 4/10, 1/10, 0/5) Use only (-, -, 4/10, 1/10. -), 4/10 @ 0.1 ml sample/tube and 1/10 @ 0.01 ml sample/tube. calculations will be:

MPN / 100 ml (approx.) = 100 × 5 / (0.69 ×1.1) ½ =

500/0.87 = 570/ 100 ml

- From (5/5, 10/10, 10/10, 0/10, 0/5) Use only (-, -, 10/10, 0/10. -), 10/10 @ 0.1 ml sample/tube and 0/10 @ 0.01 ml sample/tube. calculations will be:

MPN / 100 ml (approx.) = 100 × 10 / (0.1×1.1) ½ =

1000/0.332 = 3000/ 100 ml.

11. Quality Control:

11.1 Analytical Quality Control Batch:

- Apply the presumptive-confirmed phase of the multiple-tube procedure to all samples.
- Plates with non-selective agar medium, labeled as “Environmental Control”, will be opened along the samples analysis run time to ensure adequate environmental conditions.
- Procedural Blank: perform, under the same conditions as sample, sterile phosphate buffered dilution water after inoculation of samples series. Absence of growth indicates absence of cross contamination.
- Procedural Blank: Inoculate, under the same conditions as sample, one additional tube for each dilution with sterile phosphate buffered dilution water after inoculation of series of 10 samples. Absence of growth indicates absence of cross contamination.

11.2 Non Conformance and Corrective Action:

- If the media performance and sterility assessment are performed concurrently with the use of the media in testing and the media quality is deemed unsatisfactory, the specific use of the media in the test(s) should be investigated to determine the potential impact on the test results and the necessity for repeating the test(s).
- Testing must be repeated when unsatisfactory media and/or reagents are used for critical aspects of testing.

- If procedural blank test gives unsatisfactory results, the samples results should be investigated to determine the potential impact of contamination on the test result, if test results deemed unsatisfactory the test must be repeated.
- If dilution water gives positive result, the samples results should be investigated to determine the potential impact of dilution water on the test result, if test results deemed unsatisfactory the test must be repeated.
- If one or more of environmental control plate gives unsatisfactory results, the samples results should be investigated to determine the potential impact on the test result, if test results deemed unsatisfactory the test must be repeated.
- For all non-conformance findings, trace all media, reagents, equipment operations, and tools used in analysis to find out root cause of non-conformance.
- Take appropriate corrective action, if cause was temporary e.g. if unsterile batch of media used accidently in the analysis; discard all batch of prepared media, or preventive actions if cause was permanent e.g. re-maintenance of autoclave if actual temperature was less than adjusted value, to eliminate reoccurrence of non-conformance in the future.

12. Method Performance Data:

- A limited intra-laboratory study was conducted. Four participated microbiological analysts tested surface raw water matrix.
- Results of this intra-laboratory study, table 1, may not be typical of results for samples other than those studied, hence secondary validation (cross validation) in form of long-term study shall be carried out depending on routine samples results.
- For complete validation procedure and calculation refer to validation report for Detection and Enumeration of Total Coliform by Membrane Filter Technique.

- Table 1

Criteria	Surface Raw
Precision/ repeatability	0.515
Precision/Reproducibility	0.59
Bias %	±0.59

- Detection limit was originally established as 1.8 MPN/100 ml.
- Upper working limit was previously established for this method as 1600 MPN/100ml.
- Range of reliable estimation was previously established for this method between 1.8-1600 MPN / 100ml.

13. Reporting:

- Report fecal coliform concentration as the Most Probable Number (MPN)/100 ml.
- If sample was delayed more than permissible time, write “Delayed” in report comment.

14. References:

- Eugene W. Rice, Rodger B. Baired, Andrew D. Eaton, Lenore S. Clesceri eds.2012 Standard Methods for the Examination of Water and Wastewater. Method 9221E#, 22nd edition (on-line edition). American Public Health Association, American Water Works Association, and Water Environment Federation
- World Health Organization. 2011, Guidelines for Drinking-Water Quality, Fourth Edition. Geneva.
- Guidelines for Canadian Drinking water: Guideline Technical Document. 2006. Total Coliform. Health Canada. Ottawa. Ontario.

Appendices:

A.1 MPN index and 95% confidence limits for various combinations of positive results when five tubes are used per dilution (10 ml, 1.0 ml, 0.1 ml).

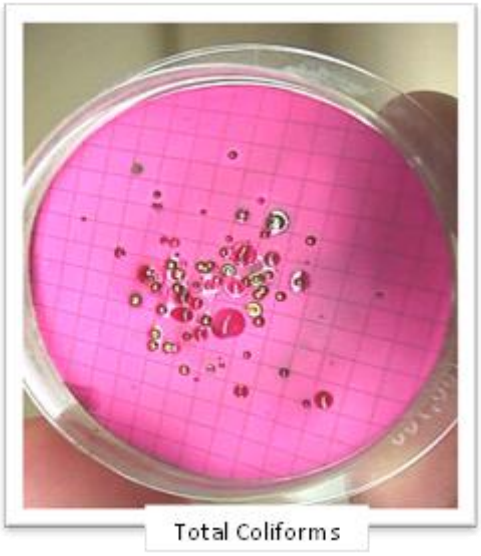
Combination of Positives	MPN Index/100 mL	Confidence Limits		Combination of Positives	MPN Index/100 mL	Confidence Limits	
		Low	High			Low	High
0-0-0	<1.8	—	6.8	4-0-3	25	9.8	70
0-0-1	1.8	0.090	6.8	4-1-0	17	6.0	40
0-1-0	1.8	0.090	6.9	4-1-1	21	6.8	42
0-1-1	3.6	0.70	10	4-1-2	26	9.8	70
0-2-0	3.7	0.70	10	4-1-3	31	10	70
0-2-1	5.5	1.8	15	4-2-0	22	6.8	50
0-3-0	5.6	1.8	15	4-2-1	26	9.8	70
1-0-0	2.0	0.10	10	4-2-2	32	10	70
1-0-1	4.0	0.70	10	4-2-3	38	14	100
1-0-2	6.0	1.8	15	4-3-0	27	9.9	70
1-1-0	4.0	0.71	12	4-3-1	33	10	70
1-1-1	6.1	1.8	15	4-3-2	39	14	100
1-1-2	8.1	3.4	22	4-4-0	34	14	100
1-2-0	6.1	1.8	15	4-4-1	40	14	100
1-2-1	8.2	3.4	22	4-4-2	47	15	120
1-3-0	8.3	3.4	22	4-5-0	41	14	100
1-3-1	10	3.5	22	4-5-1	48	15	120
1-4-0	10	3.5	22	5-0-0	23	6.8	70
2-0-0	4.5	0.79	15	5-0-1	31	10	70
2-0-1	6.8	1.8	15	5-0-2	43	14	100
2-0-2	9.1	3.4	22	5-0-3	58	22	150
2-1-0	6.8	1.8	17	5-1-0	33	10	100
2-1-1	9.2	3.4	22	5-1-1	46	14	120
2-1-2	12	4.1	26	5-1-2	63	22	150
2-2-0	9.3	3.4	22	5-1-3	84	34	220
2-2-1	12	4.1	26	5-2-0	49	15	150
2-2-2	14	5.9	36	5-2-1	70	22	170
2-3-0	12	4.1	26	5-2-2	94	34	230
2-3-1	14	5.9	36	5-2-3	120	36	250
2-4-0	15	5.9	36	5-2-4	150	58	400
3-0-0	7.8	2.1	22	5-3-0	79	22	220
3-0-1	11	3.5	23	5-3-1	110	34	250
3-0-2	13	5.6	35	5-3-2	140	52	400
3-1-0	11	3.5	26	5-3-3	170	70	400
3-1-1	14	5.6	36	5-3-4	210	70	400
3-1-2	17	6.0	36	5-4-0	130	36	400
3-2-0	14	5.7	36	5-4-1	170	58	400
3-2-1	17	6.8	40	5-4-2	220	70	440
3-2-2	20	6.8	40	5-4-3	280	100	710
3-3-0	17	6.8	40	5-4-4	350	100	710
3-3-1	21	6.8	40	5-4-5	430	150	1100
3-3-2	24	9.8	70	5-5-0	240	70	710
3-4-0	21	6.8	40	5-5-1	350	100	1100
3-4-1	24	9.8	70	5-5-2	540	150	1700
3-5-0	25	9.8	70	5-5-3	920	220	2600
4-0-0	13	4.1	35	5-5-4	1600	400	4600
4-0-1	17	5.9	36	5-5-5	>1600	700	—
4-0-2	21	6.8	40				

* Results to two significant figures.

5. Detection and Enumeration of Total Coliform by Membrane Filter Technique

1. Scope:

- This is a detailed procedure for the detection and enumeration of Total Coliform (TC) by Membrane Filter (MF) technique in water samples in 24 hours or less on the basis of the production of aldehydes from fermentation of lactose.
- This procedure can be applied for different types of water, in microbiology laboratory it will be applied for the analysis of ground water, network distribution system (drinking water), and water from different treatment process.


Total Coliforms

2. Principle:

- Volume of water to be tested is filtered through 0.45 µm and the membrane is placed on Endo type medium. The selectivity of medium is due to sodium lauryl sulphate and sodium deoxycholate that acting as inhibitor of gram positive bacteria. Coliform ferment lactose, produce acetaldehydes that reacts with sodium sulfite / basic fuchsin compound to form red colony. The development of metallic sheen occurs when the organism produce aldehyde with the rapid fermentation of lactose. If the inoculum is too heavy, the sheen will be suppressed. Lactose non fermenting bacteria form clear, colorless colonies.
- Total coliform bacteria (excluding *E. coli*) occur in both sewage and natural waters. Some of these bacteria are excreted in the faeces of humans and animals, but many coliforms are heterotrophic and able to multiply in water and soil environments.

- Total coliforms include organisms that can survive and grow in water distribution system, particularly in the presence of biofilms. They were, traditionally, regarded as belonging to the genera **Escherichia**, **Citrobacter**, **Klebsiella** and **Enterobacter**, but the group is more heterogeneous and includes a wider range of genera, such as **Serratia** and **Hafnia**. Hence, they are not useful as an index of faecal pathogens, but they can be used as an indicator of treatment effectiveness and to assess the cleanliness and integrity of distribution systems and the potential presence of biofilms. However, there are better indicators for these purposes.
- The presence of Total Coliform after disinfection indicates inadequate treatment, and presence in distribution system or stored water supplies can reveal regrowth and possible biofilm formation or contamination through ingress of foreign material, including soil or plants.
- Membrane Filter technique is highly reproducible technique which depends on sample filtration through a 47-mm, 0.45µm pore size cellulose membrane filters that retains the bacteria present in the sample. It can be used to test relatively large sample volumes and usually yields numerical results more than multiple-tube fermentation procedure.

3. Definitions:

- Total coliform bacteria in this SOP are those facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that produce red colony with metallic (golden) sheen within 24 h incubation at 35°C on an Endo-type medium containing lactose. The sheen may cover the entire colony or may appear only in a central area or on the periphery.

4. Environmental Conditions:

- All sample analysis steps will be carried out under Laminar Air Flow (LAF) to ensure aseptic environmental conditions.

5. Interference:

- Non-coliform bacteria may interfere with the recovery of coliforms when using a lactose-based medium. Data showed that the recovery of total coliforms using the MF technique decreased as the concentration of HPC bacteria increased. The greatest reduction occurred when the HPC densities exceeded 500 colony-forming units (CFU/ml). It should be noted that most water supplies maintaining a total chlorine residual of 0.2 mg/L have an HPC below 500 CFU/ml.
- Another data demonstrated that ***Pseudomonas aeruginosa*** (30 CFU/ml) and ***Aeromonas hydrophila*** (2 CFU/ml) caused significant reductions in sheen production by coliforms on m-Endo LES agar. ***Flavobacterium*** sp. and ***Bacillus*** sp., in contrast, were not inhibitory, even at concentrations above 1000 CFU/ml.
- Samples with high turbidity caused by algae, particulate, or other interfering material can clog the membrane filter, thereby preventing filtration, or cause spreading of bacterial colonies which could interfere with identification of target colonies.
- Some members of the total coliform group may produce dark red, mucoid, or nucleated colonies without a metallic sheen. When verified these are classified as atypical coliform colonies.
- Pink (non-mucoid), blue, white, or colorless colonies lacking sheen are considered non-coliforms by this technique.

6. Equipment:

- Stainless steel forceps.
- Sterile plastic petri dishes 50 mm.
- Sterile membrane filtration units.
- Vacuum pump.
- Buffered rinse water screw cap bottles.
- Measuring 100 ml Cylinder class A.
- Sterile membrane filters (47 mm diameter, 0.45 µ ± 0.02 µm pore size, white, grid- marked).
- Sterile 47-mm diameter absorbent pads (used with broth).
- Electric Gas Burner.
- Incubator: 35°C ± 0.5°C.
- Colonies counter aid.
- Indelible ink marker for labeling plates.

- Sterile plastic loop, at least 3 mm diameter in suitable holders.

7. Chemicals and Reagents:

- M-Endo LES medium.
- Lauryl Tryptose broth.
- Brilliant Green Lactose Bile Broth.
- Phosphate buffered rinse water.
- Ethyl alcohol 95% in small wide-mouth vials.

8. Precautions:

- Follow the normal safety procedures required in a microbiology laboratory.
- Mouth-pipetting is prohibited.
- Decontaminate all the used plates and materials at the end of the analyses.
- Use sterile filtration units at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is considered to be interrupted when an interval of 30 min or longer elapses between samples filtrations.
- After such interruption, treat any further samples filtration as a new filtration series and sterilize all membrane filter holders in use.
- Avoid damaging or dislodging the membrane filter when attaching funnel to base of the filter unit.
- Do not expose poured plates with culture media to direct light; refrigerate in the dark, in a suitable tight container.

9. Procedure:

9. 1 Responsibility :

- Microbiologists are responsible for the implementation of the test procedures inside the microbiology laboratory.
- Laboratory technician is responsible for preparation of the media and reagents used in this procedure under supervision of the microbiologist.

9. 2 Sample Handling:

- Analyze samples on day of receipt whenever possible. The time between sample collection and the placement of samples in the incubator must not exceed 30 hours.
- If arrival is too late for processing on same day, refrigerate overnight as long as holding time conditions still be met i.e. preservation temperature <8.0°C and time did not exceeded 30h.

9. 3 Instrument Calibration:

- Calibrate and verify balances daily using reference weights.
- Calibrate pH meter prior to use, using at least two standards points.
- Check temperature of incubators and refrigerators daily to ensure operation within stated limits of method.
- Check sterilization procedure and efficiency to ensure media sterility used in this SOP.

9. 4 Selection of sample size:

- The official volume for regulation purpose to be filtered is 100 ml.
- Use sample volumes that will yield counts between 20 and 80 total coliform colonies per membrane. Recommended volumes will be 100 and 50 ml.
- Analyze waters by filtering desired volume in same funnel, or by filtering replicate smaller sample volumes e.g. duplicate 50-ml or four replicates of 25-ml portions.
- For special monitoring purposes, such as troubleshooting water quality problems or identification of coliform breakthrough in low concentrations from treatment barriers; it can be test up to 1-L samples. If particulates prevent filtering a 1-L sample through a single filter, divide sample into four portions of 250 ml for analysis. Total the coliform counts on each membrane to report the number of coliforms per 100 ml.

9. 5 Media and Reagents preparation:

- Prepare and handle media and reagents appropriately, carry out suitable performance test to ensure sterility and suitability of each prepared batch.

9.5.1 Phosphate buffered rinse water:

- **Stock phosphate buffer solution**: Dissolve 34.0 g KH_2PO_4 in a 500 ml reagent-grade distilled water, adjust the pH of the solution to 7.2 with 1 N NaOH and bring volume to 1000 ml with reagent-grade distilled water. Sterilize by autoclave for 15 minutes at 121°C, store in the refrigerator until using (If evidence of mold or other contamination appears in the stock, discard the solution, and prepare a fresh solution).
- **$MgCl_2$ solution**: Dissolve 38 g anhydrous $MgCl_2$ (or 81.1 g $MgCl_2.6H_2O$) in one liter of reagent-grade distilled water. Sterilize by autoclave for 15 minutes at 121°C, store in the refrigerator until using (If evidence of mold or other contamination appears in the stock, discard the solution, and prepare a fresh solution).
- **Working solution**: Add 1.25 ml phosphate buffer stock and 5 ml $MgCl_2$ stock for each liter of reagent-grade distilled water prepared, mix well(Final pH 7.0 ± 0.2), and dispense in appropriate amounts for dilutions in screw-cap dilution bottles or culture tubes, and/or into larger containers for use as rinse water. Autoclave at 121°C for 15 minutes.

9.5.2 m-Endo LES medium:

- Prepare and sterilize medium according to manufacturer instructions.
- Dispense in 5 to 7 ml quantities into lower section of 50 mm Petri dishes, if dishes of any other size are used, adjust quantity to give an equivalent depth of 4 to 5 mm.
- If liquid medium is used, place a pad in the culture dish and saturate with about 2.0 ml broth medium and carefully remove excess medium by decanting the plate.
- If plates are previously prepared and stored in the refrigerator, allow them to warm at room temperature. The crystals that form on agar plates after refrigeration will disappear as the plates warm up.

- Use plates within two weeks after preparation.

9.5.3 Lauryl tryptose broth:

- Prepare and sterilize medium according to manufacturer instructions.
- Dispense 10 ml medium, before sterilization, in fermentation tubes with an inverted Durham tube.
- Alternatively, and only in case of unavailability of Durham tubes, add 0.01 g/L bromcresol purple to medium to determine acid production.
- Close tubes with heat-resistant plastic caps.
- Ensure, after sterilization, that media cover inverted tube with at least one-half to two-thirds, and completely filled with media i.e. no air space inside inverted Durham tubes.
- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes) after preparation.

9.5.4 Brilliant green broth:

- Prepare and sterilize medium according to manufacturer instructions.
- Dispense 10 ml medium, before sterilization, in fermentation tubes with an inverted Durham tube.
- Close tubes with heat-resistant plastic caps.
- Ensure, after sterilization, that media cover inverted tube with at least one-half to two-thirds, and completely filled with media i.e. no air space inside inverted Durham tubes.
- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes) after preparation.

9. 6 Sample Filtration:

- Label the bottom of plates with the sample identification number, analyst initials, and sample volume to be analyzed.
- Place a membrane filter (grid side up) using a sterile forceps on the porous plate of the filter base.
- If you have difficulties in removing the separation papers from the filters due to static electricity, place a filter with the paper on top of the funnel base and turn on the vacuum, the separation paper will curl up, allowing easier removal.

- Attach the funnel to the base of the filter unit
- Shake the sample container vigorously.
- Measure the selected volume by using sterile measuring cylinder.
- Pour selected volume or dilution of the sample into the funnel.
- Turn on the vacuum pump and allow the vacuum to operate for a time enough to filtrate all the sample volume.
- Rinse the interior surface of funnel, with filter still in place, by filtering 20 to 30 ml portions of sterile dilution water. Alternatively, rinse funnel by a flow of sterile dilution water from a squeeze bottle.
- Turn off the pump then remove the funnel from the base of the filter unit.
- Hold the membrane filter at its edge with a sterile forceps, gently lift and place the filter grid-side up on the medium plate.
- Slide the filter onto the medium, using a rolling action to avoid trapping air bubbles between the membrane filter and the underlying medium.
- Reseat the membrane if non-wetted areas occur due to air bubbles.

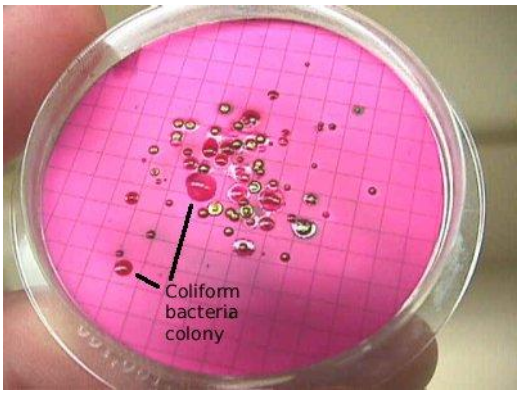
9. 7 Incubation :

- Invert the plates, and incubate at 35 ± 0.5°C for 22 to 24 h.

9. 8 Counting :

- Determine colony count on membrane filters by using a suitable optical device with a cool white fluorescent light source to provide optimal viewing of sheen.
- Count plates that have 20 to 80 coliform colonies and not more than 200 colonies of all types on a membrane-filter surface.
- The typical coliform colony has a pink to dark-red color with a metallic surface sheen.
- Atypical coliform colonies can be dark red, mucoid, or nucleated without sheen.
- Pink, blue, white, or colorless colonies lacking sheen are considered non-coliforms.
- Count both typical and atypical coliform colonies.

- Refrigerate cultures (after incubation) with high densities of non-coliforms colonies for 0.5 to 1 h before counting to deter spread of confluence and aiding sheen discernment.



9. 9 Verification :

- Verify suspect colonies by picking up to five typical and five atypical colonies from a given membrane filter culture.
- Adjust counts on the basis of verification results.

9.9.1 Verification Test :

- **Lactose Fermentation technique:**

- Pick up the selected colony (ies) and inoculate simultaneously into both Lauryl tryptose broth tube(s) and Brilliant Green broth tube(s).
- Incubate all tubes at 35.0 ± 0.5 °C for 24-48 hr.
- Colony that revealed positive reaction in Lauryl tryptose broth tube (gas formed associated with growth turbidity, or color change to yellow in case of using bromcresol purple instead of durham tube) and in brilliant Green tube (gas formation) within 24-48 hr is considered total coliform.

10. Calculations:

- Compute the count, using membrane filters with 20 to 80 coliform colonies and not more than 200 colonies of all types per membrane (see section 14.0), by the following equation:

$$\text{Total Coliform / 100 mL} = \frac{\text{Coliforms colonies counted X 100}}{\text{mL sample filtered}}$$

- For verified typical and atypical coliform counts, adjust the initial count based upon the positive verification percentage, sum both types, and report as ‘‘verified coliform count/100 ml.’’ by using equation:

$$\frac{\text{No of verified colonies}}{\text{No. of colonies subjected to verification}} \text{ X Total no. of colonies}$$

11. Quality Control:

11.1 Analytical Quality Control Batch:

- Filter each sample volume in duplicate manner, final value for that volume will be obtained by getting the arithmetic mean for both plates.
- Plate(s) with non-selective agar medium, labeled as “Environmental Control”, will be opened along the samples analysis run time to ensure adequate environmental conditions.
- Filtration control (Procedural Blank): Filter 100 ml of sterile phosphate buffered rinse water, using separate sterile funnel, after filtration of series of 10 samples; incubate under the same conditions as a sample. Absence of growth indicates the absence of cross- contamination.

11.2 Non Conformance and Corrective Action:

- If the media performance and sterility assessment are performed concurrently with the use of the media in testing and the media quality is deemed unsatisfactory, the specific use of the media in the test(s) should be investigated to determine the potential impact on the test results and the necessity for repeating the test(s).
- Testing must be repeated when unsatisfactory media and/or reagents are used for critical aspects of testing.

- If filtration control test gives unsatisfactory results, the samples results should be investigated to determine the potential impact of contamination on the test result, if test results deemed unsatisfactory the test must be repeated.
- If buffered rinse water gives positive result, the samples results should be investigated to determine the potential impact of rinse water on the test result, if test results deemed unsatisfactory the test must be repeated.
- If one or more of environmental control plate(s) gives unsatisfactory results, the samples results should be investigated to determine the potential impact on the test result, if test results deemed unsatisfactory the test must be repeated.
- For all non-conformance findings, trace all media, reagents, equipment operations, and tools used in analysis to find out root cause of non-conformance.
- Take appropriate corrective action, if cause was temporary e.g. if unsterile batch of media used accidently in the analysis; discard all batch of prepared media, or preventive actions if cause was permanent e.g. re-maintenance of autoclave if actual temperature was less than adjusted value, to eliminate reoccurrence of non-conformance in the future.
- For Invalid results, request a new sample from the same location, explaining the reason for re-sampling need.

12. Method Performance Data:

- A limited intra-laboratory study was conducted. Four participated microbiological analysts tested two water matrices (ground water and drinking water), both spiked with natural flora culture which previously analyzed five times by each analyst to determine spike volume and level of inoculation.
- Results of this intra-laboratory study, table 1, may not be typical of results for samples other than those studied, hence secondary validation (cross validation) in form of long-term study shall be carried out depending on routine samples results.

Table 1

Criteria	Drinking Water	Ground Water
Precision/ repeatability	0.10	0.168
Precision/Reproducibility	0.20	0.23
Bias %	0.02	0.80
Specificity %	94.2	92.1
Sensitivity %	97.9	88.1
Selectivity	-0.3	-0.3
Efficiency %	96	90
False Positive rate %	6.0	7.5
False Negative rate %	2.0	12.5
Uncertainty % (1-19 cfu)	26.9	33.4
Uncertainty % (20-80 cfu)	14.3	15.4

- Detection limit was originally established for any culture-based technique as one colony.
- Upper working limit was previously established for this method as 80 colonies per filter.
- Range of reliable count was previously established for this method between 20-80 colonies.

13. Reporting:

- Report results as “Total Coliform / 100 ml of sample”.
- If HPC result was >500 CFU/ml, report as “Estimated” in case of positive total coliform, or “False Negative” in case of negative result.
- If sample was delayed more than permissible time, write”Delayed” in report comment.
- If no coliform colonies are observed, report the coliform colonies counted as ‘‘<1 coliform/100 ml.’’

- If the total number of bacterial colonies, coliforms plus non-coliforms, exceeds 200 per membrane, report results as ‘‘Too Numerous To Count’’ (TNTC)
- If confluent growth occurs, covering either the entire filtration area of the membrane or a portion thereof, and colonies are not distinct enough for accurate counting, report results as ‘‘confluent growth with (or without) coliforms.’’
- Report confluent growth or TNTC without detectable coliform as ‘Invalid’’
- If sample was divided into two or more portions, Total the coliform counts on all filters and report the number of coliform per 100 ml.
- Report the largest volume filter that has a coliform count falling in the ideal range; calculate final concentration value by multiplying the count by dilution factor (if present).
- If all volumes filters have a coliform count lower than the ideal range, disregard the rule and report the result from largest volume filter count; calculate final concentration value by multiplying the count by dilution factor (if present).
- If largest volume filter has a coliform count higher than the ideal range, choose next (second) dilution volume that falls inside ideal range and calculate final concentration value by multiplying the count by dilution factor.
- If next (second) dilution volume has coliform count lower than ideal range, and largest volume count is less than 100, use the count from the largest volume rather than the count from second volume; calculate final concentration value by multiplying the count by dilution factor.
- If next (second) dilution volume has coliform count higher than ideal range, report filter count and calculate final concentration value by multiplying the count by dilution factor.

- Examples:

Examples	100 ml	50 ml	Reported Value
01	**54**	18	54
02	**10**	5	10
03	89	**52**	104
04	**90**	18	90
05	101	**18**	36
06	TNTC	**90**	180
07	TNTC	**TNTC**	TNTC

14. References:

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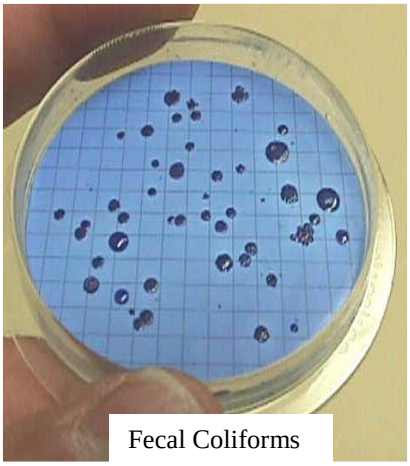
6. Detection and Enumeration of Fecal Coliform by Membrane Filter Technique

1. Scope:

- This SOP describes a detailed procedure for detection and enumeration of Fecal Coliforms (FC) by Membrane Filter (MF) technique in water samples in 24 hours on the basis of fermentation of lactose at elevated temperature. The fecal coliform test is used to distinguish those total coliform organisms that are fecal coliforms.
- This procedure can be applied for different types of water, in microbiology laboratory it will be applied for the analysis of ground water, network distribution system (drinking water), and water from different treatment process.



Fecal Coliforms

2. Principle:

- Volume of water to be tested is filtered through 0.45 µm and the membrane is placed on m-FC medium. The selectivity of medium is due to Bile salt No.3 that acting as inhibitor of gram positive bacteria in addition to elevated incubation temperature (44.5± 0.2°C).. Aniline blue is pH indicator turning into blue color in acidic medium. Fecal coliform ferment lactose, produce acid that change pH of the medium into acidic, which results in coloring colonies with blue.
- Total coliform bacteria that are able to ferment lactose at 44.5 °C are known as thermo-tolerant coliforms. Thermo-tolerant coliforms were traditionally called fecal coliforms, but they also have been documented in organically rich waters or tropical climates in the absence of recent fecal contamination. So, testing for *E. coli,* a specific indicator of fecal contamination, is recommended.

- In most waters, the predominant genus is *Escherichia*, but some types of ***Citrobacter***, ***Klebsiella*** and ***Enterobacter*** are also **thermotolerant**. They are usually found in sewage and water recently subjected to fecal pollution.
- Populations of thermo-tolerant coliforms are composed predominantly of *E. coli*; as a result, this group is regarded as a less reliable but acceptable index of faecal pollution. The presence of thermo-tolerant coliform (fecal coliform) provides evidence of recent fecal pollution.
- Membrane Filter technique is highly reproducible technique which depends on sample filtration through a 47-mm, 0.45 µm pore size cellulose membrane filters that retains the bacteria present in the sample. It can be used to test relatively large sample volumes and usually yields numerical results more than multiple-tube fermentation procedure.

3. Definitions:

- Fecal coliform bacteria in this SOP are those facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that produce blue colony within 24 h incubation at 44.5 ± 0.2°C on m FC medium.

4. Environmental Conditions:

- All sample analysis steps will be carried out under Laminar Air Flow (LAF) to ensure aseptic environmental conditions.

5. Interference:

- Samples of storm water collected during the first runoff (initial flushing) after a long dry period may have a background growth that will interfere with the recovery of fecal coliform. To eliminate such interference add 1 % rosolic acid salt reagent to the prepared media.
- Samples with high turbidity caused by algae, particulate, or other interfering material can clog the membrane filter, thereby preventing filtration, or cause spreading of bacterial colonies which could interfere with identification of target colonies.
- Non-fecal coliform colonies, a gray to cream-colored, may be observed on m FC media.

6. Modification To Standard Test Method:

- N/A.

7. Equipment:

- Stainless steel forceps.
- Sterile plastic petri dishes 50 mm.
- Sterile membrane filtration units.
- Vacuum pump.
- Buffered rinse water screwed cap bottles.
- Measuring 100 ml Cylinder class A.
- Sterile membrane filters (47 mm diameter, 0.45µ ± 0.02 µm pore size, white, grid- marked).
- Sterile 47-mm diameter absorbent pads (used with broth).
- Electric Gas Burner.
- Incubator: 44.5°C ± 0.2°C.
- Incubator: 35°C ± 0.5°C.
- Colonies counter aid.
- Indelible ink marker for labeling plates.
- Sterile plastic loop, at least 3 mm diameter in suitable holders.

8. Chemicals and Reagents:

- m-FC medium.
- Lauryl Tryptose broth.
- EC broth.
- Phosphate buffered rinse water.
- Ethyl alcohol 95% in small wide-mouth vials.

9. Precautions:

- Follow the normal safety procedures required in a microbiology laboratory.
- Mouth-pipetting is prohibited.
- Decontaminate all the used plates and materials at the end of the analyses.
- Use sterile filtration units at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is considered to be interrupted when an interval of 30 min or longer elapses between samples filtrations.

- After such interruption, treat any further samples filtration as a new filtration series and sterilize all membrane filter holders in use.
- Avoid damaging or dislodging the membrane filter when attaching funnel to base of the filter unit.
- Do not expose poured plates with culture media to direct light; refrigerate in the dark, in a suitable tight container.

10. Procedure:

10.1 Responsibility :

- Microbiologists are responsible for the implementation of the test procedures inside the microbiology laboratory.
- Laboratory technician is responsible for preparation of the media and reagents used in this procedure under supervision of the microbiologist.
- Microbiologists and technician involved in this SOP are well trained in the implementation and performance of the procedures in this SOP.

10.2 Sample Handling:

- Analyze samples on day of receipt whenever possible. The time between sample collection and the placement of samples in the incubator must not exceed 30 hours.
- If arrival is too late for processing on same day, refrigerate overnight as long as holding time conditions still be met i.e. preservation temperature <8.0°C and time did not exceeded 30 hr.

10.3 Instrument Calibration:

- Calibrate and verify balances daily using reference weights.
- Calibrate pH meter prior to use, using at least two standards points.
- Check temperature of incubators and refrigerators daily to ensure operation within stated limits of method.
- Check sterilization procedure and efficiency to ensure media sterility used in this SOP.

10.4 Selection of sample size:

- The official volume for regulation purpose to be filtered is 100 ml.
- Use sample volumes that will yield counts between 20 and 60 fecal coliform colonies per membrane. Recommended volumes will be 100 and 50 ml.
- Analyze waters by filtering desired volume in same funnel, or by filtering replicate smaller sample volumes e.g. duplicate 50-ml or four replicates of 25-ml portions.
- For special monitoring purposes, such as troubleshooting water quality problems or identification of fecal coliform breakthrough in low concentrations from treatment barriers; it can be test up to 1-L samples. If particulates prevent filtering a 1-L sample through a single filter, divide sample into four portions of 250 ml for analysis. Total the fecal coliform counts on each membrane to report the number of fecal coliforms per 100 ml.

10.5 Media and solutions preparation:

- Prepare and handle media and reagents appropriately, and carry out suitable performance test to ensure homogeneity, sterility, and suitability for each prepared batch.

9.5.1Phosphate buffered rinse water:

- **Stock phosphate buffer solution**: Dissolve 34.0 g KH_2PO_4 in a 500 ml reagent-grade distilled water, adjust the pH of the solution to 7.2 with 1 N NaOH and bring volume to 1000 ml with reagent-grade distilled water. Sterilize by autoclave for 15 minutes at 121°C, store in the refrigerator until using (If evidence of mold or other contamination appears in the stock, discard the solution, and prepare a fresh solution).
- **$MgCl_2$ solution**: Dissolve 38 g anhydrous $MgCl_2$ (or 81.1 g $MgCl_2.6H_2O$) in one liter of reagent-grade distilled water. Sterilize by autoclave for 15 minutes at 121°C, store in the refrigerator until using (If evidence of mold or other contamination appears in the stock, discard the solution, and prepare a fresh solution).
- **Working solution**: Add 1.25 ml phosphate buffer stock and 5 ml $MgCl_2$ stock for each liter of reagent-grade distilled water prepared, mix well(Final pH 7.0 ± 0.2), and dispense in

appropriate amounts for dilutions in screw-cap dilution bottles or culture tubes, and/or into larger containers for use as rinse water. Autoclave at 121°C for 15 minutes.

9.5.2M-FC medium:

- Prepare and sterilize medium according to manufacturer instructions.
- Dispense in 5 to 7 ml quantities into lower section of 50 mm Petri dishes, if dishes of any other size are used, adjust quantity to give an equivalent depth of 4 to 5 mm.
- If liquid medium is used, place a pad in the culture dish and saturate with about 2.0 ml broth medium and carefully remove excess medium by decanting the plate.
- If plates are previously prepared and stored in the refrigerator, allow them to warm at room temperature. The crystals that form on agar plates after refrigeration will disappear as the plates warm up.
- Use plates within two weeks after preparation.

9.5.3Lauryl tryptose broth:

- Prepare and sterilize medium according to manufacturer instructions.
- Dispense 10 ml medium, before sterilization, in fermentation tubes with an inverted Durham tube.
- Alternatively, and only in case of unavailability of Durham tubes, add 0.01 g/L bromcresol purple to medium to determine acid production.
- Close tubes with heat-resistant plastic caps.
- Ensure, after sterilization, that media cover inverted tube with at least one-half to two-thirds, and completely filled with media i.e. no air space inside inverted Durham tubes.
- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes).

9.5.4EC broth:

- Prepare and sterilize medium according to manufacturer instructions.
- Dispense 10 ml medium, before sterilization, in fermentation tubes with an inverted Durham tube.
- Close tubes with heat-resistant plastic caps.
- Ensure, after sterilization, that media cover inverted tube with at least one-half to two-thirds, and completely filled with media i.e. no air space inside inverted Durham tubes.
- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes).

10.6 Sample Filtration:

- Label the bottom of plates with the sample identification, analyst initials, and sample volume to be analyzed.
- Place a membrane filter (grid side up) using a sterile forceps on the porous plate of the filter base.

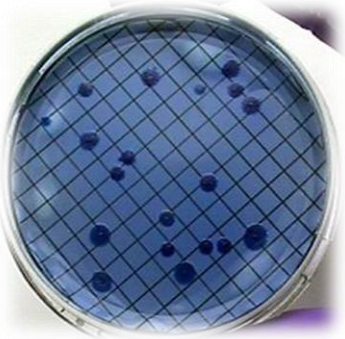
- If you have difficulties in removing the separation papers from the filters due to static electricity, place a filter with the paper on top of the funnel base and turn on the vacuum, the separation paper will curl up, allowing easier removal.
- Attach the funnel to the base of the filter unit
- Shake the sample container vigorously.
- Measure the selected volume by using sterile measuring cylinder.



- Pour selected volume or dilution of the sample into the funnel.
- Turn on the vacuum pump and allow the vacuum to operate for a time enough to filtrate all the sample volume.
- Rinse the interior surface of funnel, with filter still in place, by filtering three 20 to 30 ml portions of sterile dilution water. Alternatively, rinse funnel by a flow of sterile dilution water from a squeeze bottle.
- Turn off the pump then remove the funnel from the base of the filter unit.
- Hold the membrane filter at its edge with a sterile forceps, gently lift and place the filter grid-side up on the medium plate.
- Slide the filter onto the medium, using a rolling action to avoid trapping air bubbles between the membrane filter and the underlying medium.
- Reseat the membrane if non-wetted areas occur due to air bubbles.

10.7 Incubation :

- Invert the plates, and incubate at 44.5 ± 0.2°C for 24 ± 2 h.

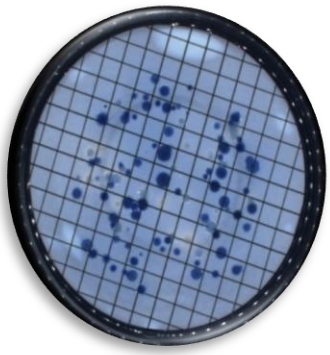


10.8 Counting :

- To determine colony count on membrane filters use a suitable optical device with a cool white fluorescent light source to provide optimal viewing of sheen.
- Count colonies with various shades of blue as typical fecal coliform, grey to green colonies as atypical fecal coliform. Non fecal coliform colonies are gray to cream-colored.
- Count both typical and a typical fecal coliform colonies.

10.9 Verification :

- Verify suspect colonies by picking up to five typical and five atypical colonies from a given membrane filter culture.
- Adjust counts on the basis of verification results.



10.9.1 Verification Test :

- **Lactose Fermentation technique:**

- Pick up the selected colony (ies) and inoculate simultaneously into both Lauryl tryptose broth tube(s) and EC tube(s).
- Incubate Lauryl tryptose broth tube(s) at 35.0 ± 0.5 °C for 24-48 hr.
- Incubate EC tube(s) at 44.5 ±0.2 °C for 24 hr.
- Colony that revealed positive reaction in Lauryl tryptose broth tube (gas formed associated with growth turbidity, or color change to yellow in case of using bromcresol purple instead of durham tube) within 48 hr and positive reaction in EC tube (gas formation) within 24 hr is considered fecal coliform.

11. Calculations:

- Compute the count, using membrane filters with 20 to 60 fecal coliform colonies by the following equation:

$$\text{Fecal coliform / 100 mL} = \frac{\text{Colonies counted} \times 100}{\text{ML sample filtered}}$$

- For verified typical and atypical fecal coliform counts, adjust the initial count based upon the positive verification percentage, sum both types, and report as ‘‘verified fecal coliform count/100 ml.’’

$$\frac{\text{No. of verified colonies}}{\text{No. of colonies subjected to verification}} \text{ X Total no. of colonies}$$

12. Quality Control:

12.1 Analytical Quality Control Batch:

- Filter each sample volume in duplicate manner, final value for that volume will be obtained by getting the arithmetic mean for both plates.
- Plate(s) with non-selective agar medium, labeled as “Environmental Control”, will be opened along the samples analysis run time to ensure adequate environmental conditions.
- Filtration control (Procedural Blank): Filter 100 ml of sterile phosphate buffered rinse water, using separate sterile funnel, after filtration of series of 10 samples; incubate under the same conditions as a sample. Absence of growth indicates absence of cross-contamination.

12.2 Non Conformance and Corrective Action:

- If the media performance and sterility assessment are performed concurrently with the use of the media in testing and the media quality is deemed unsatisfactory, the specific use of the media in the test(s) should be investigated to determine the potential impact on the test results and the necessity for repeating the test(s).
- Testing must be repeated when unsatisfactory media and/or reagents are used for critical aspects of testing.
- If filtration control test gives unsatisfactory results, the samples results should be investigated to determine the potential impact

of contamination on the test result, if test results deemed unsatisfactory the test must be repeated.

- If buffered rinse water gives positive result, the samples results should be investigated to determine the potential impact of rinse water on the test result, if test results deemed unsatisfactory the test must be repeated.
- If one or more environmental control plate(s) gives unsatisfactory results, the samples results should be investigated to determine the potential impact on the test result, if test results deemed unsatisfactory the test must be repeated.
- For all non-conformance findings, trace all media, reagents, equipment operations, and tools used in analysis to find out root cause of non-conformance.
- Take appropriate corrective action, if cause was temporary e.g. if unsterile batch of media used accidently in the analysis; discard all batch of prepared media, or preventive actions if cause was permanent e.g. re-maintenance of autoclave if actual temperature was less than adjusted value, to eliminate reoccurrence of non-conformance in the future.
- For Invalid results, request a new sample from the same location, explaining the reason for re-sampling need.

13. Method Performance Data:

- A limited intra-laboratory study was conducted. Four participated microbiological analysts tested two water matrices (ground water and drinking water), both spiked with natural flora culture which previously analyzed five times by each analyst to determine spike volume and level of inoculation.
- Results of this intra-laboratory study, Table 1, may not be typical of results for samples other than those studied, hence secondary validation (cross validation) in form of long-term study shall be carried out depending on routine samples results.

Table 1

Criteria	Drinking Water	Ground Water
Precision/ repeatability	0.106	0.082
Precision/Reproducibility	0.10	0.08
Bias %	0.03	0.12
Specificity %	94.7	89.7
Sensitivity %	90.5	87.8
Selectivity	-0.3	-0.3
Efficiency %	92.5	88.8
False Positive rate %	5	10
False Negative rate %	10	12.5
Uncertainty % (0-19 cfu)	29.7	41.7
Uncertainty % (20-60 cfu)	17.9	14.8

- Detection limit was originally established for any culture-based technique as one colony.
- Upper working limit was previously established for this method as 60 colonies per filter.
- Range of reliable count was previously established for this method between 20-60 colonies.

14. Reporting:

- Report results as " Fecal Coliform / 100 ml of sample "
- If sample was delayed more than permissible time, write "Delayed" in report comment.
- If no fecal coliform colonies are observed, report the fecal coliform colonies as ''<1 fecal coliform/100 ml.''
- If the total number of fecal coliforms colonies exceeds 60 per membrane, report results as ''Too Numerous To Count with (or without) fecal coliforms'' (TNTC).
- If confluent growth occurs, covering either the entire filtration area of the membrane or a portion thereof, and colonies are not distinct enough for accurate counting, report results as ''confluent growth with (or without) fecal coliforms.''

- Report confluent growth or TNTC without detectable fecal coliform as ‘Invalid’’.
- If sample was divided into two or more portions, Total the fecal coliform counts on all filters and report the number of fecal coliform per 100 ml.
- Report the largest volume filter that has a fecal coliform count falling in the ideal range; calculate final concentration value by multiplying the count by dilution factor (if present).
- If all volumes filters have a fecal coliform count lower than the ideal range, disregard the rule and report the result from largest volume filter count. Calculate final concentration value by multiplying the count by dilution factor (if present).
- If largest volume filter has a fecal coliform count higher than the ideal range, choose next (second) dilution volume that falls inside ideal range and calculate final concentration value by multiplying the count by dilution factor.
- If next (second) dilution volume has fecal coliform count lower than ideal range, and largest volume count is less than 100, use the count from the largest volume rather than the count from second volume; calculate final concentration value by multiplying the count by dilution factor.

Examples	100 ml	50 ml	Reported Value
01	54	18	54
02	10	5	10
03	89	52	104
04	90	18	90
05	101	18	36
06	TNTC	90	180
07	TNTC	TNTC	TNTC

- Examples:

- If next (second) dilution volume has fecal coliform count higher than ideal range, report filter count and calculate final concentration value by multiplying the count by dilution factor.

15. References:

- World Health Organization. 2011, Guidelines for Drinking-Water Quality, Fourth Edition. Geneva,
- U.S. Environmental Protection Agency.2005. Manual of The Certification of laboratories Analyzing Drinking Water, Criteria and Procedures Quality Assurance. EPA 815-R-05-054. Office of Ground Water and Drinking Water. Cincinnati, Ohio.
- U.S Environmental Protection Agency, Office of Research and Development, National Exposure research laboratory. 2003. Standard Operating Procedure for Choosing the best membrane filter count for the calculation of final concentration of Microorganisms per 100 ml. Number MERP-041.01

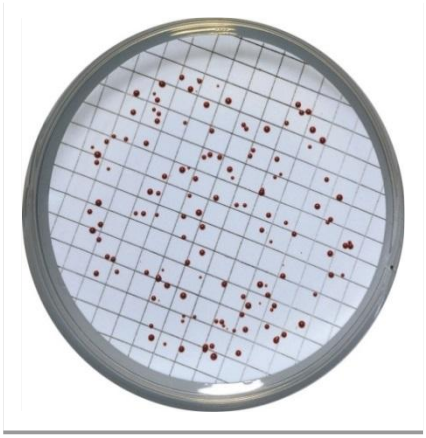
7. Detection and Enumeration of Fecal Streptococcus and Enterococcus Groups by Membrane Filter Technique

1. Scope:

- This is a detailed procedure for detection and enumeration of Fecal Streptococcus group (FS) and Enterococcus group by Membrane Filter (MF) technique in water samples in 48 hours or less on the basis of reduction of Tri-phenyl Tetra-zolium Chloride (TTC).
- This procedure can be applied for different types of water, in microbiology laboratory it will be applied for the analysis of ground water, network distribution system (drinking water), and water from different treatment process.

2. Principle:

- Volume of water to be tested is filtered through 0.45 µm and the membrane is placed on m-Enterococcus medium. The selectivity of medium is due to sodium azide that acts as inhibitor of gram negative bacteria. Bacteria reduce Triphenyl Tetrazolium Chloride (TTC) to the insoluble formazan inside the bacterial cell, resulting in the production of red colonies.



Fecal streptococcus colonies

- The fecal streptococcus group consists of a number of species of the genus Streptococcus, such as *S. faecalis*, *S. faecium*, *S. avium*, *S. bovis*, *S. equinus*, and *S. gallinarum*. The normal habitat of fecal streptococci is the gastrointestinal tract of warm-blooded animals and humans.
- Enterococcus group is a subgroup of the fecal streptococci that includes *S.* faecalis, *S. faecium*, *S. gallinarum*, and *S. avium*. Enterococci group can be used as an index of fecal pollution, important advantages of this group are that they tend to survive longer in water environment than fecal coliforms, more resistant to drying and are more resistant to chlorination.
- Membrane Filter technique is highly reproducible technique which depends on sample filtration through a 47-mm, 0.45 or 0.22-µm pore size cellulose membrane filters that retains the bacteria present in the sample. It can be used to test relatively large sample volumes and usually yields numerical results.

3. Definitions:

- Fecal streptococcus bacteria in this SOP are those gram-positive, cocci-shaped, facultative anaerobic, catalase-negative, able to grow on bile esculin agar, and at 45°C in brain-heart infusion broth producing red colony on m-Enterococcus agar.
- Enterococcus bacteria in this SOP are those gram-positive, cocci-shaped, facultative anaerobic, catalase-negative, able to grow on bile esculin agar, 6.5% NaCl broth, and at 45°C in brain-heart infusion broth producing red colony on m-Enterococcus agar.

4. Environmental Conditions:

- All sample analysis steps will be carried out under Laminar Air Flow (LAF) to ensure aseptic environmental conditions.

5. Interference:

- Samples with high turbidity caused by algae, particulate, or other interfering material can clog the membrane filter, thereby preventing filtration, or cause spreading of bacterial colonies which could interfere with identification of target colonies.

6. Equipment:

- Stainless steel forceps.
- Presterilized plastic petri dishes 50 mm.
- Sterile membrane filtration units.
- Vacuum pump.
- Buffered rinse water screw cap bottles.
- Measuring 100 ml Cylinder class A.
- Sterile membrane filters (47 mm diameter, 0.45 µ ± 0.02 µm pore size, white, grid- marked).
- Sterile 47-mm diameter absorbent pads (used with broth).
- Electric Gas Burner.
- Incubator: 35°C ± 0.5°C.
- Incubator: 45°C ± 0.5°C.
- Colonies counter aid.
- Indelible ink marker for labeling plates.
- Sterile plastic loop, at least 3 mm diameter in suitable holders.

7. Chemicals and Reagents:

- M-Enterococcus agar.
- Brain-heart infusion broth.
- Brain-heart infusion broth with 6.5 % NaCl.
- Brain-heart infusion agar.
- Bile esculin agar.
- Phosphate buffered rinse water.
- Hydrogen Peroxide 3.0 %
- Sodium Chloride (NaCl)
- Gram Stain reagents set.

8. Precautions:

- Follow the normal safety procedures required in a microbiology laboratory.
- Mouth-pipetting is prohibited.
- Decontaminate all the used plates and materials at the end of the analyses.
- Use sterile filtration units at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is considered to be interrupted when an interval of 30 min or longer elapses between samples filtrations.
- After such interruption, treat any further samples filtration as a new filtration series and sterilize all membrane filter holders in use.
- Avoid damaging or dislodging the membrane filter when attaching funnel to base of the filter unit.
- Do not expose poured plates with culture media to direct light; refrigerate in the dark, in a suitable tight container.

9. Procedure:

9.1 Responsibility :

- Microbiologists are responsible for the implementation of the test procedures inside the microbiology laboratory.
- Laboratory technician is responsible for preparation of the media and reagents used in this procedure under supervision of the microbiologist(s).
- Microbiologists and technician involved in this SOP are well trained in the implementation and performance of the procedures in this SOP.

9.2 Sample Handling:

- Analyze samples on a day of receipt whenever possible. The time between sample collection and the placement of samples in the incubator must not exceed 30 hours.
- If arrival is too late for processing on same day, refrigerate overnight as long as holding time conditions still be met i.e. preservation temperature <8.0°C and time did not exceeded 30h.

9.3 Instrument Calibration:

- Calibrate and verify balances daily using reference weights.
- Calibrate pH meter prior to use, using at least two standards points.
- Check temperature of incubators and refrigerators daily to ensure operation within stated limits of method.
- Check sterilization procedure and efficiency to ensure media sterility used in this SOP.

9.4 Selection of sample size:

- The official volume for regulation purpose to be filtered is 100 ml.
- Use sample volumes that will yield counts between 20 and 60 fecal streptococci colonies per membrane. Recommended volumes will be 100 and 50 ml.
- Analyze waters by filtering desired volume in same funnel, or by filtering replicate smaller sample volumes e.g. duplicate 50-ml or four replicates of 25-ml portions.
- For special monitoring purposes, such as troubleshooting water quality problems or identification of fecal streptococcus or enterococcus breakthrough in low concentrations from treatment barriers; it can be test up to 1-L samples. If particulates prevent filtering a 1-L sample through a single filter, divide sample into four portions of 250 ml for analysis. Total the counts on each membrane to report the number of fecal streptococcus or enterococcus per 100 ml.

9.5 Media and solutions preparation:

- Prepare and handle media and reagents appropriately, carry out suitable performance test to ensure sterility and suitability of each prepared batch.

9.5.1 Phosphate buffered rinse water:

- **Stock phosphate buffer solution**: Dissolve 34.0 g KH_2PO_4 in a 500 ml reagent-grade distilled water, adjust the pH of the solution to 7.2 with 1 N NaOH and bring volume to 1000 ml with reagent-grade distilled water. Sterilize by autoclave for 15 minutes at 121°C, store in the refrigerator until using (If

evidence of mold or other contamination appears in the stock, discard the solution, and prepare a fresh solution).

- **$MgCl_2$ solution**: Dissolve 38 g anhydrous $MgCl_2$ (or 81.1 g $MgCl_2.6H_2O$) in one liter of reagent-grade distilled water. Sterilize by autoclave for 15 minutes at 121°C, store in the refrigerator until using (If evidence of mold or other contamination appears in the stock, discard the solution, and prepare a fresh solution).
- **Working solution**: Add 1.25 ml phosphate buffer stock and 5 ml $MgCl_2$ stock for each liter of reagent-grade distilled water prepared, mix well(Final pH 7.0 ± 0.2), and dispense in appropriate amounts for dilutions in screw-cap dilution bottles or culture tubes, and/or into larger containers for use as rinse water. Autoclave at 121°C for 15 minutes.

9.5.2 m-Enterococcus agar medium:

- Prepare and sterilize medium according to manufacturer instructions.
- Dispense in 5 to 7 ml quantities into lower section of 50 mm Petri dishes, if dishes of any other size are used, adjust quantity to give an equivalent depth of 4 to 5 mm.
- If plates are previously prepared and stored in the refrigerator, allow them to warm at room temperature. The crystals that form on agar plates after refrigeration will disappear as the plates warm up.
- Use plates within two weeks after preparation.

9.5.3 Brain-heart infusion broth:

- Prepare medium according to manufacturer instructions.
- Dispense 10 ml medium in tubes, before sterilization.
- Close tubes with metal or heat-resistant plastic caps.
- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes).

9.5.4 Brain-heart infusion broth with 6.5 % NaCl:

- Prepare medium according to manufacturer instructions.
- Add 60.0 g of NaCl to the ingredient of brain heart infusion broth.
- Close tubes with metal or heat-resistant plastic caps.
- Use tubes within two weeks (if loose plastic cap used) and within three months (for tight screw cap tubes) after preparation.

9.5.5 Brain-heart infusion agar:

- Prepare medium according to manufacturer instructions.
- Add 15.0 g agar to the ingredient of brain heart infusion broth.
- Dispense 8 to 10 ml in screw-capped tubes, before sterilization.
- Place tubes in an inclined position, immediately after sterilization, so that the agar will solidify with a sloped surface.
- Tighten screw caps after cooling and store in a protected, cool storage area.
- Use tubes within three months after preparation.

9.5.6 Bile esculin agar:

- Prepare medium according to manufacturer instructions
- Dispense 8 to 10 ml in screw-capped tubes, before sterilization.
- Place tubes in an inclined position, immediately after sterilization, so that the agar will solidify with a sloped surface.
- Tighten screw caps after cooling and store in a protected, cool storage area.
- Use tubes within three months after preparation.

9.6 Sample Filtration:

- Label the bottom of plates with the sample identification, analyst initials, and sample volume to be analyzed.
- Place a membrane filter (grid side up) using a sterile forceps on the porous plate of the filter base.
- If you have difficulties in removing the separation papers from the filters due to static electricity, place a filter with the paper on top of the funnel base and turn on the vacuum, the separation paper will curl up, allowing easier removal.
- Attach the funnel to the base of the filter unit
- Shake the sample container vigorously.

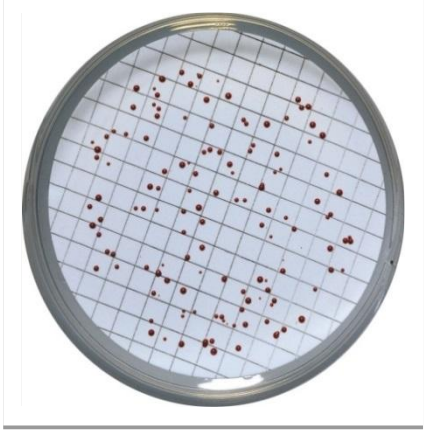
- Measure the selected volume by using sterile measuring cylinder.
- Pour selected volume or dilution of the sample into the funnel.
- Turn on the vacuum pump and allow the vacuum to operate for a time enough to filtrate all the sample volume.
- Rinse the interior surface of funnel, with filter still in place, by filtering three 20 to 30 ml portions of sterile dilution water. Alternatively, rinse funnel by a flow of sterile dilution water from a squeeze bottle.
- Turn off the pump then remove the funnel from the base of the filter unit.
- Hold the membrane filter at its edge with a sterile forceps, gently lift and place the filter grid-side up on the medium plate.
- Slide the filter onto the medium, using a rolling action to avoid trapping air bubbles between the membrane filter and the underlying medium.
- Reseat the membrane if non-wetted areas occur due to air bubbles.

9.7 Incubation :

- Let the plates stand for 30 min, invert the plates, and incubate at 35 ± 0.5°C for 48 h

9.8 Counting :

- Determine colony count on membrane filters by using a suitable optical device with a cool white fluorescent light source to provide optimal viewing of sheen.
- Count plates that have 20 to 60.
- The typical colony has red color.
- Count all light and dark red colonies as fecal streptococci.



Fecal streptococcus colonies

9.9 Verification:

- Verify suspect colonies by picking up to 5 typical colonies.
- Adjust counts on the basis of verification results.

9.9.1 Verification Test :

- **Biochemical Characteristics** :

- Pick selected typical colony from a membrane and streak for isolation onto the surface of a brain-heart infusion agar.
- Incubate at 35 ± 0.5°C for 24 to 48 h.
- Transfer a loop full of growth from a well-isolated colony on brain-heart infusion agar into a brain-heart infusion broth tube and to each of two clean glass slides.
- Incubate the brain-heart infusion broth at 35 ± 0.5°C for 24 h.
- Add a few drops of freshly prepared 3% hydrogen peroxide to the smear on a slide. The appearance of bubbles constitutes a positive catalase test and indicates that the colony is not a member of the fecal streptococcus group. If the catalase test is negative, i.e., no bubbles, make a Gram stain of the second slide.
- Transfer a loop full of growth from the brain-heart infusion broth to each of the following media:
  - **bile esculin agar** (incubate at 35 ± 0.5°C for 48 h);
  - **brain-heart infusion broth** (incubate at 45 ± 0.5°C for 48 h);
  - **brain-heart infusion broth with 6.5% NaCl** (incubate at 35± 0.5°C for 48 h).
- Growth of catalase-negative, gram-positive cocci on bile esculin agar and at 45°C in brain-heart infusion broth verifies that the colony is of the fecal streptococcus group.

- Growth at 45°C and in 6.5% NaCl broth indicates that the colony belongs to the **enterococcus group**.

10. Calculations:

- Compute the count, using membrane filters with 20 to 60 colonies, by the following equation:

$$\text{Fecal Streptococci/100 mL} = \frac{\text{Colonies counted} \times 100}{\text{ML sample filtered}}$$

- For verified fecal streptococci and enterococci counts, adjust the initial count based upon the positive verification percentage, sum both types, and report as ‘‘verified fecal streptococcus /100 ml.’’ by using equation:

$$= \frac{\text{Number of verified colonies} \times 100}{\text{Total number of colonies subjected to verification}}$$

11. Quality Control:

11.1 Analytical Quality Control Batch:

- Filter each sample volume in duplicate manner, final value for that volume will be obtained by getting the arithmetic mean for both plates.
- Plate(s) with non-selective agar medium, labeled as “Environmental Control”, will be opened along the samples analysis run time to ensure adequate environmental conditions.
- Filtration control (Procedural Blank): Filter 100 ml of sterile phosphate buffered rinse water, using separate sterile funnel, after filtration of series of 10 samples; incubate under the same conditions as a sample. Absence of growth indicates absence of cross-contamination.

11.2 Non Conformance and Corrective Action:

- If the media performance and sterility assessment are performed concurrently with the use of the media in testing and the media quality is deemed unsatisfactory, the specific use of the media in the test(s) should be investigated to determine the potential impact on the test results and the necessity for repeating the test(s).

- Testing must be repeated when unsatisfactory media and/or reagents are used for critical aspects of testing.
- If filtration control test gives unsatisfactory results, the samples results should be investigated to determine the potential impact of contamination on the test result, if test results deemed unsatisfactory the test must be repeated.
- If rinse water gives positive result, the samples results should be investigated to determine the potential impact of dilution water on the test result, if test results deemed unsatisfactory the test must be repeated.
- If one or more of environmental control plate(s) gives unsatisfactory results, the samples results should be investigated to determine the potential impact on the test result, if test results deemed unsatisfactory the test must be repeated.
- For all non-conformance findings, trace all media, reagents, equipment operations, and tools used in analysis to find out root cause of non-conformance.
- Take appropriate corrective action, if cause was temporary e.g. if unsterile batch of media used accidently in the analysis; discard all batch of prepared media, or preventive actions if cause was permanent e.g. re-maintenance of autoclave if actual temperature was less than adjusted value, to eliminate reoccurrence of non-conformance in the future.
- For Invalid results, request a new sample from the same location, explaining the reason for re-sampling need.

12. Method Performance Data:

- A limited intra-laboratory study was conducted. Four participated microbiological analysts tested two water matrices (ground water and drinking water), both spiked with natural flora culture which previously analyzed five times by each analyst to determine spike volume and level of inoculation.
- Results of this intra-laboratory study, tables one and two, may not be typical of results for samples other than those studied, hence secondary validation (cross validation) in form of long-term study shall be carried out depending on routine samples results.

Table 1: Performance Criteria for Fecal Streptococcus

Criteria	Drinking Water	Ground Water
Precision/ repeatability	0.057	0.106
Precision/Reproducibility	0.064	0.11
Bias %	0.080	0.065
Specificity %	N/A	N/A
Sensitivity %	100	100
Selectivity	0	0
Efficiency %	100	100
False Positive rate %	0	0
False Negative rate %	N/A	N/A
Uncertainty % (0-19 cfu)	22.2	28.29
Uncertainty % (20-80 cfu)	10.65	13.43

Table 2: Performance Criteria for Fecal Enterococcus

Criteria	Drinking Water	Ground Water
Specificity %	N/A	N/A
Sensitivity %	100	100
Selectivity	0	0
Efficiency %	65	92.5
False Positive rate %	35	7.5
False Negative rate %	N/A	N/A
Uncertainty % (0-19 cfu)	22.2	28.29
Uncertainty % (20-80 cfu)	10.65	13.43

- Detection limit was originally established for any culture-based technique as one colony.
- Upper working limit was previously established for this method as 60 colonies per filter.
- Range of reliable count was previously established for this method between 20-60 colonies.

13. Reporting:

- Report the result as “Fecal Streptococci / 100 ml”
- If sample was delayed more than permissible time, write “Delayed” in report comment.
- If no colonies are observed, report the fecal streptococci colonies counted as ‘‘<1 fecal streptococci/100 ml.’’
- If no filter has count falling in the ideal range, total the counts (disregarding the lower limit of 20 cited above) and use the formula given above to obtain fecal streptococci density.
- If confluent growth occurs, covering either the entire filtration area of the membrane or a portion thereof, and colonies are not distinct enough for accurate counting, report results as ‘‘confluent growth with (or without) fecal streptococci.’’
- If the total number of bacterial colonies exceeds 200 per membrane, report results as ‘‘Too Numerous To Count’’ (TNTC)
- If sample was divided into two or more portions, Total the counts on all filters and report the number of fecal streptococci per 100 ml.
- Report the largest volume filter that has count falling in the ideal range; calculate final concentration value by multiplying the count by dilution factor (if present).
- If all volumes filter have count lower than the ideal range, disregard the rule and report the result from largest volume filter count. Calculate final concentration value by multiplying the count by dilution factor (if present).
- If largest volume filter has count higher than the ideal range, choose next (second) dilution volume that falls inside ideal range and calculate final concentration value by multiplying the count by dilution factor.
- If next (second) dilution volume has count lower than ideal range, and largest volume count is less than 100, use the count from the largest volume rather than the count from second

volume; calculate final concentration value by multiplying the count by dilution factor.

- If next (second) dilution volume has count higher than ideal range, report filter count and calculate final concentration value by multiplying the count by dilution factor.
- Examples:

Examples	100 ml	50 ml	Reported Value
01	**54**	18	54
02	**10**	5	10
03	89	**52**	104
04	**90**	18	90
05	101	**18**	36
06	TNTC	**90**	180
07	TNTC	TNTC	TNTC

14. References:

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المراجع

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