Kathmandu Upatyaka Khanepani Limited **Standard Operating Procedure** **Multiple Tube Fermentation Technique for Total coliforms, Fecal coliforms and *Escherichia coli***	SOP Code No:	**MB 1.1**
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1. Objectives and Scope

This document is Kathmandu Upatyaka Khanepani Limited (KUKL) Standard Operating Procedure (SOP) for the analysis of Total coliform, Fecal Coliform and *E. coli* in drinking water and Waste-water using Multiple Tube Fermentation Technique.

2. Applicability

This SOP includes the procedure for media preparation, quality control, sample collection, transportation and detection and enumeration of total, fecal coliform and *E. coli* by inoculation in liquid medium.

3. Definitions

Coliform: It is a group of aerobic and facultative anaerobic, gram negative, non-sporing bacilli that are present in the intestine of warm-blooded animals and can ferment lactose with the production of acid and gas within 48 hours at 37±1°C. It includes members of *Enterobacteriaceae* viz., the genera *Klebsiella, Escherichia, Enterobacter and Citrobacter*.

Fecal coliform: It is a subgroup of coliform which grows at higher temperature and is only associated with the fecal matter of warm-blooded animals.

4. Responsibilities

Laboratory technicians, Microbiologists

5. Equipment, Reagents, and Supplies

- Autoclave
- Incubator
- Sterile Sampling bottles
- Sampling bottle labels and waterproof markers
- Ice box
- MacConkey broth
- Brilliant green bile lactose broth
- Kovac's reagent
- Nutrient agar
- Culture bottles, pipettes

6. Operation Procedure

I. <u>Sampling, transportation, and storage:</u>

- Turn the tap on full, and allow the water to run to waste for 1 minute.
- Hold the sterile bottle by the base in one hand.
- Use the other hand to remove the stopper and cover together.
- Do not touch the screw thread of the bottle neck or the inside of the cap.
- Replace the stopper and cover.
- Label the bottles properly.
- Transport the samples within 6 hours to the testing laboratory at room temperature. If delay is unavoidable, place the sample in icebox and transport to the laboratory at 4°C within 24 hours.

II. *Neutralizing chlorine in water samples*: When the water to be examined is likely to contain chlorine or chloramine, add 0.1–0.2 ml sodium thiosulphate 30 g/l (3% w/v)

to each bottle of 200 ml capacity before the bottle is sterilized to neutralize those substances**.**

III. Media preparation:

Media should be prepared in accordance with the manufacturer's instructions, as follows:

- Dissolve the stated amount of the dehydrated medium in distilled water to obtain the double-strength or single-strength presumptive medium.
- For single strength, use the normal amount of broth powder as instructed by the manufacturer strength.
- For double strength, use twice the normal amount of broth powder.
- Dispense the requisite volume into culture tubes containing an inverted Durham tube, and cap the culture tubes.
- Make sure the inverted tube is full of broth and there is no air bubble inside it.
- If an air bubble is trapped in the tube, invert the bottle of medium to allow the bubble to rise out of the tube.
- Sterilize in an autoclave. Use the autoclave tape for confirmation of sterilization with each batch of the items sterilized.
- Store the medium at 2-8°C.
- Warm the medium to room temperature before use to ensure that all components have been dissolved.

IV. Quality control and assurance:

- For sterility control, a representative sample of each lot/batch of medium is incubated 37°C for 24 hours. As a general rule, for a lot of 100 or less units a 3-5% sample should be tested. For a larger lot, 10 random plates or tubes are taken. There should be no evidence of microbial growth after incubation. Discard all sterility samples when the tests have been completed.

- For utility control, inoculate *Escherichia coli* ATCC 00013 and incubate at 37°C for 24 hours.
- Stability test: Periodically perform the above procedures on stored prepared media in order to determine whether the storage conditions will give optimal results.

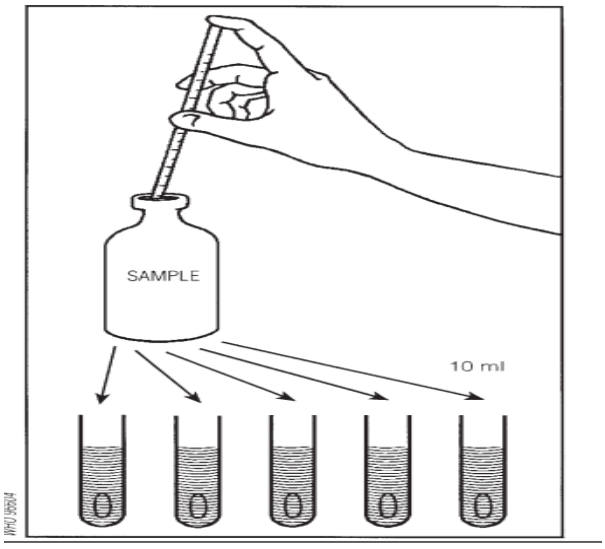
V. Sample processing

a. Presumptive test

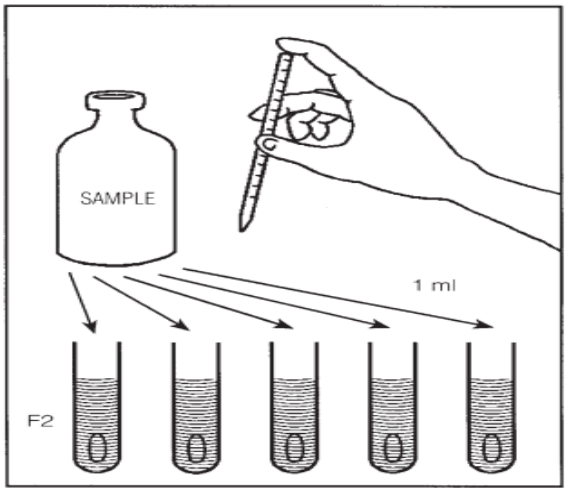
- Prepare MacConkey broth (purple) for this test.
- Depending on the type of water sample (treated/untreated) the number of tubes used is as follows:

	No. of tubes used	Vol. of broth per tube	Strength of broth
Treated sample	1	50	Double
	5	10	Double
Untreated sample	1	50	Double
	5	10	Double
	5	5	Single

- Label each culture tube with the sample code number.
- Mix thoroughly the sample of water by inverting the bottle several times.
- Remove the bottle cap and cover, flame the mouth of the bottle, and inoculate the bottles of sterile broth as follows:
  - ✓ Add 50 ml of water to the bottle containing 50 ml of broth (by pouring direct into the bottle of broth up to a 100 ml graduation mark scratched previously on the bottle).
  - ✓ Using a sterile pipette, add 10 ml of water to each of the five bottles containing 10 ml of broth.

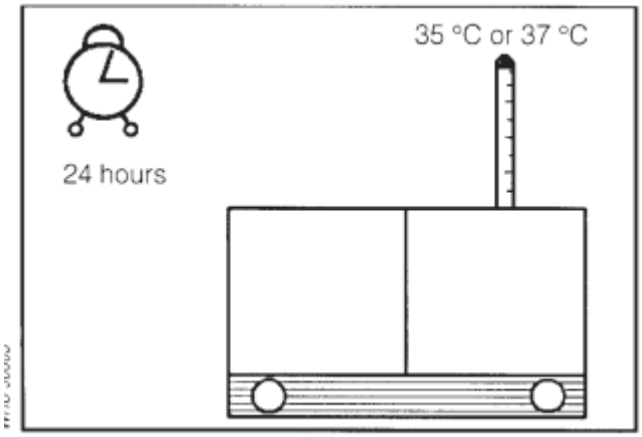


✓ For untreated samples, pipette 1 ml of water into each of five bottles containing 5 ml of broth.



(Note: the total volume of water inoculated is 100 ml for treated samples and 105 ml for untreated poor quality samples)

- Incubate the tubes at 35°C or 37°C for 24 hours.

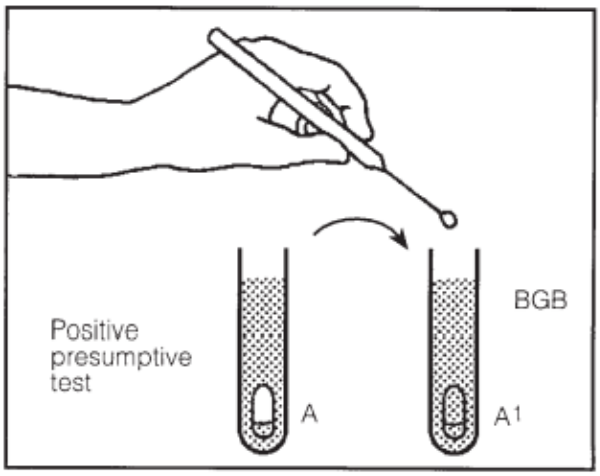


- At the end of the 24-hour incubation period, examine each tube for the presence of gas and acid production.
- Gas can be seen in Durham's tube and acid production is indicated by change in color of the medium (purple color turns yellow).
- Record the number of positive tubes after 24 hours.

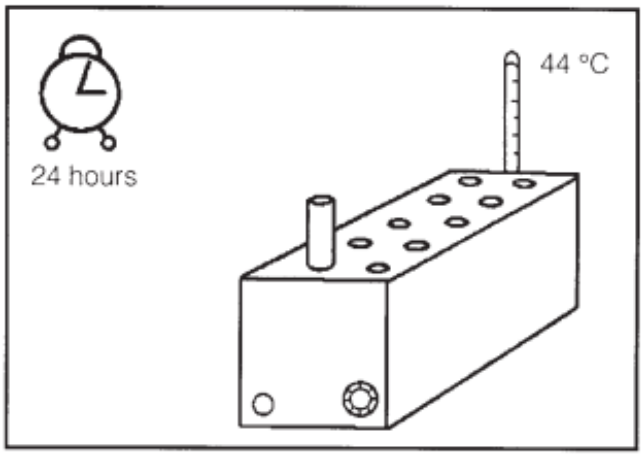
- Re-incubate negative tubes for a further 24-hour period, and check the tubes again for gas and acid production.
- Gas and acid production indicate the presence of coliform in that sample.

b. Confirmatory test

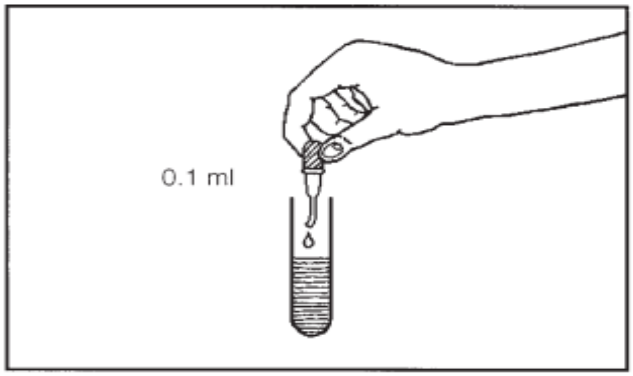
- It should be carried out at the end of both the 24-hour and the 48-hour incubation.
- Using a sterile loop, transfer one or two drops from each presumptive positive tube into two tubes containing respectively Brilliant Green Lactose Bile broth and tryptone water (Sterilize the inoculation loop before each transfer by flaming and allow cooling).



- To confirm the presence of fecal coliforms, incubate the subculture tubes from each presumptive positive tube for 24 hours at 44.6±0.5°C.



- At the end of 24 hours' incubation, examine each broth tube for growth and the presence of gas in the Durham tube.
- To each tube of tryptone water, add approximately 0.1ml of Kovacs reagent and mix gently (Red color in the Kovacs reagent, forming a film over the aqueous phase of the medium indicates presence of indole).



- Confirmatory tests positive for indole, growth, and gas production show the presence of *E. coli.*
- Growth and gas production in the absence of indole confirms fecal coliforms.

c. Completed test

- Sub-culture on LB with Durham tube and nutrient agar slant.
- After incubation for 24 hrs. at 37°C, the lactose broth is examined for gas production.
- A gram stained slide is made from the slant and slide observed under oil.
- If the organism proves to be Gram-negative non-spore forming rod that ferments lactose we conclude that coliform is present in the water sample.

VI. Determination of MPN

- For **treated water**, where one 50-ml and five 10-ml portions are inoculated, the MPN can be found from the test results by means of Table V1.
- For **untreated water**, where one 50-ml, five 10-ml and five 1-ml portions are inoculated, the MPN can be found from the test results by means of Table V2.

Table V1: MPN values per 100ml of sample for various combinations of positive and negative results (when one 50-ml and five 10-ml test portions are used)

No. of tubes giving a positive reaction		MPN per 100 ml
1 of 50 ml	5 of 10 ml	
0	0	<1
0	1	1
0	2	2
0	3	4
0	4	5
0	5	7
1	0	2
1	1	3
1	2	6
1	3	9
1	4	16
1	5	>18

Table V2: MPN values per 100ml of sample for various combinations of positive and negative results (when one 50-ml, five 10-ml and five 1ml test portions are used)

No. of tubes giving a positive reaction			MPN per 100ml
1 of 50 ml	5 of 10 ml	5 of 1 ml	
0	0	0	0
0	0	0	1
0	0	1	2
0	1	2	1
0	1	0	2
0	1	1	3
0	2	2	2
0	2	0	3
0	2	1	4
0	3	0	3
0	3	0	5
0	4	1	5
1	0	2	1
1	0	3	3
1	0	0	4
1	0	1	6
1	1	2	3
1	1	3	5
1	1	0	7
1	1	1	9
1	2	2	5
1	2	3	7
1	2	0	10
1	2	1	12
1	3	2	8
1	3	3	11
1	3	4	14
1	3	0	18
1	3	1	20
1	4	2	13
1	4	3	17
1	4	4	20
1	4	5	30
1	4	0	35
1	4	1	40
1	5	2	25
1	5	3	35
1	5	4	50
1	5	5	90
1	5		160
1	5		>180

VII. Interpretation of Results

The following guidelines are suggested for result interpretation.

SN	Presumptive coliform count per 100 ml	*E. coli* count per 100 ml	Class	Remarks
1.	0	0	I	**Excellent**
2.	1-3	0	II	**Satisfactory**
3.	4-10	0	III	**Suspicious**
4.	More than10	0 or more	IV	**Unsatisfactory**

7. References

- Cheesbrough M. (1984). Collection, transport, and examination of specimens. Medical laboratory manual for tropical countries, elbs, london.
- WHO guidelines
- Lippincots Illustrated Microbiology Reviews
- Essentials of Microbiology, Chapter 83: Bacteriology of Water, Milk and Air, Page 576-577. Surinder Kumar