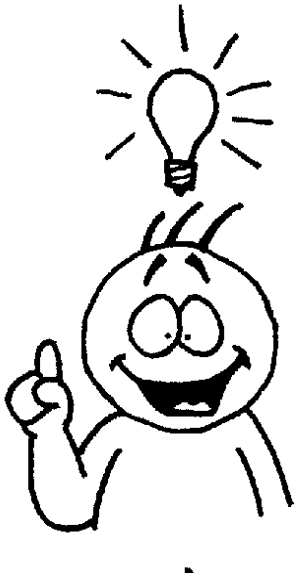
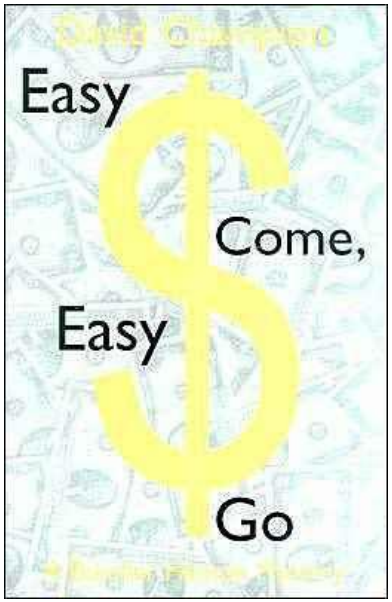
Estimation of *Coliform group* by Multiple Tube Fermentation Technique

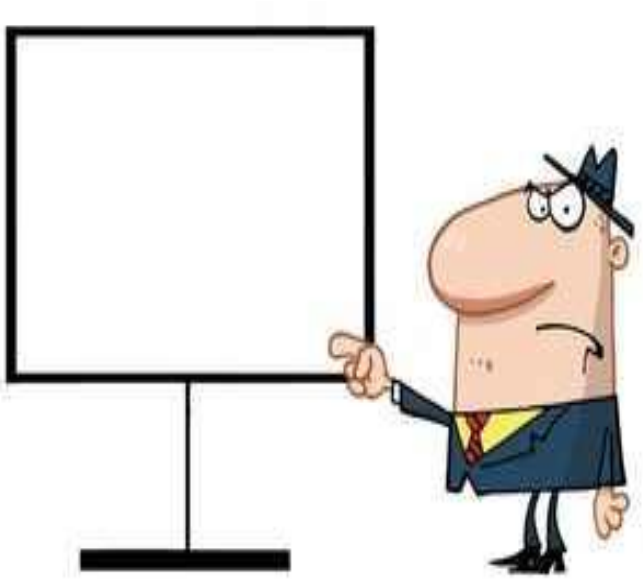
(For other than Drinking Water)



Understand ..
Not Memorize



Easy Come..
Easy Go ..



You..
Big Boss..

Index

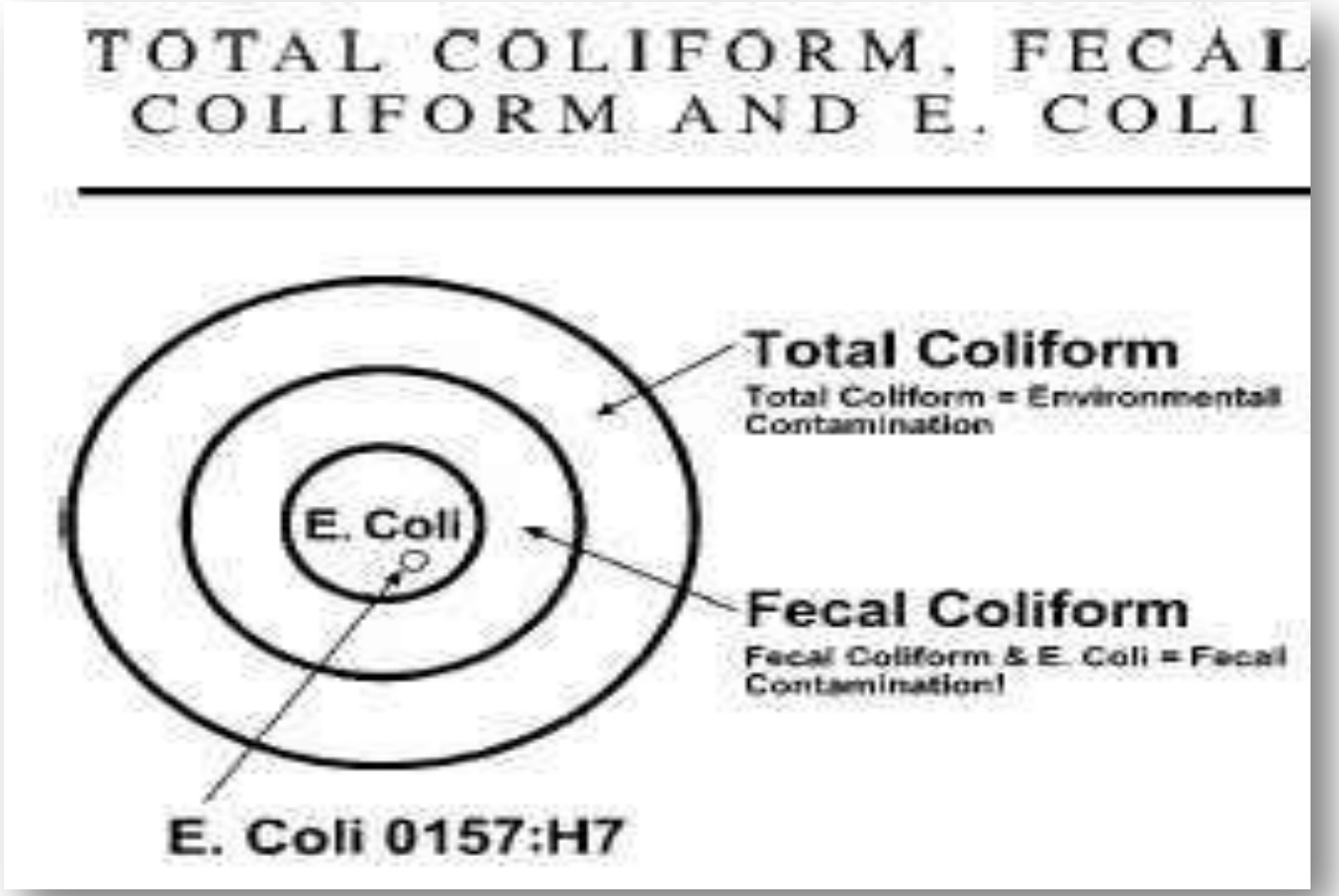
Scope :

This is a detailed procedure for:

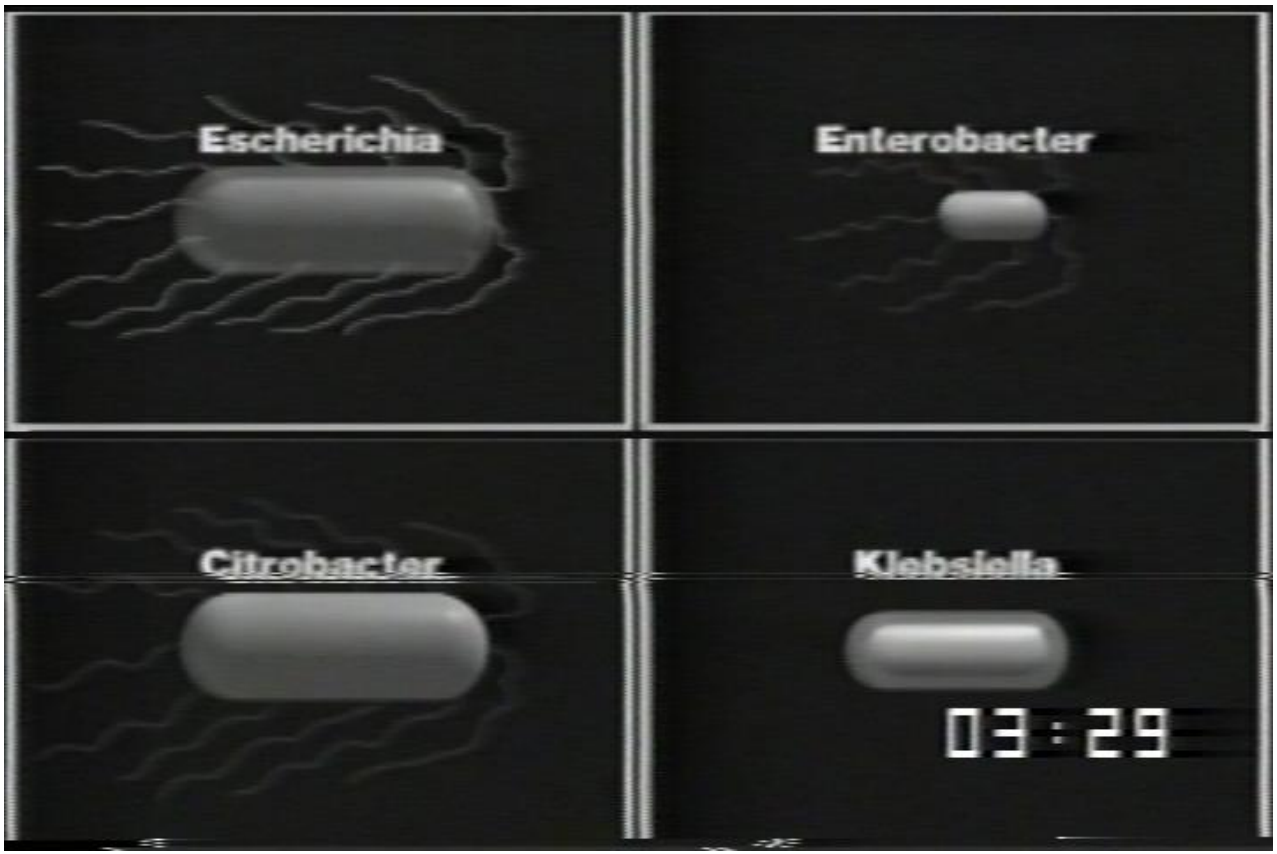
- Estimation of Coliforms by Multiple-Tube Fermentation technique (**MTF**),

- 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.

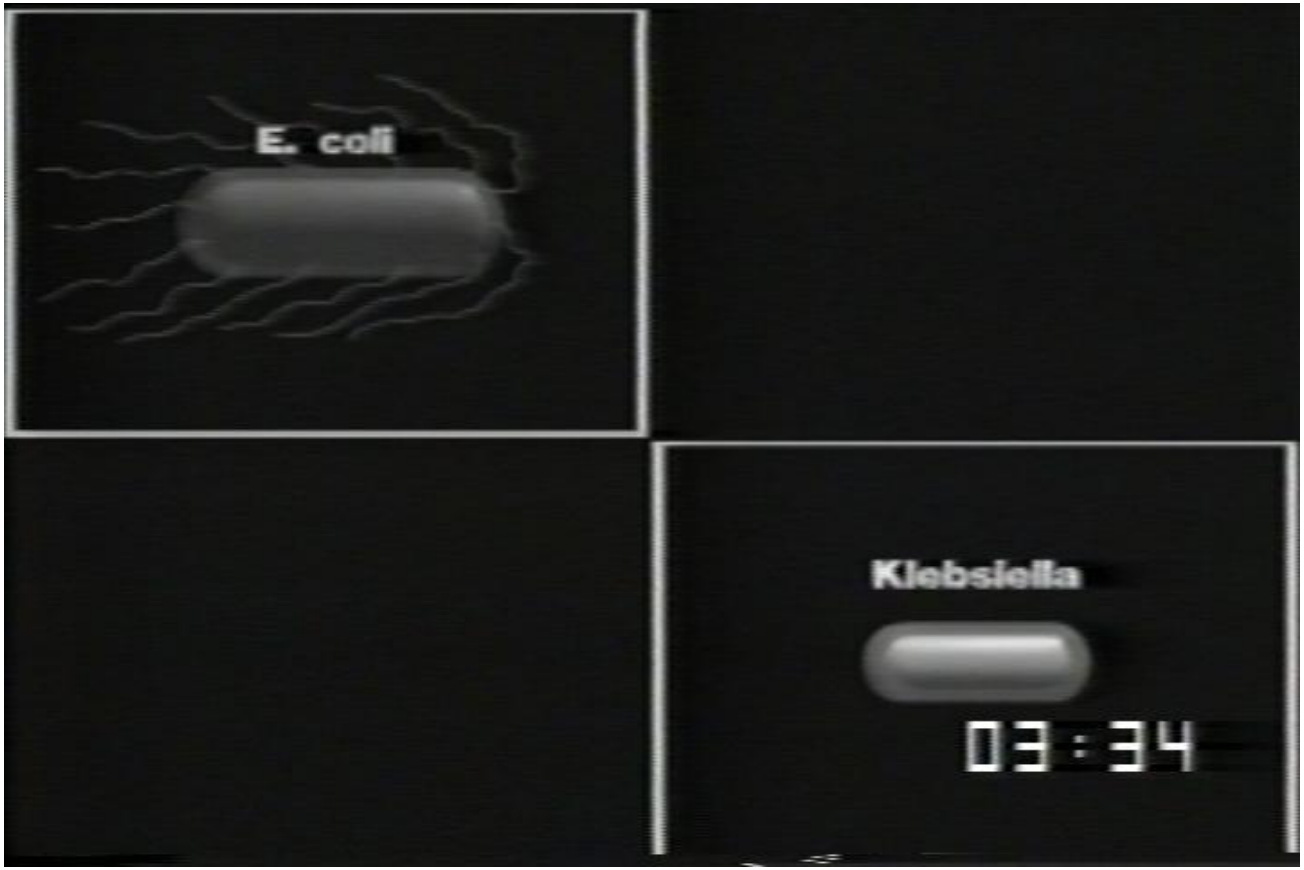

Coliforms
:
Total
Coliform
Fecal
Coliform


TOTAL COLIFORM, FECAL COLIFORM AND E. COLI
Total Coliform
Total Coliform = Environmental Contamination
E. Coli
Fecal Coliform
Fecal Coliform & E. Coli = Fecal Contamination!
E. Coli 0157:H7

Total Coliform (TC)



Fecal Coliform (FC)

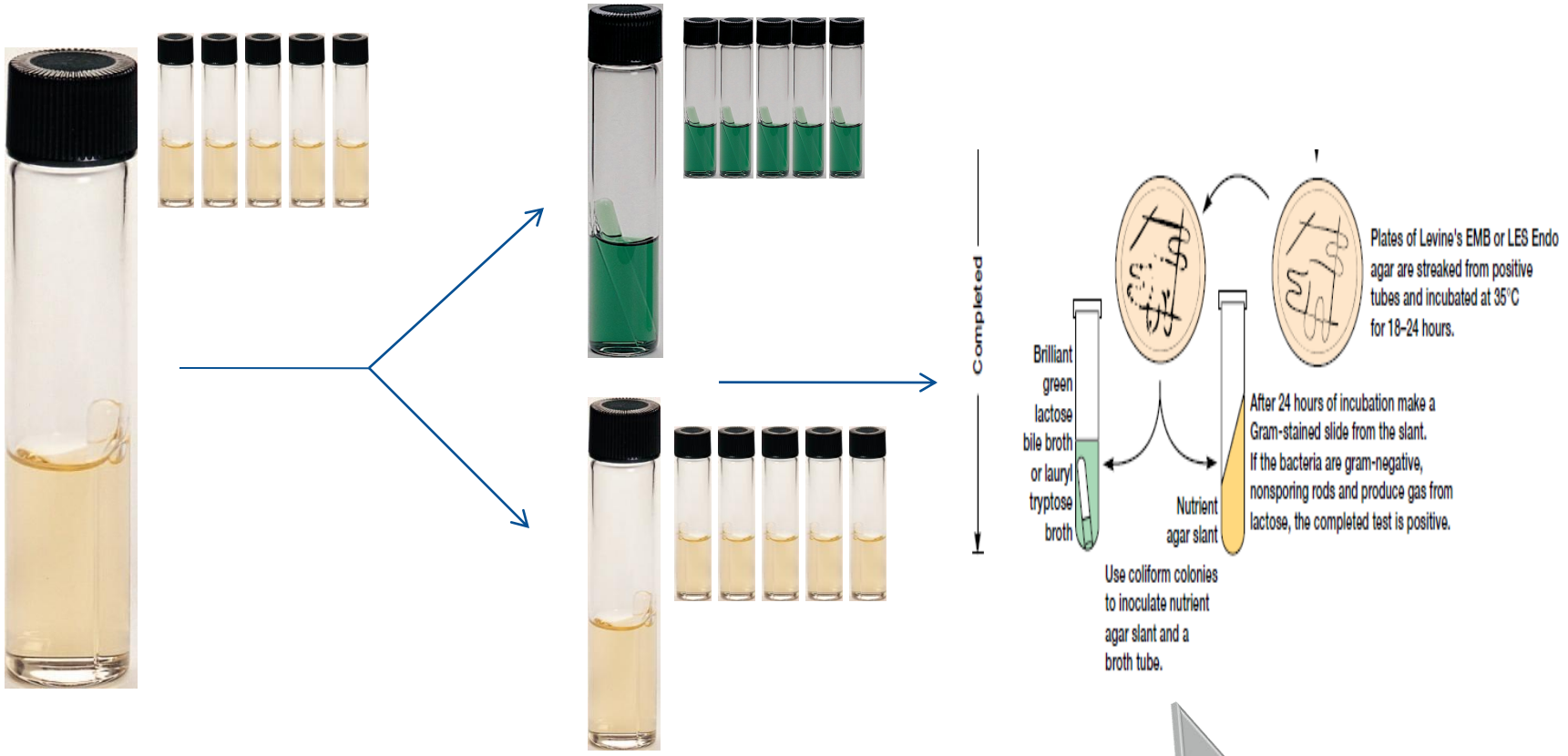


Scope :

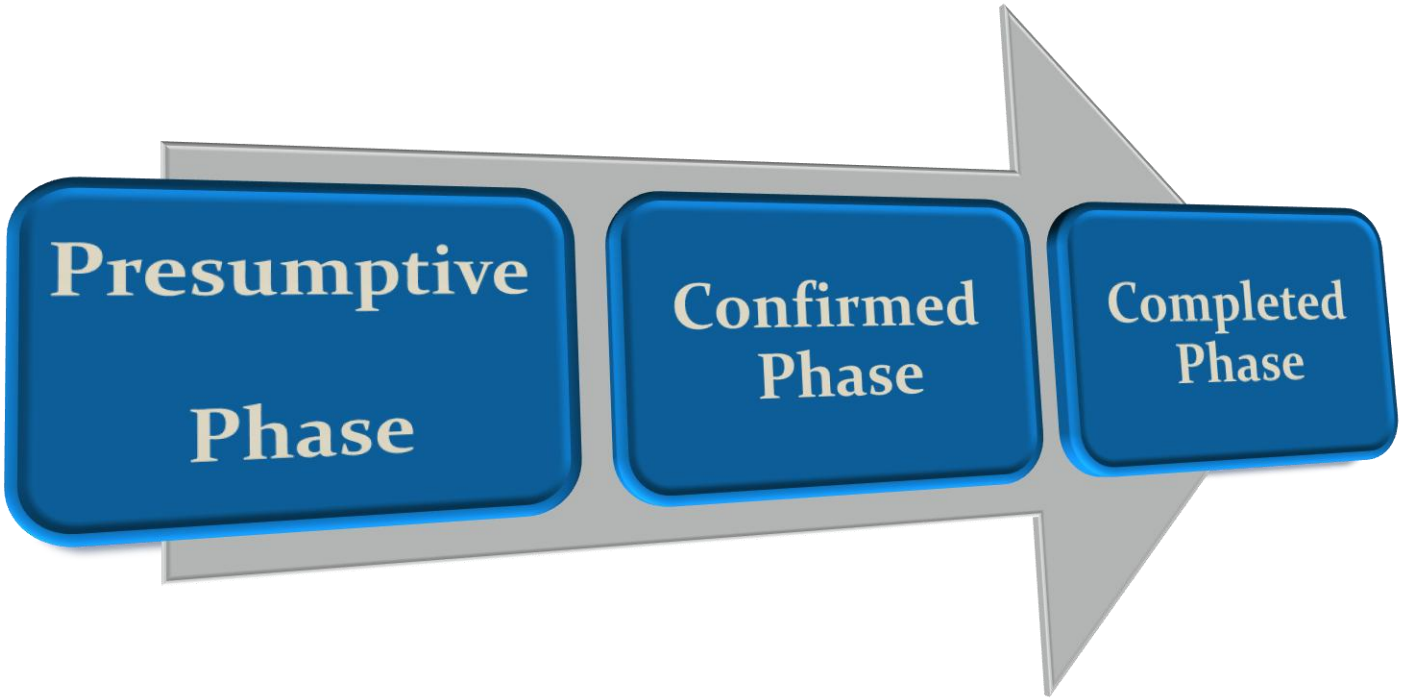
- This procedure can be applied for non-potable water.

Principle :

- Volumes of water sample to be tested are added to tubes containing the presumptive media (5 tubes per dilution) with inverted vials and incubated at 35°C for 48 hours.
- After incubation, the tubes are examined for growth (turbidity) and gas formation.
- An additional confirmatory is required to confirm the result.



Presumptive Phase → **Confirmed Phase** → **Completed Phase**


Presumptive Phase
Confirmed Phase
Completed Phase

Principle

Total coliforms

- 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.

Principle

Total coliforms

- 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.

Principle

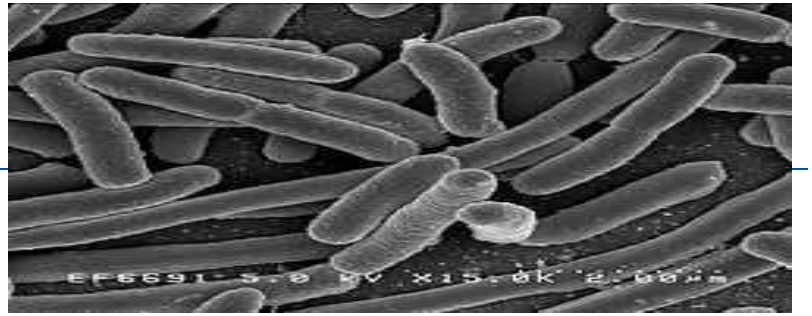
Fecal coliforms

- include species that may inhabit the intestines of warm-blooded animals and human. 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.

Fecal coliforms

- Populations of thermotolerant coliforms are composed predominantly of ***E. coli***; as a result, this group is regarded as a less reliable but acceptable index of faecal pollution, their presence in drinking water indicates the possible presence of pathogens.



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.**

- **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 48 hours **at 44.5°C.**

Interference :

- Since the MPN indexes are based on a Poisson distribution, if the sample is not adequately mixed to ensure equal bacterial cell distribution before portions are removed, the MPN value will be a misrepresentation of the bacterial density.

Interference

- 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.

Procedure

Procedure

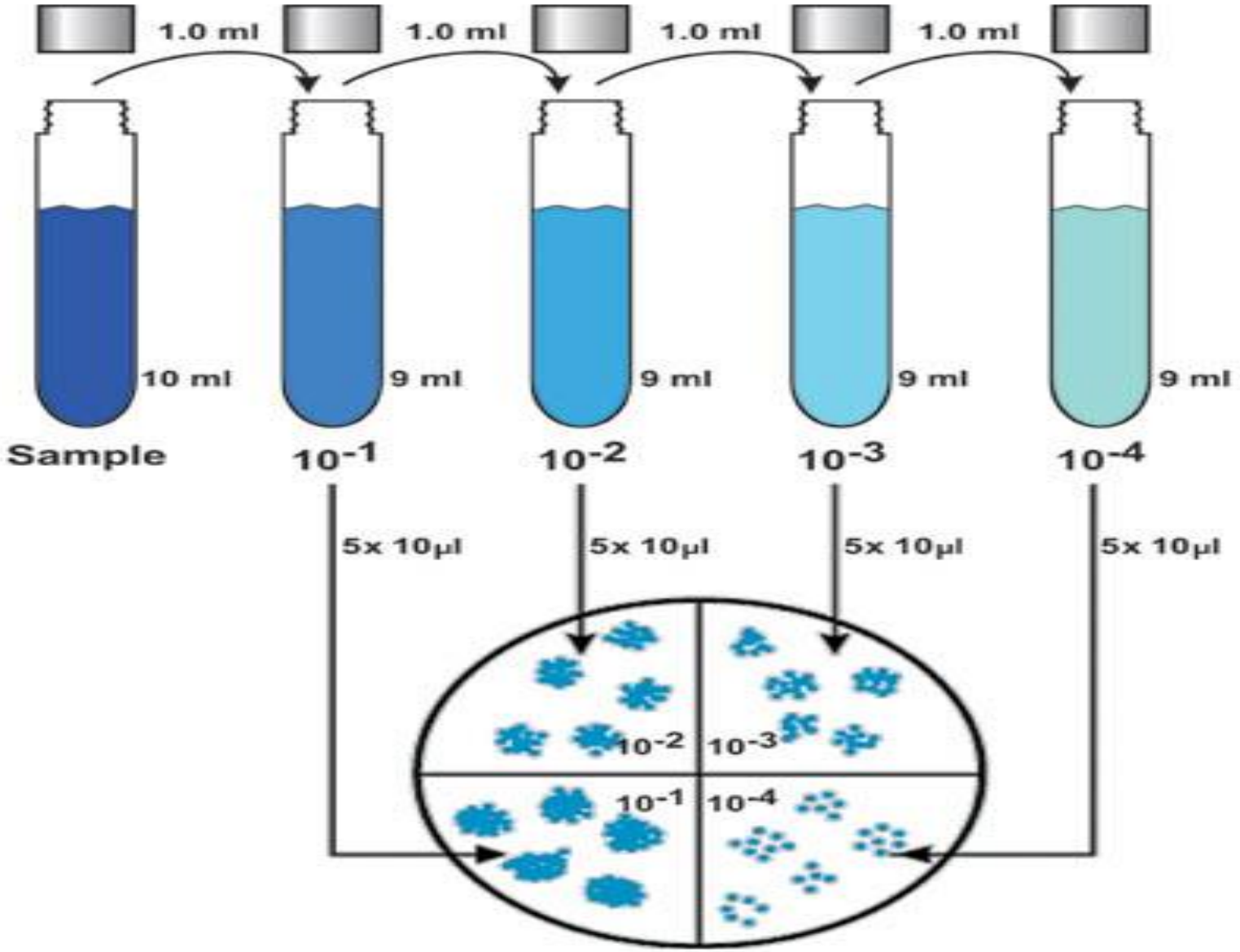
Sample Handling:

Sample Holding Time and Limitations:

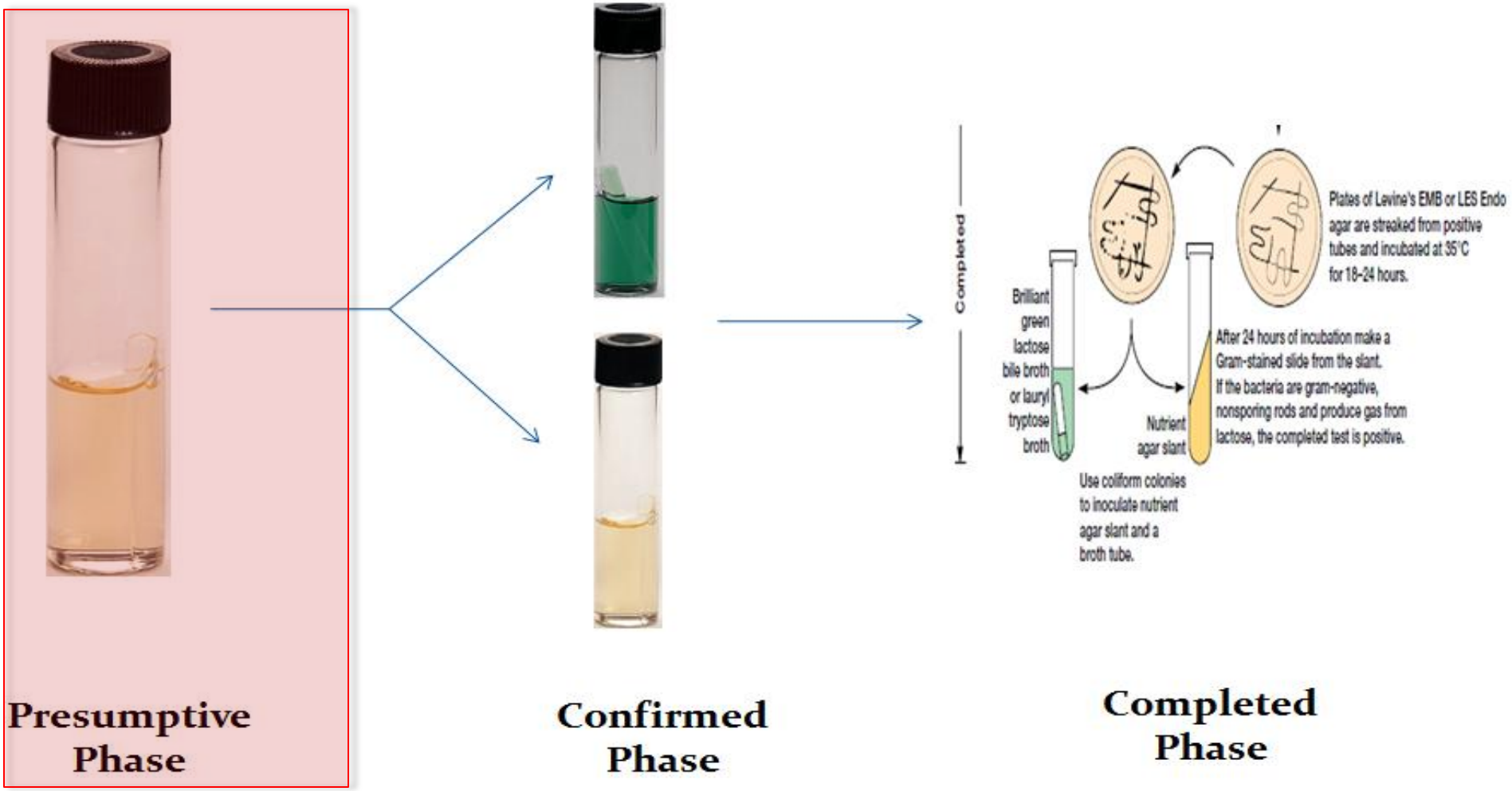
Analyze samples **on day of receipt whenever possible**, and **refrigerate at 3±2.0°c overnight if arrival is too late** for processing on same day.

Selection of sample size:

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


1.0 ml
1.0 ml
1.0 ml
1.0 ml
10 ml
9 ml
9 ml
9 ml
9 ml
Sample
10-1
10-2
10-3
10-4
5x 10µl
5x 10µl
5x 10µl
5x 10µl
10-2
10-3
10-1
10-4

Presumptive Phase



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.

Lauryl Tryptose broth:

- 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).

Samples Dilutions :

- Mix the sample by vigorously shakes the bottle.
- Use a sterile tip to transfer **10** ml of sample to **90** 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.

Inoculation :

1. Presumptive Phase:

    1.1. Use lauryl tryptose broth for presumptive phase.

    1.2. Arrange fermentation tubes in rows

    1.3. Prepare three set of serial dilutions (e.g. 0.1, 0.01, 0.001 ml) using five tubes per dilution.

    1.4. Shake sample or dilutions vigorously.

    1.5. Inoculate each tube in the set or bottle with sample or dilution volume.

    1.6. Mix test portions in the medium by gentle agitation.

1. Presumptive Phase:

Incubation :

1.7. Incubate inoculated tubes or bottles at 35 ± 0.5C.

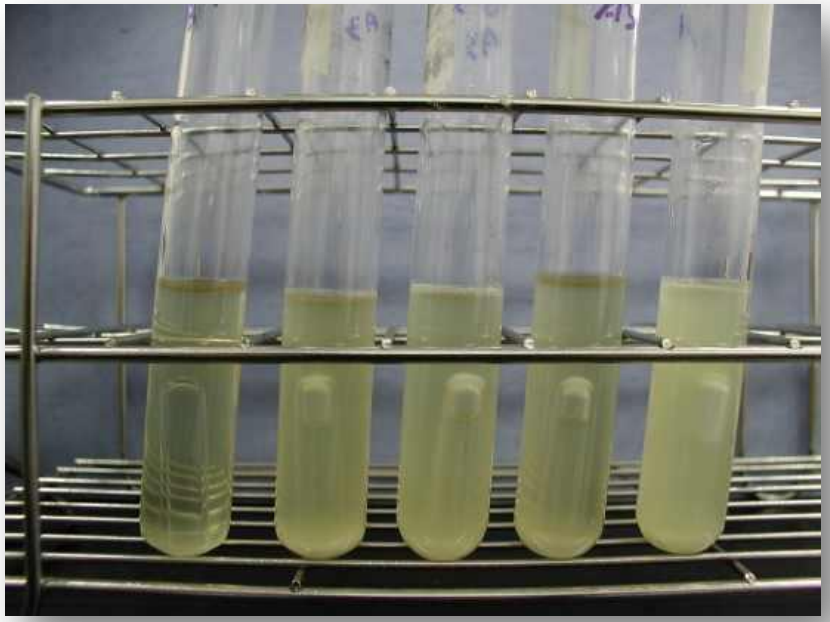
1.8. After 24 ± 2 h swirl each tube or bottle gently and examine it for growth, gas, and acidic reaction (shades of yellow color)

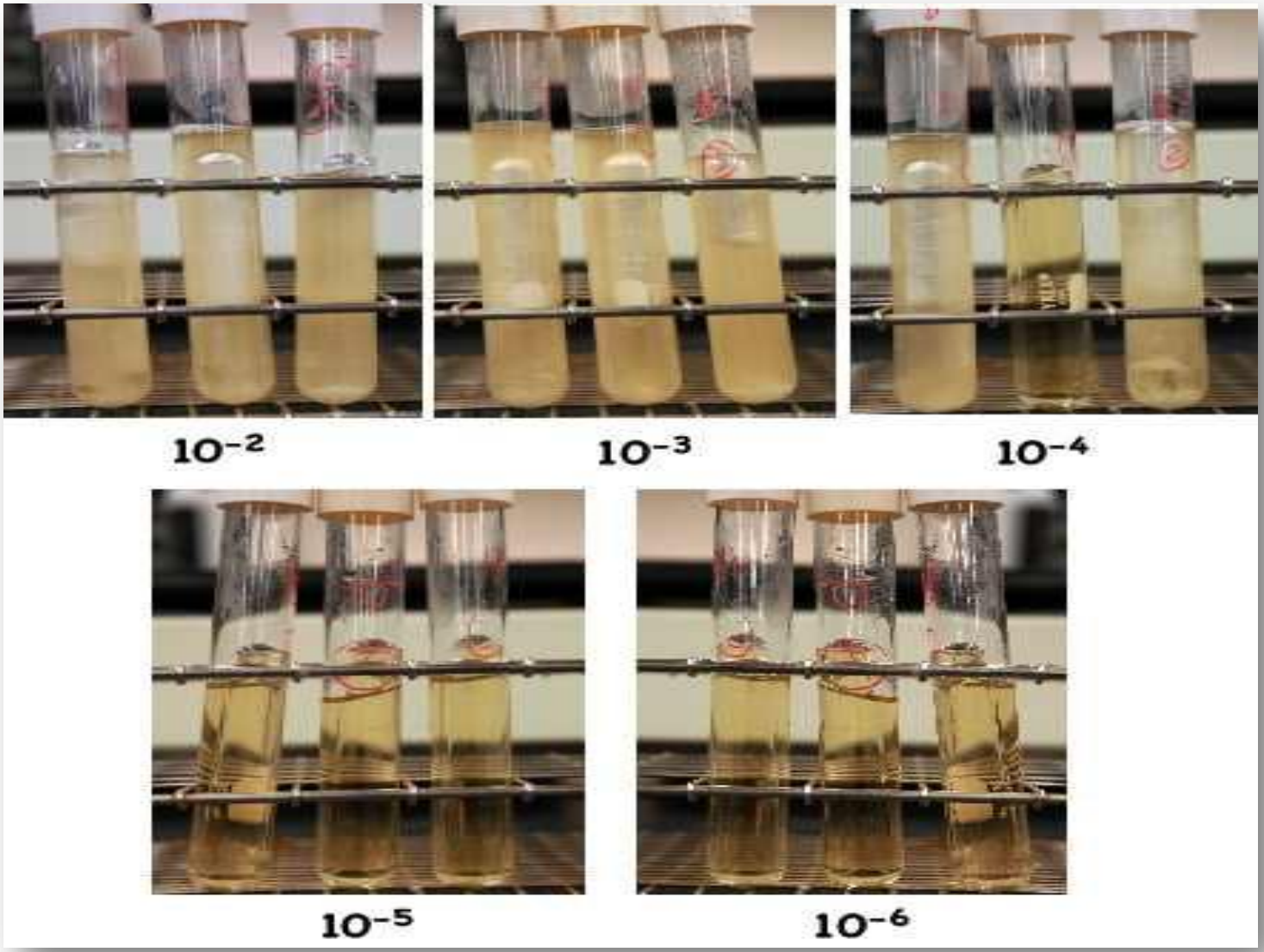
1.9. If no gas or acidic reaction is evident, reincubate and reexamine at the end of 48 ± 3 h.

1.10 If the inner vial is omitted, growth with acidity signifies a positive presumptive reaction

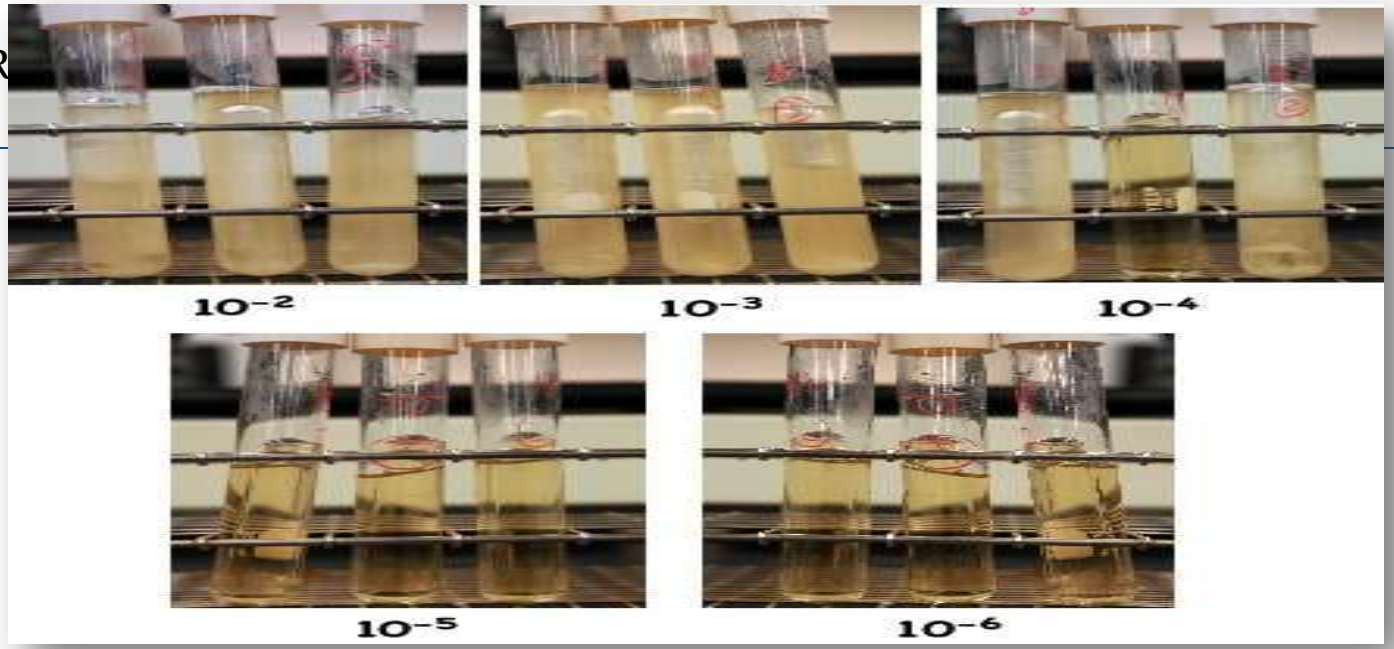
Interpretation :

- Submit tubes produced an acidic reaction or gas 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.




10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}

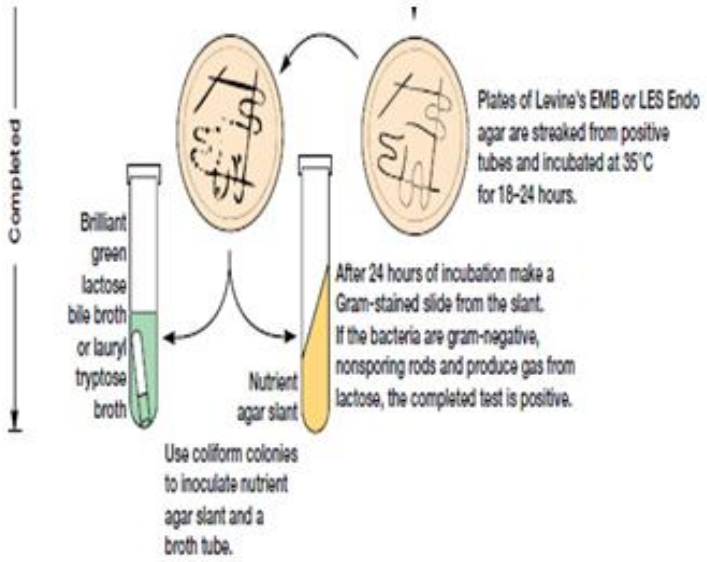
R



Example	1 ml	0.1 ml	0.01 ml	Combination	MPN index/100 ml	Result
Lauryl Tryptose broth	5	1	o	5-1-0	330	
Brilliant Green	3		0			
EC			0			


B. sub
S.
E. C
S. aureus
S. avr

Confirmed Phase



Presumptive Phase

Confirmed Phase

Completed Phase

2. Confirmed Phase:

2.1. Use **brilliant green lactose bile broth** fermentation tubes for the confirmed phase for **Total Coliform**.

2.2. Use **EC broth** fermentation tubes for the confirmed phase for **Fecal Coliform.**

Inoculation

1. Arrange fermentation tubes in rows.

2. Gently shake or rotate positive presumptive tubes or bottles to resuspend the organisms.

3. Use a sterile loop 3.0 to 3.5 mm in diameter to transfer three loopfuls of culture to a fermentation tube containing brilliant green lactose bile broth and EC broth.

4. 2.6. Repeat for all other positive presumptive tubes.

Confirmed Phase:

Incubation:

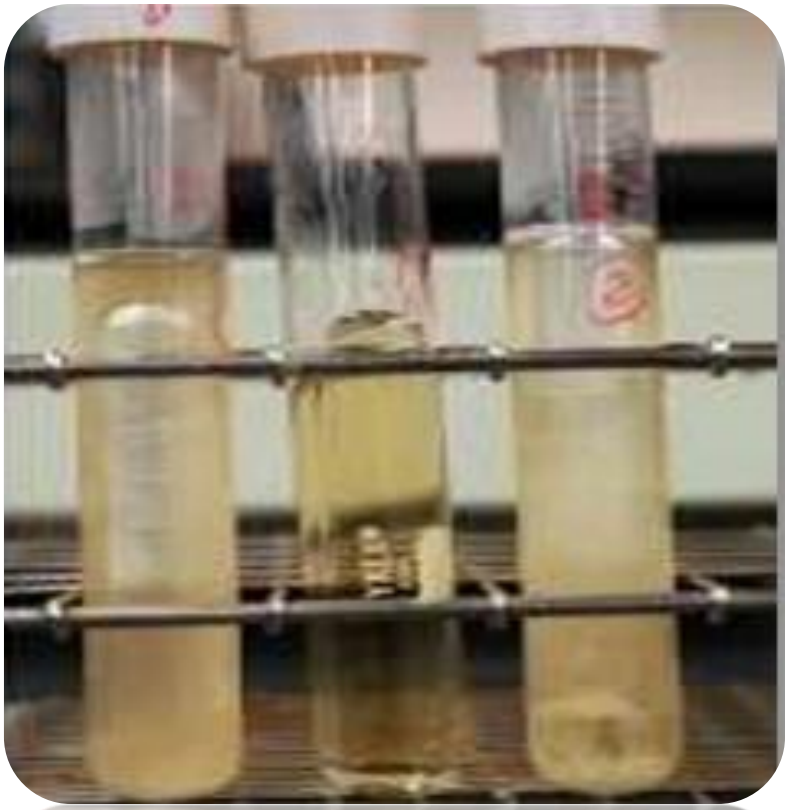
- Incubate Brilliant Green tubes at 35 ± 0.5°C for 48 ± 3 h.
- Incubate EC tubes at 44.5 ± 0.2°C for 24 ± 2 h..

Confirmed Phase:

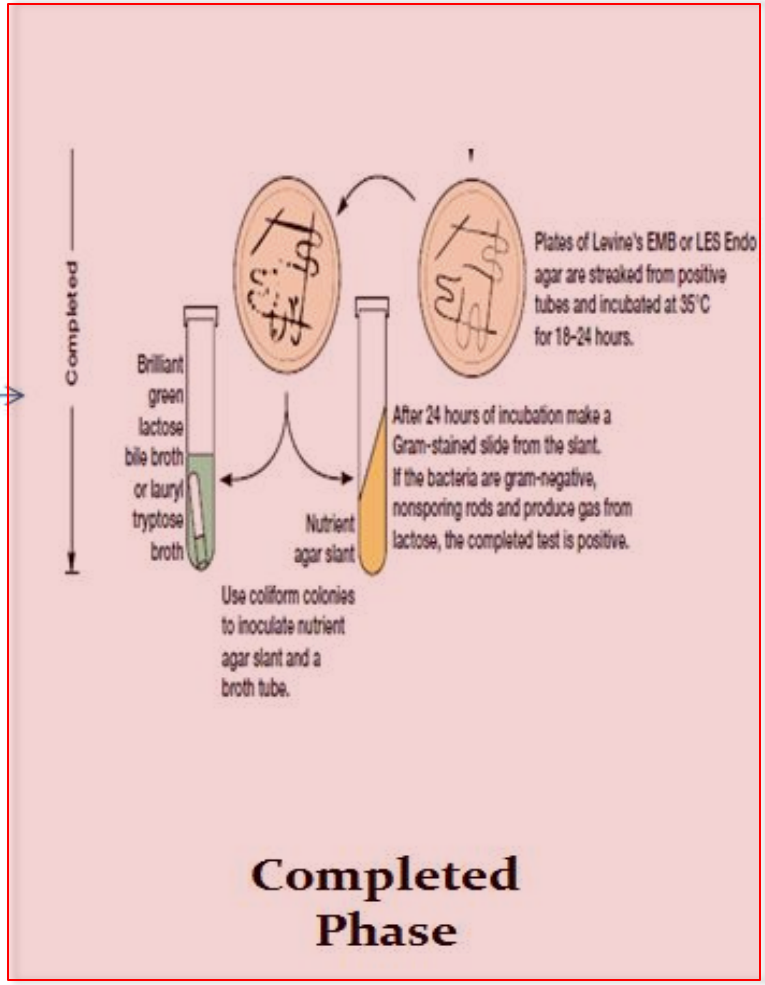
Incubation:

- **Formation of gas in any amount** in the inverted vial constitutes a positive confirmed result.

- Calculate the MPN value of the number of positive tubes from MPN index.



Completed Phase



Presumptive Phase

Confirmed Phase

Completed Phase

3. Completed Phase (In case of Analyzing Total Coliform Only):

3.1. Use **LES Endo agar** plates and **Nutrient agar slants** for completed phase

3.2. Streak one LES Endo agar plate from each tube of brilliant green lactose bile broth showing gas, as soon as possible after the observation of gas.

3.3. Streak plates in a manner to insure presence of some discrete colonies separated.

3.4. Incubate plates (inverted) at 35 ± 0.5°C for 24 ± 2 h.

Completed Phase:

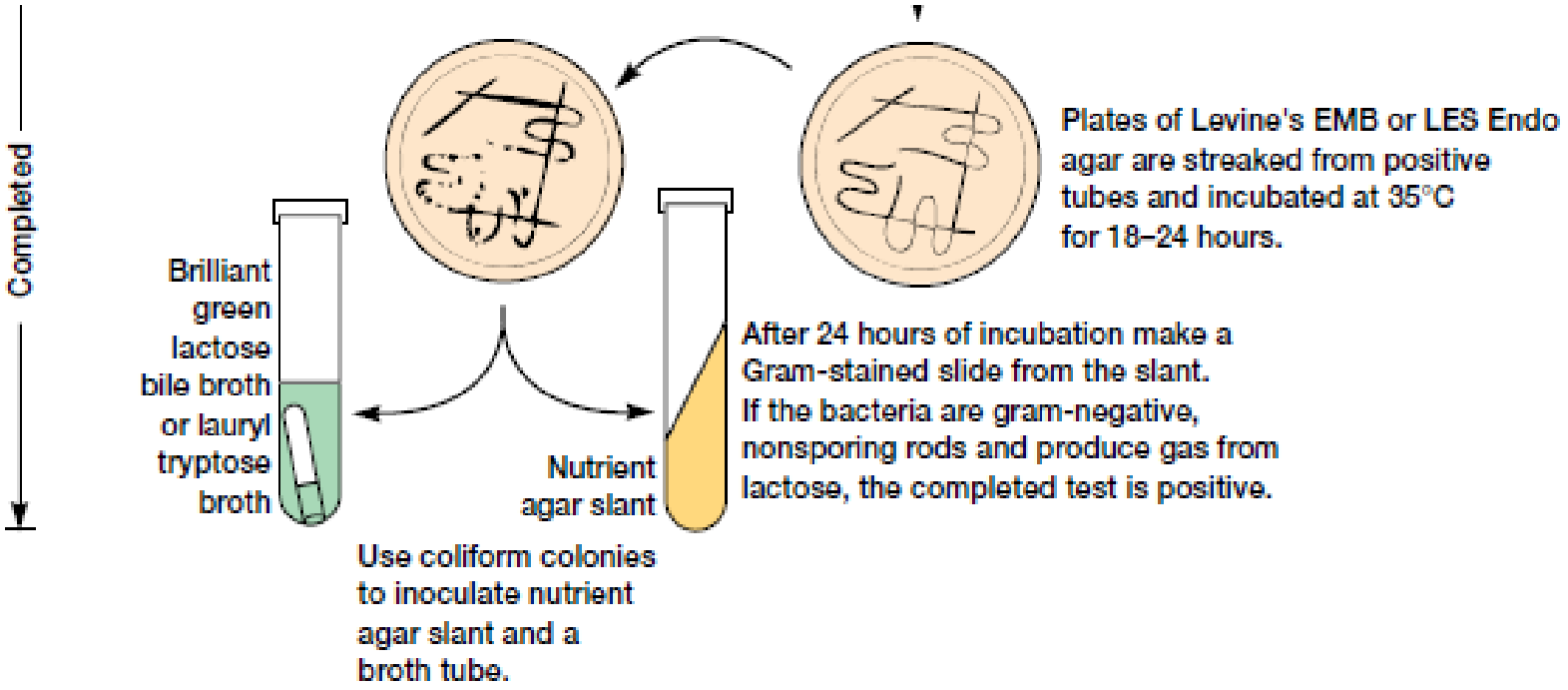
3.5 Pick from each plate one or more typical (pink to dark red with a green metallic surface sheen), well-isolated coliform colonies.

3.6 Transfer growth from each isolate to a **single-strength lauryl tryptose broth** fermentation tube and onto a **nutrient agar slant**.

3.7 Incubate nutrient agar slants at 35 ± 0.5°C for 24 ± 2 h.

3.8 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.

Completed Phase:



Completed Phase:

3.9 Examine microscopically **Gram-stained preparations** from those 24-h nutrient agar slant cultures corresponding to the secondary tubes that show gas.

3.10 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.

Calculations

Calculations :

- 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.

MPN Index

Example	1 ml	0.1 ml	0.01 ml	Combination	MPN index/100 ml	Result
A	5	1	0	5-1-0	330	3300
B	4	3	1	4-3-1	48	480
C	4	4	1	4-4-1	40	400
E	5	5	2	5-5-2	540	5400

Index numbers in the table above are for example only and not necessarily indicate true values

Examples illustrating correct selection are listed below :

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	**0**	**0**	**1**	0	0	0-0-1	1.8
D	5	**4**	**4**	**1**	0	4-4-1	400
E	5	**4**	**4**	0	**1**	4-4-1	400
F	5	5	**5**	**5**	**2**	5-5-2	54000

Examples illustrating correct selection are listed below :

•If MPN value for combinations not appearing in the table, or for other combinations of tubes or dilutions, estimate the result as follows:

1. Select the lowest dilution that does not have all positive results.
2. Select the highest dilution with at least one positive result.
3. 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.

- Report coliform concentration as the Most Probable Number (MPN)/100 ml.

Examples :

MPN / 100 ml (approx.) = 100 × P / (N × T) ½

- 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

Examples :

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

- From (5/5, 10/10, 10/10, 0/10, 0/5) Use only (-, -, 10/10, 0/10. -), take :

  10/10 @ 0.1 ml sample/tube and

  0/10 @ 0.01 ml sample/tube.

  calculations will be:

$$\text{MPN / 100 ml (approx.)} = 100 \times 10 / (0.1 \times 1.1)^{½} =$$

$$1000/0.332 = 3000/\ 100\ \text{ml}.$$

Thank you