Amended, Jan 1, 2022
MOHWM0023.02

# Methods of Test for Food Microorganisms -Test of *Escherichia coli*

**1. Scope:** This method is applicable for detection and enumeration of *Escherichia coli (E. coli)* in food.

**2. Method:** Samples are serially diluted and selectively cultured, and E. coli is enumerated with either most probable number (MPN) method or direct plate count method.

**2.1.** Work environment: The working platform needs to be spacious, clean and well-lit with illumination of cabinet over 100 cd. The air in the closed room is well-ventilated, with as little dust and flowing air as possible. Colonies must not exceed 15 CFU/dish for every 15 min.

**2.2.** Equipment and materials

**2.2.1.** Sterilization oven (Hot air sterilizer): capable of operating at 170 ± 10°C.

**2.2.2.** Autoclave: capable of operating at 121°C or higher temperature.

**2.2.3.** Refrigerator: capable of operating at 5 ± 3°C.

**2.2.4.** Incubator: capable of controlling temperature at ± 1.0°C.

**2.2.5.** Water bath: capable of controlling water temperature at ± 0.2°C.

**2.2.6.** Blender or stomacher: appropriate for aseptic operation.

**2.2.7.** Light microscope: with maximum magnification of 1000×.

**2.2.8.** Balances: with sensitivities of 0.1 g and 1 mg for capacities of 2,000 g and 100 g, respectively.

**2.2.9.** Stirrer

**2.2.10.** Vortex mixer

**2.2.11.** pH meter

**2.2.12.** Heater

**2.2.13.** Shaker

**2.2.14.** Pipette aid and micropipettes

**2.2.15.** Graduated pipettes: sterile, 1 mL pipette with scale of 0.01 mL; 5 mL and 10 mL pipettes with scale of 0.1 mL.

**2.2.16.** Containers: screw-capped flasks, glass bottles or wide-mouth jars made of glass, polyethylene, Teflon or other materials that can be sterilized at 121°C for more than 20 min, or sterilized bags.

**2.2.17.** Petri dishes: sterile, 90 × 15 mm, surface of the dish should be flat

and contain no bubbles or scratches.

**2.2.18.** Inoculating needle and inoculating loop (3 mm i.d.): made of nichrome, platinum-iridium or chromel wire, or disposable products.

**2.2.19.** Test tube: 16 × 150 mm or a comparable product.

**2.2.20.** Durham fermentation tube: 9 × 22 mm or a comparable product.

**2.2.21.** Slide and coverslip: suitable for microbiological staining and microscopy.

**2.2.22.** Spatula, scissors, scalpel and forceps: sterilizable or disposable.

**2.2.23.** Filter paper and brown reagent bottles.

**2.2.24.** Mortar and pestle: for grinding reagents.

**2.2.25.** Chemicals:

Reagent grade: methylene blue, bile salts No.3, glucose, lactose, sodium lauryl sulfate, eosin Y, sodium ammonium hydrogen phosphate ($NaNH_4HPO_4 \cdot 4H_2O$), magnesium sulfate ($MgSO_4 \cdot 7H_2O$), sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), sodium chloride (NaCl), potassium dihydrogen phosphate ($KH_2PO_4$), dipotassium hydrogen phosphate ($K_2HPO_4$), crystal violet, ammonium oxalate ($C_2H_8N_2O_4$), potassium iodide (KI), iodine, safranin O, *p*-dimethylaminobenzaldehyde ($C_9H_{11}NO$), methyl red, α-Naphthol ($C_{10}H_8O$), dehydrated ethanol ($C_2H_5OH$), sodium hydroxide (NaOH), potassium hydroxide (KOH), creatine, 95% ethanol, amyl alcohol ($C_5H_{12}O$), polysorbate 80 (Tween 80), 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (cyclohexylammonium salt), hydrochloric acid (HCl);
Microbiological grade: peptone, tryptose, yeast extract, tryptone, trypticase, buffered peptone-water powder, agar.

**2.2.26.** Reagents

**2.2.26.1.** Diluents

**2.2.26.1.1.** Normal saline

Dissolve 8.5 g of sodium chloride in distilled water and bring the volume to 1000 mL. Dispense and autoclave the solution at 121°C for 15 min.

**2.2.26.1.2.** Butterfield's phosphate-buffered dilution water (BPBW)

Dissolve 34 g of potassium dihydrogen phosphate in 500 mL

of distilled water. Adjust the pH to 7.2 with 1 N sodium hydroxide solution, and add distilled water to 1000 mL. Autoclave the solution at 121°C for 15 min and, after cooling, store it in a refrigerator as a stock solution. Dilute 1.25 mL of the stock solution with distilled water to 1000 mL. Dispense and autoclave the diluted solution at 121°C for 15 min before use.

**2.2.26.1.3.** Peptone diluent, 0.1%

Dissolve 1 g of peptone in 1000 mL of distilled water. Dispense and autoclave the solution at 121°C for 15 min. Final pH of the solution is 7.0 ± 0.2.

**2.2.26.2.** Gram stain solutions

**2.2.26.2.1**. Hucker's crystal violet solution (primary dye)

Solution A: dissolve 2 g of crystal violet in 20 mL of 95% ethanol.

Solution B: dissolve 0.8 g of ammonium oxalate in 80 mL of distilled water.

Mix solution A and solution B and let the mixture stand at room temperature for 24 hr. Filter the mixture with a filter paper, and collect the filtrate as the primary dye.

**2.2.26.2.2.** Gram iodine solution (mordant)

Put 2 g of potassium iodide and 1 g of iodine into a mortar. Grind the crystals initially with a pestle for 5 to 10 sec, and then grind with sequential addition of 1 mL, 5 mL, and 10 mL of distilled water until the crystals are completely dissolved. Pour the solution into a brown bottle, and wash the mortar and pestle with an appropriate amount of distilled water. Pool the washings and the initial solution and add distilled water to bring the volume to 300 mL.

**2.2.26.2.3.** Hucker's counterstain

Dissolve 2.5 g of safranin O in 100 mL of 95% ethanol to make counterstain stock solution. Working solution is prepared by diluting 10 mL of the stock solution with 90 mL of distilled water.

Note 1: Gram stain solutions deteriorate after prolonged

storage. Note the expiry date of the commercial products. Users preparing the staining solutions themselves should regularly check the staining quality.

**2.2.26.3.** Kovacs' reagent

Dissolve 5 g of *p*-dimethylaminobenzaldehyde in 75 mL of amyl alcohol and slowly add 25 mL of concentrated hydrochloric acid. The solution appears yellow after mixed thoroughly. Store it in a refrigerator.

**2.2.26.4.** Methyl red indicator

Dissolve 0.1 g of methyl red in 300 mL of 95% ethanol and bring the volume to 500 mL with distilled water.

**2.2.26.5.** Voges-Proskauer reagents (VP reagents)

Solution A: dissolve 5 g of α-naphthol in 100 mL of dehydrated ethanol.

Solution B: dissolve 40 g of potassium hydroxide in 100 mL of distilled water.

**2.2.27.** Medium

**2.2.27.1.** Lauryl sulfate tryptose (LST) broth

Tryptose or trypticase……20 g
Lactose……5 g
$KH_2PO_4$ ……2.75 g
$K_2HPO_4$ ……2.75 g
NaCl……5 g
Sodium lauryl sulfate……0.1 g
Distilled water……1000 mL

Dissolve the components by heating and dispense the solution into 10-mL aliquots in test tubes containing a fermentation tube. Sterilize the aliquots by autoclaving at 121°C for 15 min. Final pH is 6.8 ± 0.2.

**2.2.27.2.** EC Broth

Tryptose or trypticase……20 g
Lactose……5 g
Bile salts No. 3……1.5 g
$KH_2PO_4$ ……1.5 g

$K_2HPO_4$ ……4 g
NaCl……..5 g
Distilled water……..……..1000 mL
Dissolve the components by heating and dispense the solution into 8-mL aliquots in test tubes containing a fermentation tube. Sterilize the aliquots by autoclaving at 121°C for 15 min. Final pH is 6.9 ± 0.2.

**2.2.27.3.** Levine's eosin methylene blue agar (L-EMB)
Peptone……...10 g
Lactose……..……10 g
$K_2HPO_4$ ……..……2 g
Agar……..…….....15 g
Eosin Y…….……0.4 g
Methylene blue…….……..0.065 g
Distilled water……...1000 mL
Dissolve the components by heating and autoclave the solution at 121°C for 15 min. Final pH is 7.1 ± 0.2. Cool the medium to 50°C, gently shake it to evenly suspend the flocculent precipitate without bubble generation, and pour 15-20 mL of the medium into a Petri dish. Allow the medium to solidify and the agar surface to dry with the lid of the Petri dish one-fourth to half open.

**2.2.27.4.** Plate count agar (PCA)
Tryptone……..5 g
Yeast extract……..……2.5 g
Glucose…….……....1 g
Agar…….……...……15 g
Distilled water……..1000 mL
Dissolve the components by heating and autoclave the solution at 121°C for 15 min. Final pH is 7.0 ± 0.2. Aliquot the medium into test tubes to make agar slants, or pour the medium into Petri dishes (12-15 mL per dish) to make agar plates. Ensure that the agar surface is dry before use.

**2.2.27.5.** Tryptone or tryptophane broth
Tryptone or trypticase……..……10 g

Distilled water……1000 mL

Dissolve the powder by heating and dispense the solution into 5-mL aliquots in test tubes, and sterilize the aliquots by autoclaving at 121°C for 15 min. Final pH is 6.9 ± 0.2.

**2.2.27.6.** MR-VP broth

Buffered peptone-water powder……7 g

Glucose……5 g

$K_2HPO_4$……5 g

Distilled water……1000 mL

Dissolve the components by heating and dispense the solution into 5-mL aliquots in test tubes, and sterilize the aliquots by autoclaving at 121°C for 15 min. Final pH is 6.9 ± 0.2.

**2.2.27.7.** Koser's citrate broth

$NaNH_4HPO_4 \cdot 4H_2O$……1.5 g

$KH_2PO_4$ ……1 g

$MgSO_4 \cdot 7H_2O$ ……0.2 g

$Na_3C_6H_5O_7 \cdot 2H_2O$……3 g

Distilled water……1000 mL

Dissolve the components by heating and dispense the solution into 10-mL aliquots in screwcapped tubes, and sterilize the aliquots by autoclaving at 121°C for 15 min. Final pH is 6.7 ± 0.2.

**2.2.27.8.** Tryptone bile X-glucuronide agar (TBX)

Tryptone……20 g

Bile salts No. 3……1.5 g

5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid (cyclohexyl-ammonium salt) ……0.075 g

Agar……15 g

Distilled water……1000 mL

Dissolve 0.075 g of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (cyclo-hexylammonium salt) in 3 mL of sodium hydroxide-ethanol solution (2.5 mL of 95% ethanol and 0.5 mL of 1N NaOH). Mix this solution with other components and heat to dissolve. Autoclave the medium at 121°C for 15 min. Final pH is 7.2 ± 0.2.

## 2.3. Preparation of test

**2.3.1.** Solid samples

Mince and mingle the sample properly, take 50 g of the sample, and mix well with 450 mL of diluent as the 10-fold diluted test solution.

**2.3.2.** Powder, granule or other easily smashed samples

Smash the sample with sterilized spatulas or other utensils, and mix well. Take 50 g of the sample, and mix well with 450 mL of diluent as the 10-fold diluted test solution.

**2.3.3.** Liquid samples

Mix the sample thoroughly, take 50 mL of the sample, and mix well with 450 mL of diluent as the 10-fold diluted test solution.

**2.3.4.** Frozen samples

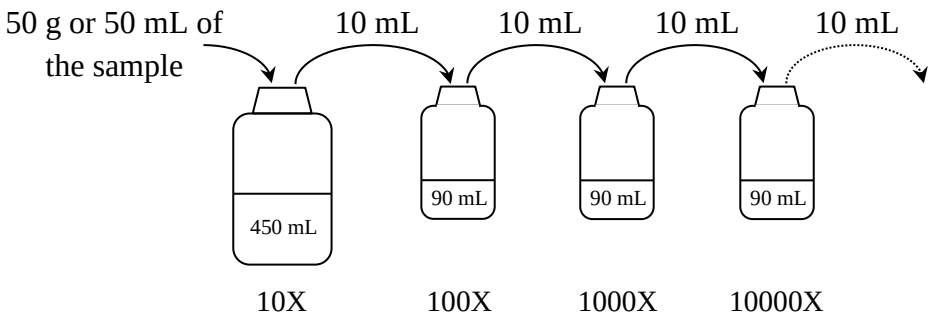
Completely thaw the samples, such as frozen fish meat, poultry meat, fruits, vegetables and dumplings, etc., at refrigerated temperatures (e.g. at 2-5°C within 18 hr), or quickly thaw them at higher temperatures (e.g. in a water bath at 45°C for samples that could be thawed within 15 min). Shake the sample frequently to accelerate thawing. After thawing, chop the sample and mix evenly. Make the sample which do not need to be thawed, such as ice cubes, popsicles and ice cream, etc., into appropriate small pieces, take 50 g of the sample, and mix well with 450 mL of diluent as the 10-fold diluted test solution.

**2.3.5.** Condensed and thick liquid samples

Stir and mix thoroughly the sample, such as pudding, condensed milk, etc. Take 50 g of the sample, and mix well with 450 mL of diluent as the 10-fold diluted test solution.

**2.3.6.** Serially diluted test solutions

Using a sterilized pipette, add 10 mL of the 10-fold diluted test solution to 90 mL of diluent. Prepare a series of decimal dilutions, such as 100-fold, 1000-fold, and 10000-fold test solutions as shown in the following figure.




Note 2: Use 0.1% peptone as the diluent for meat products. For other types of sample, use mainly Butterfield's phosphate-buffered dilution water or alternatively normal saline as the diluent.

Note 3: As the total amount of the sample is less than 50 g (mL), add an appropriate amount of diluent, according to the amount of the sample, to prepare a 10-fold diluted test solution.

Note 4: In the case of the greasy sample that is difficult to disperse and easy to foam, it is necessary to add appropriate amount of sterilized emulsifier (such as Tween 80 at the final concentration in the test solution of 1%), and shake well to emulsify.

**2.4.** Identification

**2.4.1.** Most probable number (MPN) method

**2.4.1.1.** Presumptive test

**2.4.1.1.1.** Shake to well mix the diluted test solutions prepared in section 2.3. Pipette 1 mL of the test solution to inoculate a test tube containing 10 mL of LST broth. Inoculate three tubes per dilution for three consecutive 10-fold dilutions, such as 10X-100X-1000X or 100X-1000X-10000X. Inoculation should be completed within 15 min after sample preparation. Incubate the culture tubes at 35 °C for 24 ± 2 hr and observe whether gas is produced. Cultures producing gas are suspected of *E.coli* and are selectively cultured per Section 2.4.1.1.2. Those producing no gas are further incubated for 24 hr. If still no gas is produced, the culture is determined as *E. coli*

negative. If gas is produced, it is suspected of *E. coli* and is selectively cultured per Section 2.4.1.1.2.

**2.4.1.1.2.** For each LST tube producing gas per Section 2.4.1.1.1, take a loopful of the broth to inoculate a tube of EC broth. Incubate the EC tubes at 44.5°C for 24 ± 2 hr in a covered water bath. Cultures producing gas are suspected of *E. coli* and are selectively cultured per Section 2.4.1.2. Those producing no gas are further incubated for 24 ± 2 hr. If still no gas is produced, the culture is determined to be *E. coli* negative. If gas is produced, it is suspected of *E. coli* and is selectively cultured per Section 2.4.1.2.

Note 5: Gas production is determined by gently shaking the culture tube and observing replacement of the medium by air in the fermentation tube.

**2.4.1.2.** Selective culture

For each EC tube producing gas per Section 2.4.1.1.2, take a loopful of the broth to streak an L-EMB agar plate. Incubate the plates at 35°C for 18 to 24 hr and observe the morphology of the colonies. A typical colony of *E. coli* is flat with a dark purple center and with or without metallic sheen. For each L-EMB plate containing suspected *E. coli* colonies, pick five colonies to inoculate non-selective media such as PCA slants or plates. Incubate the PCA culture at 35°C for 18-24 hr for subsequent confirmation tests.

Note 6: If less than five typical colonies are present in each L-EMB plate, pick all of the suspected colonies to inoculate PCA slants or plates, and incubate the culture at 35°C for 18-24 hr for confirmation tests. Only one colony confirmed to be *E. coli* is needed for each L-EMB plate to determine the EC tube (from which the colony is grown on the L-EMB plate) as positive.

**2.4.1.3.** Confirmation tests

**2.4.1.3.1.** Gram Staining

(1) Preparation of a smear

Place a drop of sterile 0.85% normal saline on a glass slide.

Pick a part of the isolated colony to be stained using an inoculating needle (or loop), mix it with normal saline on the slide, and then smear the mixture into a thin film. Allow the smear to air dry and heat-fix it by quick passing over a flame three to four times. Don't put the slide on the fire.

(2) Primary Staining

Stain the fixed smear with Hucker's crystal violet solution for 1 min and then wash with water.

(3) Mordant Staining

Cover the smear with Gram iodine solution for 1 min and then wash with water.

(4) Decoloring

Decolor with 95% ethanol until no further fading of the blue-violet stains (about 30 sec, longer for thicker smears), and then rinse with water.

(5) Counterstaining

Stain with Hucker's counterstain solution for 30 sec and then wash with water.

(6) Air-dry

(7) Microscopic examination

Under the microscope, Gram-positive bacteria appear dark purple and Gram-negative bacteria appear pink. *E. coli* is Gram-negative and rod- or short rod-shaped without spores.

**2.4.1.3.2.** Indole test

Pick the colony to be tested from the PCA slant or plate to inoculate a tube of tryptone broth, and incubate the tube at 35°C for 24 ± 2 hr. Add 0.2 - 0.3 mL of Kovacs' reagent to the culture tube and shake gently. Stand the tube at room temperature for 10 min and read the color of the top layered solution. Red color represents positive reaction, otherwise negative reaction. *E. coli* usually gives positive reactions but sometimes negative reaction.

**2.4.1.3.3.** Voges-Proskauer (VP) Test

Pick the colony to be tested from the PCA slant or plate to inoculate a tube of MR-VP broth, and incubate the tube for 48

± 2 hr at 35°C. Pipette 1 mL of the broth into a sterilized tube, and add 0.6 mL of VP solution A, 0.2 mL of VP solution B, and a small amount of creatine. Shake the tube gently, stand the mixture at room temperature for 2 hr, and read the color of the solution. Pink color represents positive reaction, otherwise negative reaction. *E. coli* gives negative reaction.

**2.4.1.3.4.** Methyl Red (MR) Test

Incubate the MR-VP broth cultured per Section 2.4.1.3.3. at 35°C for further 48 ± 2 hr. Add 5 drops of methyl red indicator with gentle shaking and read the color of the medium. Red color represents positive reaction, otherwise negative reaction. *E. coli* gives positive reaction.

**2.4.1.3.5.** Citrate utilization test

Pick the colony to be tested from the PCA slant or plate to inoculate a tube of Koser's citrate broth and incubate the tube at 35°C for 96 hr. Appearance of turbidity in the broth is a positive reaction, and remaining clear in appearance represents negative reaction. *E. coli* gives negative reaction.

**2.4.1.3.6.** Gas production from lactose

Pick the colony to be tested from the PCA slant or plate to inoculate a tube of LST broth and incubate the tube at 35°C for 48 ± 2 hr. Production of gas is a positive reaction, otherwise negative reaction. *E. coli* gives positive reaction.

**2.4.1.4.** Determination

Positivity for *E. coli* should be in accordance with the results listed in the following table.

| Test | Positive (+) | Negative (−) | Reaction of *E. coli* |
|---|---|---|---|
| Gram Staining | Dark purple | Pink | − |
| Indole test | Red | Original color | + or − |
| MR test | Red | Yellow | + |
| VP test | Pink | Original color | − |

| | | | |
|---|---|---|---|
| Citrate utilization test | Turbid | Clear | − |
| Gas production from lactose | Producing gas | No gas production | + |

**2.4.1.5.** Estimation of most probable number (MPN)

Determine the *E. coli* positivity per section 2.4.1.4. and count the number of positive tubes per three tubes inoculated for each test dilution in the presumptive test (Section 2.4.1.1). Use the count values of three consecutive dilutions and the MPN table below to calculate the most probable number of *E. coli* (MPN/g or MPN/mL) in the sample.

Three-tube most probable number (MPN) estimation

| No. of positive tubes | | | MPN per g or mL | 95% Confidence limits | | No. of positive tubes | | | MPN per g or mL | 95% Confidence limits | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.10* | 0.01* | 0.001* | | Lower limit | Upper limit | 0.10* | 0.01* | 0.001* | | Lower limit | Upper limit |
| 0 | 0 | 0 | < 3.0 | -- | 9.5 | 2 | 2 | 0 | 21 | 4.5 | 42 |
| 0 | 0 | 1 | 3.0 | 0.15 | 9.6 | 2 | 2 | 1 | 28 | 8.7 | 94 |
| 0 | 1 | 0 | 3.0 | 0.15 | 11 | 2 | 2 | 2 | 35 | 8.7 | 94 |
| 0 | 1 | 1 | 6.1 | 1.2 | 18 | 2 | 3 | 0 | 29 | 8.7 | 94 |
| 0 | 2 | 0 | 6.2 | 1.2 | 18 | 2 | 3 | 1 | 36 | 8.7 | 94 |
| 0 | 3 | 0 | 9.4 | 3.6 | 38 | 3 | 0 | 0 | 23 | 4.6 | 94 |
| 1 | 0 | 0 | 3.6 | 0.17 | 18 | 3 | 0 | 1 | 38 | 8.7 | 110 |
| 1 | 0 | 1 | 7.2 | 1.3 | 18 | 3 | 0 | 2 | 64 | 17 | 180 |
| 1 | 0 | 2 | 11 | 3.6 | 38 | 3 | 1 | 0 | 43 | 9 | 180 |
| 1 | 1 | 0 | 7.4 | 1.3 | 20 | 3 | 1 | 1 | 75 | 17 | 200 |
| 1 | 1 | 1 | 11 | 3.6 | 38 | 3 | 1 | 2 | 120 | 37 | 420 |
| 1 | 2 | 0 | 11 | 3.6 | 42 | 3 | 1 | 3 | 160 | 40 | 420 |
| 1 | 2 | 1 | 15 | 4.5 | 42 | 3 | 2 | 0 | 93 | 18 | 420 |
| 1 | 3 | 0 | 16 | 4.5 | 42 | 3 | 2 | 1 | 150 | 37 | 420 |
| 2 | 0 | 0 | 9.2 | 1.4 | 38 | 3 | 2 | 2 | 210 | 40 | 430 |
| 2 | 0 | 1 | 14 | 3.6 | 42 | 3 | 2 | 3 | 290 | 90 | 1,000 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 0 | 2 | 20 | 4.5 | 42 | 3 | 3 | 0 | 240 | 42 | 1,000 |
| 2 | 1 | 0 | 15 | 3.7 | 42 | 3 | 3 | 1 | 460 | 90 | 2,000 |
| 2 | 1 | 1 | 20 | 4.5 | 42 | 3 | 3 | 2 | 1100 | 180 | 4,100 |
| 2 | 1 | 2 | 27 | 8.7 | 94 | 3 | 3 | 3 | >1100 | 420 | -- |

* Quantity of sample (g or mL) inoculated into each tube.

Description:

1. The present table is suitable for estimating the MPN for the dilution series of 10X-100X-1000X (i.e., 0.1, 0.01 and, 0.001 gram or mL of sample inoculation per tube for each dilution, respectively). For different dilution series the MPN is corrected with the following formula:
   MPN/g (or MPN/mL) = $MPN_0/(Q_1 \times 10)$
   $MPN_0$: MPN for standard dilution series
   $Q_1$: quantity of sample inoculation for the first dilution of the test dilution series
2. Example: Numbers of positive tubes 3-1-0 for a dilution series correspond to an MPN of 43.
   (1) When the tested dilution series is 1X-10X-100X (sample quantity: 1, 0.1, 0.01 g or mL), the number of *E. coli* in the sample is calculated as $43/(1 \times 10) = 4.3$ MPN/g (or MPN/mL).
   (2) If the sample is tested in a standard dilution series as 10X-100X-1000X (sample quantity: 0.1, 0.01, 0.001 g or mL), the number of *E. coli* in the sample is calculated as $43/(0.1\times 10) = 43$ MPN/g (or MPN/mL).
   (3) If the tested dilution series is 100X-1000X-10000X (sample quantity: 0.01, 0.001, 0.0001 g or mL), the number of *E. coli* in the sample is calculated as $43/(0.01 \times 10) = 4.3 \times 10^2$ MPN/g (or MPN/mL).

**2.4.2.** Direct plate count method

**2.4.2.1.** Mix thoroughly the diluted test solutions prepared per Section 2.3.

**2.4.2.2.** Pipette 1 mL of the test solution into a Petri dish. Make at least two replicates for the original and each diluted solution.

**2.4.2.3.** Pour 15 mL of 47-50°C TBX medium into the dish, rotate to thoroughly mix the test solution and medium, and allow the mixture to jellify at room temperature. Invert the dish and incubate it at 37°C for 4 hr and then 44°C for 20-24 hr.

**2.4.2.4.** Select the plates grown with 15-150 typical colonies of *E. coli* (blue or blue-green) to count the numbers of colony.

Note 7: When the numbers of colony are less than 15 per plate for all the dilutions of sample cultured, count the colonies only for plates of the lowest dilution. If the numbers of colony are greater than 150 per plate for all the dilutions, count the colonies only for plates of the highest dilution.

**2.4.2.5.** Calculation of colonies

**2.4.2.5.1.** When plates containing 15-150 typical colonies exist in only one test dilution of the sample cultured, count the total number of colony on all plates for that dilution and calculate the concentration of *E. coli* in the sample as CFU/g or CFU/mL with the following formula:

$$\text{Concentration of } E.\ coli\ (\text{CFU/g or CFU/mL}) = \Sigma a \times \frac{A}{V_A}$$

$\Sigma a$: total number of typical colonies on all plates for the test solution with dilution factor A

$V_A$: total volume of the test solution with dilution factor A used in all plates

A: dilution factor of the test solution

**2.4.2.5.2.** When plates containing 15-150 typical colonies exist in two test dilutions of the sample cultured, calculate the concentration of *E. coli* for each dilution per Section 2.4.2.5.1 and average the concentrations with the following formula:

$$\text{Concentration of } E.\ coli\ (\text{CFU/g or CFU/mL}) = \left[\Sigma a \times \frac{A}{V_A} + \Sigma b \times \frac{B}{V_B}\right] \times \frac{1}{2}$$

$\Sigma a$: total number of typical colonies on all plates for the test solution with dilution factor A

$\Sigma b$: total number of typical colonies on all plates for the test solution with dilution factor B

$V_A$: total volume of the test solution with dilution factor A used in all plates

$V_B$: total volume of the test solution with dilution factor B used in all plates

A: dilution factor of test solution A
B: dilution factor of test solution B

**Remark:** It is allowed to use validated commercial media, biochemical test kits or biochemical identification systems. However, when the test results are disputed, this test method shall prevail.

## References

1. Feng, P., Weagant, S. D., Grant M. A. and Burkhardt W. 2017. Chapter 4 Enumeration of Escherichia coli and the Coliform Bacteria. Bacteriological Analytical Manual. [https://www.fda.gov/food/laboratory-methods-food/bam-4-enumeration-escherichia-coli-and-coliform-bacteria].
2. International Organization for Standardization. 2001. Microbiology of food and animal feeding stuffs–Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli*–Part 2: Colony-count technique at 44ºC using 5-bromo-4-chloro-3-indolyl β-D-glucuronide. ISO 16649-2.
3. International Organization for Standardization. 2018. Microbiology of the food chain–Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli*–Part 1: Colony-count technique at 44ºC using membranes and 5-bromo-4-chloro-3-indolyl beta-D-glucuronide. ISO 16649-1.

## Test flow chart