BAM 4: Enumeration of Escherichia coli and the Coliform Bacteria

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Revision History:

- July 2017 - Chap. 4 Sec. I. E. For the completed phase of testing for *E. coli*, the incubation temperature of EC tubes has been changed from 45.5 ± 0.2°C to 44.5 ± 0.2°C. The change was made in part due to the poor ability of the control strain ATCC25922 to grown and ferment lactose to produce acid and gas at 45.5 ± 0.2°C. The use of 44.5 ± 0.2°C would also make it consistent with that used for fecal Coliform analysis in shellfish and shellfish meats (Sec. VI) as well as conditions used for *E. coli* testing by other International organizations.
- February 2013 - Shellfish analysis method revised to be consistent with the APHA Examination of seawater and shellfish, 4th ed.
- February 2013 - Membrane filter methods added to water analysis.

Chapter Contents

Escherichia coli, originally known as *Bacterium coli* commune, was identified in 1885 by the German pediatrician, Theodor Escherich (14, 29). *E. coli* is widely distributed in the intestine of humans and warm-blooded animals and is the predominant facultative anaerobe in the bowel and part of the essential intestinal flora that maintains the physiology of the healthy host (9, 29). *E. coli* is a member of the family *Enterobacteriaceae* (15), which includes many genera,

including known pathogens such as *Salmonella*, *Shigella*, and *Yersinia*. Although most strains of *E. coli* are not regarded as pathogens, they can be opportunistic pathogens that cause infections in immunocompromised hosts. There are also pathogenic strains of *E. coli* that when ingested, causes gastrointestinal illness in healthy humans (see Chap. 4A).

In 1892, Shardinger proposed the use of *E. coli* as an indicator of fecal contamination. This was based on the premise that *E. coli* is abundant in human and animal feces and not usually found in other niches. Furthermore, since *E. coli* could be easily detected by its ability to ferment glucose (later changed to lactose), it was easier to isolate than known gastrointestinal pathogens. Hence, the presence of *E. coli* in food or water became accepted as indicative of recent fecal contamination and the possible presence of frank pathogens. Although the concept of using *E. coli* as an indirect indicator of health risk was sound, it was complicated in practice, due to the presence of other enteric bacteria like *Citrobacter*, *Klebsiella* and *Enterobacter* that can also ferment lactose and are similar to *E. coli* in phenotypic characteristics, so that they are not easily distinguished. As a result, the term "coliform" was coined to describe this group of enteric bacteria. Coliform is not a taxonomic classification but rather a working definition used to describe a group of Gram-negative, facultative anaerobic rod-shaped bacteria that ferments lactose to produce acid and gas within 48 h at 35°C. In 1914, the U.S. Public Health Service adopted the enumeration of coliforms as a more convenient standard of sanitary significance.

Although coliforms were easy to detect, their association with fecal contamination was questionable because some coliforms are found naturally in environmental samples (6). This led to the introduction of the fecal coliforms as an indicator of contamination. Fecal coliform, first defined based on the works of Eijkman (12) is a subset of total coliforms that grows and ferments lactose at elevated incubation temperature, hence also referred to as thermotolerant coliforms. Fecal coliform analyses are done at 45.5°C for food testing, except for water, shellfish and shellfish harvest water analyses, which use 44.5°C (1, 3, 30). The fecal coliform group consists mostly of *E. coli* but some other enterics such as *Klebsiella* can also ferment lactose at these temperatures and therefore, be considered as fecal coliforms. The inclusion of *Klebsiella* spp in the working definition of fecal coliforms diminished the correlation of this group with fecal contamination. As a result, *E. coli* has reemerged as an indicator, partly facilitated by the introduction of newer methods that can rapidly identify *E. coli*.

Currently, all 3 groups are used as indicators but in different applications. Detection of coliforms is used as an indicator of sanitary quality of water or as a general indicator of sanitary condition in the food-processing environment. Fecal coliforms remain the standard indicator of choice for shellfish and shellfish harvest waters; and *E. coli* is used to indicate recent fecal contamination or unsanitary processing. Almost all the methods used to detect *E. coli*, total coliforms or fecal coliforms are enumeration methods that are based on lactose fermentation (4). The Most Probable Number (MPN) method is a statistical, multi-step assay consisting of presumptive, confirmed and completed phases. In the assay, serial dilutions of a sample are

inoculated into broth media. Analysts score the number of gas positive (fermentation of lactose) tubes, from which the other 2 phases of the assay are performed, and then uses the combinations of positive results to consult a statistical table (Appendix 2 (/food/laboratory-methods/bam-appendix-2-most-probable-number-serial-dilutions)), to estimate the number of organisms present. Typically only the first 2 phases are performed in coliform and fecal coliform analysis, while all 3 phases are done for *E. coli*. The 3-tube MPN test is used for testing most foods. Analysis of seawater using a multiple dilution series should not use less than 3 tubes per dilution (5 tubes are recommended); in certain instances a single dilution series using no less than 12 tubes may also be acceptable. (For additional details, see: FDA. National Shellfish Sanitation Program, Manual of Operations. 2009 Revision. DHHS/PHS/FDA, Washington DC). Likewise, analysis of bivalve molluscan shellfish should be performed using a multiple dilution MPN series whereby no fewer than 5- tubes per dilution should be used, see section IV. There is also a 10-tube MPN method that is used to test bottled water or samples that are not expected to be highly contaminated (3). Analysis of citrus juice for *E. coli* is performed as an absence/presence method, see section V.

Also, there is a solid medium plating method for coliforms that uses Violet Red Bile Agar, which contains neutral red pH indicator, so that lactose fermentation results in formation of pink colonies. There are also membrane filtration tests for coliform and fecal coliform that measure aldehyde formation due to fermentation of lactose. This chapter also includes variations of above tests that use fluorogenic substrates to detect *E. coli* (18), special tests for shellfish analysis, a brief consideration of bottled water testing and a method for testing large volumes of citrus juices for presence of *E. coli* in conjunction with the Juice HACCP rule.

I. Conventional Method for coliforms, fecal coliforms and *E. coli*

A. Equipment and materials

1. Covered water bath, with circulating system to maintain temperature of 44.5 ± 0.2°C. The temperature for water baths for the shellfish program is 44.5°C ± 0.2°C. Water level should be above the medium in immersed tubes.
2. Immersion-type thermometer, 1-55°C, about 55 cm long, with 0.1°C subdivisions, certified by National Institute of Standards and Technology (NIST), or equivalent
3. Incubator, 35 ± 1.0°C. The incubator temp for the shellfish program is 35°C ± 0.5°C
4. Balance with capacity of ≥2 kg and sensitivity of 0.1 g
5. Blender and blender jar (**see** Chapter 1)
6. Sterile graduated pipets, 1.0 and 10.0 mL
7. Sterile utensils for sample handling (**see** Chapter 1)

8. Dilution bottles made of borosilicate glass, with polyethylene screw caps equipped with Teflon liners. Commercially prepared dilution bottles containing sterile Butterfield's phosphate buffer can also be used.
9. Quebec colony counter, or equivalent, with magnifying lens
10. Longwave UV light [~365 nm], not to exceed 6 W.
11. pH meter

B. **Media** (/food/laboratory-methods/media-index-bam) **and Reagents (/food/laboratory-methods/reagents-index-bam)**

1. Brilliant green lactose bile (BGLB) broth, 2% (M25 (/food/laboratory-methods/bam-media-m25-brilliant-green-lactose-bile-broth))
2. Lauryl tryptose (LST) broth (M76 (/food/laboratory-methods/bam-media-m76-lauryl-tryptose-lst-broth))
3. Lactose Broth (M74 (/food/laboratory-methods/bam-media-m74-lactose-broth))
4. EC broth (M49 (/food/laboratory-methods/bam-media-m49-ec-broth))
5. Levine's eosin-methylene blue (L-EMB) agar (M80 (/food/laboratory-methods/bam-media-m80-levines-eosin-methylene-blue-l-emb-agar))
6. Tryptone (tryptophane) broth (M164 (/food/laboratory-methods/bam-media-m164-tryptone-tryptophane-broth-1))
7. MR-VP broth (M104 (/food/laboratory-methods/bam-media-m104-mr-vp-broth))
8. Koser's citrate broth (M72 (/food/laboratory-methods/bam-media-m72-kosers-citrate-broth))
9. Plate count agar (PCA) (standard methods) (M124 (/food/laboratory-methods/bam-media-m124-plate-count-agar-standard-methods))
10. Butterfield's phosphate-buffered water (R11 (/food/laboratory-methods/bam-r11-butterfields-phosphate-buffered-dilution-water)) or equivalent diluent

    (Note: This same formulation is referred to as Buffered Dilution Water in American Public Health Association. 1970. Recommended Procedures for the Examination of Seawater and Shellfish, 4th ed. APHA, Washington, DC., p14-15)
11. Kovacs' reagent (R38 (/food/laboratory-methods/bam-r38-kovacs-reagent))
12. Voges-Proskauer (VP) reagents (R89 (/food/laboratory-methods/bam-r89-voges-proskauer-vp-test-reagents))
13. Gram stain reagents (R32 (/food/laboratory-methods/bam-r32-gram-stain))
14. Methyl red indicator (R44 (/food/laboratory-methods/bam-r44-methyl-red-indicator))

15. Violet red bile agar (VRBA) (M174 (/food/laboratory-methods/bam-media-m174-violet-red-bile-agar-vrba))
16. VRBA-MUG agar (M175 (/food/laboratory-methods/bam-media-m175-violet-red-bile-mug-agar))
17. EC-MUG medium (M50 (/food/laboratory-methods/bam-media-m50-ec-mug-medium))
18. Lauryl tryptose MUG (LST-MUG) broth (M77 (/food/laboratory-methods/bam-media-m77-lauryl-tryptose-mug-lst-mug-broth))
19. Peptone Diluent, 0.5% (R97 (/food/laboratory-methods/bam-r97-peptone-diluent-05))

C. **MPN - Presumptive test for coliforms, fecal coliforms and *E. coli***

Weigh 50 g of food into sterile high-speed blender jar (see Chapter 1 and current FDA compliance programs for instructions on sample size and compositing) Frozen samples can be softened by storing for <18 h="" at="" 2-5°c,="" but="" do="" not="" thaw.="" add="" 450="" ml="" of="" butterfield's="" phosphate-buffered="" water="" and="" blend="" for="" 2="" min.="" if=""><50 g="" of="" sample="" are="" available,="" weigh="" portion="" that="" is="" equivalent="" to="" half="" of="" the="" sample="" and="" add="" sufficient="" volume="" of="" sterile="" diluent="" to="" make="" a="" 1:10="" dilution.="" the="" total="" volume="" in="" the="" blender="" jar="" should="" completely="" cover="" the="">

Prepare decimal dilutions with sterile Butterfield's phosphate diluent or equivalent. Number of dilutions to be prepared depends on anticipated coliform density. Shake all suspensions 25 times in 30 cm arc or vortex mix for 7 s. Using at least 3 consecutive dilutions, inoculate 1 mL aliquots from each dilution into 3 LST tubes for a 3 tube MPN analysis (other analysis may require the use of 5 tubes for each dilution; See IV). Lactose Broth may also be used. For better accuracy, use a 1 mL or 5 mL pipet for inoculation. Do not use pipets to deliver <10% of="" their="" total="" volume;="" eg.="" a="" 10="" ml="" pipet="" to="" deliver="" 0.5="" ml. ="" hold="" pipet="" at="" angle="" so="" that="" its="" lower="" edge="" rests="" against="" the="" tube.="" not="" more="" than="" 15="" min="" should="" elapse="" from="" time="" the="" sample="" is="" blended="" until="" all="" dilutions="" are="" inoculated="" in="" appropriate="">

Incubate LST tubes at 35°C ± 0.5°C . Examine tubes and record reactions at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 3 h. Perform confirmed test on all presumptive positive (gas) tubes.

D. **MPN - Confirmed test for coliforms**

From each gassing LST or lactose broth tube, transfer a loopful of suspension to a tube of BGLB broth, avoiding pellicle if present. (a sterile wooden applicator stick may also be used for these transfers). Incubate BGLB tubes at 35°C ± 0.5°C and examine for gas production at 48 ± 3 h. Calculate most probable number (MPN) (see Appendix 2 (/food/laboratory-methods/bam-appendix-2-most-probable-number-serial-dilutions)) of coliforms based on proportion of confirmed gassing LST tubes for 3 consecutive dilutions.

E. **MPN - Confirmed test for fecal coliforms and *E. coli***

From each gassing LST or Lactose broth tube from the Presumptive test, transfer a loopful of each suspension to a tube of EC broth (a sterile wooden applicator stick may also be used for these transfers). Incubate EC tubes 24 ± 2 h at 44.5°C and examine for gas production. If negative, reincubate and examine again at 48 ± 2 h. Use results of this test to calculate fecal coliform MPN. To continue with *E. coli* analysis, proceed to Section F below. The EC broth MPN method may be used for seawater and shellfish since it conforms to recommended procedures (1).

F. **MPN - Completed test for *E. coli*.**

To perform the completed test for *E. coli*, gently agitate each gassing EC tube, remove a loopful of broth and streak for isolation on a L-EMB agar plate and incubate for 18-24 h at 35°C ± 0.5°C . Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen. Transfer up to 5 suspicious colonies from each L-EMB plate to PCA slants, incubate them for 18-24 h at 35°C ± 0.5°C and use for further testing.

NOTE: Identification of any 1 of the 5 colonies as *E. coli* is sufficient to regard that EC tube as positive; hence, not all 5 isolates may need to be tested.

Perform Gram stain. All cultures appearing as Gram-negative, short rods should be tested for the IMViC reactions below and also re-inoculated back into LST to confirm gas production.

Indole production. Inoculate tube of tryptone broth and incubate 24 ± 2 h at 35°C ± 0.5°C . Test for indole by adding 0.2-0.3 mL of Kovacs' reagent. Appearance of distinct red color in upper layer is positive test.

Voges-Proskauer (VP)-reactive compounds. Inoculate tube of MR-VP broth and incubate 48 ± 2 h at 35°C± 0.5°C . Transfer 1 mL to 13 × 100 mm tube. Add 0.6 mL α-naphthol solution and 0.2 mL 40% KOH, and shake. Add a few crystals of creatine. Shake and let stand 2 h. Test is positive if eosin pink color develops.

Methyl red-reactive compounds. After VP test, incubate MR-VP tube additional 48 ± 2 h at 35°C± 0.5°C . Add 5 drops of methyl red solution to each tube. Distinct red color is positive test. Yellow is negative reaction.

Citrate. Lightly inoculate tube of Koser's citrate broth; avoid detectable turbidity. Incubate for 96 h at 35°C ± 0.5°C . Development of distinct turbidity is positive reaction.

Gas from lactose. Inoculate a tube of LST and incubate 48 ± 2 h at 35°C ± 0.5°C . Gas production (displacement of medium from inner vial) or effervescence after gentle agitation is positive reaction.

Interpretation: All cultures that (a) ferment lactose with gas production within 48 h at 35°C, (b) appear as Gram-negative nonsporeforming rods and (c) give IMViC patterns of ++-- (biotype 1) or -+-- (biotype 2) are considered to be *E. coli*. Calculate MPN (see Appendix 2) of *E. coli* based on proportion of EC tubes in 3 successive dilutions that contain *E. coli*.

NOTE: Alternatively, instead of performing the IMViC test, use API20E or the automated VITEK biochemical assay to identify the organism as *E. coli*. Use growth from the PCA slants and perform these assays as described by the manufacturer.

G. **Solid medium method - Coliforms**

Prepare violet red bile agar (VRBA) according to manufacturer's instructions. Cool to 48°C before use. Prepare, homogenize, and decimally dilute sample as described in section I. C above so that isolated colonies will be obtained when plated. Transfer two 1 mL aliquots of each dilution to petri dishes, and use either of the following two pour plating methods, depending on whether injured or stressed cells are suspected to be present (1).

Pour 10 mL VRBA tempered to 48°C into plates, swirl plates to mix, and let solidify. To prevent surface growth and spreading of colonies, overlay with 5 mL VRBA, and let solidify. If resuscitation is necessary, pour a basal layer of 8-10 mL of tryptic soy agar tempered to 48°C. Swirl plates to mix, and incubate at room temperature for 2 ± 0.5 h. Then overlay with 8-10 mL of melted, cooled VRBA and let solidify.

Invert solidified plates and incubate 18-24 h at 35°C. Incubate dairy products at 32°C (2). Examine plates under magnifying lens and with illumination. Count purple-red colonies that are 0.5 mm or larger in diameter and surrounded by zone of precipitated bile acids. Plates should have 25-250 colonies. To confirm that the colonies are coliforms, pick at least 10 representative colonies and transfer each to a tube of BGLB broth. Incubate tubes at 35°C. Examine at 24 and 48 h for gas production.

NOTE: If gas-positive BGLB tube shows a pellicle, perform Gram stain to ensure that gas production was not due to Gram-positive, lactose-fermenting bacilli.

Determine the number of coliforms per gram by multiplying the number of suspect colonies by percent confirmed in BGLB by dilution factor.

Alternatively, *E. coli* colonies can be distinguished among the coliform colonies on VRBA by adding 100 µg of 4-methyl-umbelliferyl-β-D-glucuronide (MUG) per mL in the VRBA overlay. After incubation, observe for bluish fluorescence around colonies under longwave UV light. (see LST-MUG section II for theory and applicability.)

H. **Membrane Filtration (MF) Method - coliforms: see Section III. Bottled Water.**

Food homogenates will easily clog filters, hence MF are most suitable for analysis of water samples; however, MF may be used in the analysis of liquid foods that do not contain high levels of particulate matter such as bottled water (see Section III for application of MF).

II. LST-MUG Method for Detecting *E. coli* in Chilled or Frozen Foods Exclusive of Bivalve Molluscan Shellfish

The LST-MUG assay is based on the enzymatic activity of β-glucuronidase (GUD), which cleaves the substrate 4-methylumbelliferyl β-D-glucuronide (MUG), to release 4-methylumbelliferone (MU). When exposed to longwave (365 nm) UV light, MU exhibits a bluish fluorescence that is easily visualized in the medium or around the colonies. Over 95% of *E. coli* produces GUD, including anaerogenic (non-gas-producing) strains. One exception is enterohemorrhagic *E. coli* (EHEC) of serotype O157:H7, which is consistently GUD negative (11, 17). The lack of GUD phenotype in O157:H7 is often used to differentiate this serotype from other *E. coli*, although GUD positive variants of O157:H7 do exist (24, 26). The production of GUD by other members of the family *Enterobacteriaceae* is rare, except for some shigellae (44 -58%) and salmonellae (20-29%) (18, 27). However, the inadvertent detection of these pathogens by GUD-based assays is not considered a drawback from a public health perspective. Expression of GUD activity is affected by catabolite repression (8) so on occasion, some *E. coli* are GUD-negative, even though they carry the *uid*A gene (*gus*A) that encodes for the enzyme (19). In most analyses however, about 96% of *E. coli* isolates tested are GUD-positive without the need for enzyme induction (27).

MUG can be incorporated into almost any medium for use in detecting *E. coli*. But some media such as EMB, which contain fluorescent components, are not suitable, as they will mask the fluorescence of MU. When MUG is incorporated into LST medium, coliforms can be enumerated on the basis of gas production from lactose and *E. coli* are presumptively identified by fluorescence in the medium under longwave UV light, thus it is capable of providing a presumptive identification of *E. coli* within 24 h (18, 28). The LST-MUG method described below has been adopted as Official Final Action by the AOAC for testing for *E. coli* in chilled or frozen foods, exclusive of shellfish (28). See Sec. IV.4. D. for precautions in using MUG in testing shellfish. For information on MUG assay contact, Dr. Peter Feng (mailto:peter.feng@fda.hhs.gov) FDA, CFSAN, College Park, MD, 20740; 240-402-1650.

CAUTION: To observe for fluorescence, examine inoculated LST-MUG tubes under longwave (365 nm) UV light in the dark. A 6-watt hand-held UV lamp is adequate and safe. When using a more powerful UV source, such as a 15-watt fluorescent lamp, wear protective glasses or goggles. Also, prior to use in MUG assays, examine all glass tubes for auto fluorescence. Cerium oxide, which is sometimes added to glass as a quality control measure, will fluoresce under UV light and interfere with the MUG test (25). The use of positive and negative control strains for MUG reaction is essential.

A. **Equipment and material: see section I.A above and in addition**,

1. New, disposable borosilicate glass tubes (100 × 16 mm)
2. New, disposable borosilicate glass Durham vials (50 × 9 mm) for gas collection
3. Longwave UV lamp, not to exceed 6-watt

B. **Media and reagents: see section I.B above**

C. **Presumptive LST-MUG test for *E. coli*.**

Prepare food samples and perform the MPN Presumptive test as described in section I.C. above, except use LST-MUG tubes instead of LST. Be sure to inoculate one tube of LST-MUG with a known GUD-positive *E. coli* isolate as positive control (ATCC 25922). In addition, inoculate another tube with a culture of *Enterobacter aerogenes* (ATCC 13048) culture of Enterobacter aerogenes (ATCC 13048) or a *Klebsiella pneumoniae* strain as negative control, to facilitate differentiation of sample tubes that show only growth from those showing both growth and fluorescence. Incubate tubes for 24 to 48 ± 2 h at 35°C. Examine each tube for growth (turbidity, gas) then examine tubes in the dark under longwave UV lamp (365 nm). A bluish fluorescence is a positive presumptive test for *E. coli*. Studies by Moberg et al. (28) show that a 24 h fluorescence reading is an accurate predictor of *E. coli* and can identify 83-95% of the *E. coli*-positive tubes. After 48 h of incubation, 96-100% of *E. coli*-positive tubes can be identified (28). Perform a confirmed test on all presumptive positive tubes by streaking a loopful of suspension from each fluorescing tube to L-EMB agar and incubate 24 ± 2 h at 35°C. Follow protocols outlined in I. F, above, for Completed test for *E. coli*. Calculate MPN of *E. coli* based on combination of confirmed fluorescing tubes in 3 successive dilutions.

III. Examination of Bottled Water

Consumption of bottled water is increasing rapidly worldwide. In the U.S. alone, over 3.6 billion gallons of bottled water were consumed in 1998 (International Bottled Water Association, Alexandria, VA). Unlike potable water, which is regulated by the U.S. EPA, bottled water is legally classified as food in the U.S. and regulated by the FDA (Federal Register. 1995. 21 CFR Part 103 et al. beverages: bottled water; final rule. 60(218) 57076-57130). FDA defines bottled water as "water that is intended for human consumption and that is sealed in bottles or other containers with no added ingredients except that it may contain safe and suitable antimicrobial agents" and, within limitations, some added fluoride. Bottled water may be used as a beverage by itself or as an ingredient in other beverages. These regulations do not apply to soft drinks or similar beverages. In addition to "bottled water" or "drinking water", in 21 CFR Part 103 FDA also defines various types of bottled water that meet certain criteria. These identities include "artesian or artesian well water", "ground water", mineral water", "purified or demineralized water", "sparkling bottled water", "spring water" and "well water". Additionally "sterile water" is defined as water that meets the requirements under the "Sterility Test" in the United States Pharmacopeia.

Coliform organisms are not necessarily pathogens and are rarely found in bottled water, however, they serve as an indicator of insanitation or possible contamination. Surveys have shown that coliforms are useful indicators of bottled water quality, but some countries also monitor additional microbial populations as indicators of bottle water quality (10, 33). Under the current bottled water quality standard, FDA has established a microbiological quality requirement that is based on coliform detection levels. These levels may be obtained by membrane filtration (MF) or by 10-tube MPN analysis of ten 10-mL analytical units. For information on bottled water methods contact Dr. Peter Feng (mailto:peter.feng@fda.hhs.gov), FDA, CFSAN, College Park, MD, 20740; 240-402-1650.

A. **Equipment and Materials.**

1. Incubator at 35° ± 0.5°C.
2. Membrane filtration units (filter base and funnels): glass, plastic, or stainless steel; wrapped in foil or paper and sterilized.
3. Ultraviolet sterilization chamber for sterilizing filter base and funnels (optional).
4. Filter manifold or vacuum flask to hold filter funnels.
5. Vacuum source (line vacuum, electric vacuum pump or water aspirator).
6. Membrane filters; sterile, white, gridded, 47 mm diameter, 0.45 µm pore size (or equivalent, as specified by the manufacturer) for enumeration of bacteria.
7. Petri dishes, sterile, plastic, 50 × 12 mm, with tight fitting lids.
8. Forceps designed to transfer membranes without damage.

B. **Culture media.**

1. Lauryl sulfate tryptose (LST) broth (M-76 (/food/laboratory-methods/bam-media-m76-lauryl-tryptose-lst-broth)).
2. Brilliant green lactose bile broth (BGLB) (M-25 (/food/laboratory-methods/bam-media-m25-brilliant-green-lactose-bile-broth)).
3. M-Endo Medium (BD#274930) (M-196 (/food/laboratory-methods/bam-media-m-196-mendo-mf-medium-bd-274930)).
4. LES-Endo Agar (BD#273620) (M-197 (/food/laboratory-methods/bam-media-m-197-les-endo-agar-bd-273620)).

C. **Ten tube MPN coliform test - Presumptive and Confirmed procedures.**

For routine examination of bottled water, take 100 mL of sample and inoculate 10 tubes of 2X LST (10 mL of medium) with 10 mL of undiluted sample each. Incubate tubes at 35°C. Examine tubes at 24 ± 2 h for growth and gas formation as evidenced by displacement of medium in fermentation vial or effervescence when tubes are gently agitated. If negative at 24 h, reincubate tubes for an additional 24 h and examine again for gas. Perform a

confirmed test on all presumptive positive (gassing) tubes as follows: gently agitate each positive LST tube and, using a 3.0 - 3.5 mm sterile loop, transfer one or more loopfuls of suspension to a tube of BGLB broth. Sterile wooden applicator sticks may also be used for transfer by inserting it at least 2.5 cm into the broth culture. Incubate BGLB tubes for 48 ± 2 h at 35°C. Examine for gas production and record. Calculate MPN using 10 tube MPN Table (9221.III), p. 9-52, Standard Methods for the Examination of Water and Wastewater (3).

NOTE: if a sample is found to contain coliforms (at any level) follow procedure outlined in Sec. I. F. above to determine if it is *E. coli*. Bottled water is not permitted to contain *E. coli*.

D. **Membrane filter method for coliforms.**

Filter 100 mL of test sample and transfer the filter to M-Endo medium (M-196 (/food/laboratory-methods/bam-media-m-196-mendo-mf-medium-bd-274930)) or LES Endo Agar (M-197 (/food/laboratory-methods/bam-media-m-197-les-endo-agar-bd-273620)) and incubate at 35 °C± 0.5°C for 22-24 h. Count colonies that are pink to dark red with a green metallic surface sheen. The sheen may vary from pinpoint to complete coverage of the colony. Use of a low power, dissecting-type microscope to examine filters is recommended.

Confirmation - If there are 5 to 10 sheen colonies on the filter, confirm all by inoculating growth from each sheen colony into tubes of LST and incubate at 35 °C± 0.5°C for 48 h. If the number of sheen colonies exceeds 10, randomly select and confirm 10 colonies that are representative of all sheen colonies. Any gas positive LST tubes should be sub cultured to BGLB and incubated at 35°C± 0.5°C for 48 hr. Gas production in BGLB within 48 h is a confirmed coliform test. Report results as number of coliform colonies per 100 mL. NOTE: Standard Method, 1998, 20th ed, p. 9-60 (3), allows for simultaneous inoculation of LST and BGLB during verification. However, BGLB is somewhat inhibitory so the method described above, where samples are sub cultured from LST into BGLB is regarded as a more sensitive verification assay and therefore, recommended.

NOTE: if a sample is found to contain coliforms (at any level) follow procedure outlined in Sec. I. F. above to determine if it is *E. coli*. Bottled water is not permitted to contain *E. coli*.

IV. Examination of Shellfish and Shellfish Meats

The official FDA procedure for bacteriological analysis of domestic and imported bivalve molluscan shellfish is fully and properly described in the APHA's Recommended Procedures for the Examination of Sea Water and Shellfish, 4th ed. 1970 (1). The methods, including the conventional 5-tube MPN for coliform, fecal coliform and standard total plate count for bacteria (see Part III, APHA's Recommended Procedures the Examination of Sea Water and Shellfish, 4th ed. 1970 (1), are described below for examining shell stock, fresh-shucked meats, fresh-shucked frozen shellfish, and shellfish frozen on the half shell. These procedures do not apply to the examination of crustaceans (crabs, lobsters, and shrimp) or to processed shellfish meats

such as breaded, shucked, pre-cooked, and heat-processed products (see section I. C. this chapter). Also, there are many methods that are used for testing for shellfish harvest and environmental water for fecal coliforms. One example, the mTEC agar (M-198 (/food/laboratory-methods/bam-media-m198-mtec-agar-bd-233410)) is a suitable membrane filter medium for enumerating fecal coliforms in marine and estuarine waters. Briefly, following the filtration of 100 ml of water, the filter funnels should be rinsed twice with approx. 20 ml of PBS. The filter is then transferred onto mTEC agar and incubated for 22-24 h at 44.5°C in Ethyfoam. All yellow, yellow-green or yellow-brown colonies are counted as fecal coliforms. Only plates having fewer than 80 colonies are counted. However, analysis of environmental waters will not be covered in detail here, as environmental water analyses are done by the U.S. EPA (3) and the quality of shellfish harvest waters are mainly the responsibilities of each State's Shellfish Control Authorities (20).

A. **Sample Preparation**

Using 10-12 shellfish, obtain 200 g of shellfish liquor and meat. Blend 2 min, with 200 mL sterile phosphate buffered dilution water or 0.5% peptone water (R97 (/food/laboratory-methods/bam-r97-peptone-diluent-05)) to yield a 1:2 dilution of sample. Analysis of the ground sample must begin within 2 min after blending. Make serial dilutions in 0.5% sterile peptone water or sterile phosphate buffered dilution water.

B. **MPN - Presumptive and Confirmed Test for Coliform**

Use Lactose Broth (M74 (/food/laboratory-methods/bam-media-m74-lactose-broth)) or Lauryl Tryptose Broth (M76 (/food/laboratory-methods/bam-media-m76-lauryl-tryptose-lst-broth)), at single strength in 10 ml volumes. For 5-tube MPN analysis, inoculate the 5 tubes at each dilution as follows:

To each of 5 tubes, add 2 mL of the blended homogenate (equivalent to 1 g of shellfish).

To each of 5 tubes, add 1 mL of 1:10 dilution of homogenate (0.1 g shellfish).

To each of 5 tubes, add 1 mL of 1:100 dilution of homogenate (0.01 g shellfish).

To each of 5 tubes, add 1 mL of 1:1000 dilution of homogenate (0.001 g shellfish).

Further dilutions may be necessary to avoid indeterminate results. Incubate tubes at 35°C ± 0.5°C then follow instructions in section 1.C and perform Confirmed test as in 1.D above, under "Conventional Method for Coliforms, fecal coliforms and *E. coli*". Calculate MPN as described in section 1.D above, except that shellfish analysis specifies that the coliform density be expressed as MPN per 100 g of sample rather than per g.

C. **MPN - Presumptive and Confirmed Test for Fecal Coliforms in Shellfish**

Perform presumptive test as described in section II above. To confirm positive tubes, transfer one loopful from gas positive LST tubes to EC broth and incubate in a covered circulating waterbath at 44.5°±0.2°C for 24 ± 2 hr. Gas production in EC is a positive confirmed test for fecal coliforms. Calculate the MPN per 100 g for fecal coliforms as described above for coliform.

D. MPN - EC-MUG Method for Determining *E. coli* in Shellfish Meats

The MUG assay for β-glucuronidase (GUD) described above for detecting *E. coli* in chilled and frozen food can also be used for testing for *E. coli* in shellfish meats; but with slight modifications. This is due to the fact that foods such as shellfish meats contain natural GUD activity (32). As a result, oyster homogenate inoculated directly into LST-MUG tubes in the Presumptive phase of the MPN test can cause false positive fluorescence reactions. Hence, in the analysis of *E. coli* in shellfish meats, the MUG reagent is added to the EC medium and used in the confirmatory phase of the assay. The EC-MUG tubes, incubated at 44.5°C ± 0.2°C, can be used in the confirmatory phase of a conventional 5-tube MPN assay to determine fecal coliform levels in shellfish meats, then by examining tubes for fluorescence under longwave UV, an *E. coli* MPN can also be readily obtained (32).

See section 1.A and 1.B above for materials and reagents required. Use commercially prepared dehydrated EC-MUG, or prepare medium by adding MUG to EC broth (0.05 g/L) (M50 (/food/laboratory-methods/bam-media-m50-ec-mug-medium)). Several sources of MUG compound are suitable: Marcor Development Corp., Carlstadt, NJ; Biosynth International, Itasca, IL; Sigma Chemical Co., St. Louis, MO and Hach Chemical, Loveland, CO. Dispense 5 mL into new disposable borosilicate glass tubes (100 × 16 mm) containing, new disposable borosilicate glass Durham vials (50 × 9 mm) for gas collection. Sterilize EC-MUG broth tubes at 121°C for 15 min; store up to 1 week at room temperature or up to a month under refrigeration.

Perform the 5-tube MPN Presumptive and Confirmed Test for Fecal Coliforms in Shellfish as described above in Section 3, except use EC-MUG tubes instead of EC for the confirmed test. Determination of fluorescence in EC-MUG broth requires the use of 3 control tubes, one inoculated with *E. coli* as positive control; one with *Enterobacter aerogenes* (ATCC 13048) or *K. pneumoniae* as negative control; and an uninoculated tube as EC-MUG medium batch control. Inoculate the positive and negative controls at the time when Confirmed test is being performed and incubate all tubes at 44.5°C ± 0.2°C for 24 h.

Read fluorescence as described above under LST-MUG assay. Note that some (<10%)>*E. coli* are anaerogenic (gas-negative), but should be MUG-positive. Include all fluorescence positive tubes in the *E. coli* MPN calculations. Determine *E. coli* MPN/100g from the tables in the BAM (Appendix 2) using combination of fluorescence positive tubes at each dilution.

NOTE: If analysis is to determine compliance with established *E. coli* limits, it will be necessary to confirm the presence of *E. coli* in MUG positive tubes.

V. Analysis for *E. coli* in citrus juices

Analysis for *E. coli* has been implemented to identify potentially contaminated juices or for verifying the effectiveness of HACCP during processing of unpasteurized juices (21 CFR Part 120, Vol. 66, No. 13, January 19, 2001). The standard method commonly used for testing for *E. coli* is the MPN however, it does not seem adequate for juice testing because of the acidity (pH 3.6 to 4.3) of juices, which can interfere with the test, plus it only allows for testing 3.33 mL of sample. Unlike most *E. coli* methods, which are enumeration assays, the following method is a simple Presence/Absence test that can examine 10-mL volume of juices (34, 35). This assay, designated as modified ColiComplete (CC) Method, is a modification of AOAC Official Method 992.30, which uses MUG for detection of *E. coli* (see Section on LST-MUG Method for details).

A. **Equipment and materials**

1. Covered water bath, with circulating system to maintain temperature of 44.5 ± 0.2°C.
   Water level should be above the medium in immersed tubes.
2. Incubator, 35 ± 0.5°C
3. Longwave UV light [~365 nm], not to exceed 6 W.

B. **Media and reagents:**

1. Universal Preenrichment Broth (UPEB) (M188 (/food/laboratory-methods/bam-media-m188-universal-preenrichment-broth)) or can be purchased from BD(#223510)
2. EC medium (M49 (/food/laboratory-methods/bam-media-m49-ec-broth))
3. ColiComplete (CC) discs (#10800) - BioControl, Bellevue, WA

C. **Sample preparation, enrichment and analysis**

Perform assay in duplicate. Aseptically, inoculate 10-mL portion of juice into 90 mL of UPEB and incubate at 35°C ± 0.5°C for 24 h. After enrichment, mix and transfer 1-mL from each UPEB enrichment broth into 9 mL of EC broth containing a CC disc. Incubate EC/CC broth tubes at 44.5± 0.2°C in a circulating water bath for 24 ± 2 h. Include a tube inoculated with a MUG (+) *E. coli* strain as positive control and another with *K. pneumoniae* or *Enterobacter aerogenes* (ATCC 13048) as negative control. Examine tubes in the dark and under long wave UV light. The presence of blue fluorescence in either tube is indicative that *E. coli* is present in the sample. Note: The CC discs also contain X-gal, which when cleaved by β-galactosidase will yield blue color on or around the disc. This reaction is analogous to measuring acid/gas production from fermentation of lactose hence, the presence of blue color is indicative of coliforms.

VI. Other Methods for Enumerating Coliforms and *E. coli*

There are many other methods for enumerating coliforms and *E. coli* , including several that uses fluorogenic reagents like MUG or other chromogenic substrates for presumptive detection and identification of coliform and *E. coli* in foods. Many of these tests, such as the Petrifilm dry rehydratable film, the hydrophobic grid membrane filter/MUG (HGMF/MUG) method (13), ColiComplete disc (16), Colilert (AOAC 991.15), have been evaluated by collaborative studies and adopted as official first or final action by the AOAC. There are also many modifications of the membrane filtration assays that have been developed for testing for coliform, fecal coliform and *E. coli* and some of these may be useful in testing foods such as milk and beverages, but they are used mostly for water, environmental waters, and shellfish harvest waters analysis (5, 7, 20, 22, 23, 31).

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