Fecal Coliform (Membrane Filter) Colony Verification Technical Assistance

With regards to Fecal Coliform by Standard Methods, 9222 D-2006, the Code of Federal Regulations, Title 40, Part 136; Federal Register Vol. 82, No. 165, August 28, 2017; 136.3. Table IA, Footnote 30 and Table IH, Footnote 27 state: On a monthly basis, at least ten blue colonies from the medium must be verified using Lauryl Tryptose Broth and EC broth, followed by count adjustment based on these results; and representative non-blue colonies should be verified using Lauryl Tryptose Broth. Where possible, verifications should be done from randomized sample sources.

Standard Methods, 9020 B-2005. (10) states: Verification is a general process used to determine whether the microbiological analytical method is performing as expected to provide reliable data. If a laboratory finds a low percentage of verification with a certain water supply or matrix, another test method must be chosen. To determine false negatives, pick representative atypical colonies of different morphological types and verify as in Presumptive Phase [Lauryl Tryptose Broth], Section 9221B.

If samples do not routinely produce 10 or more blue colonies, verify the first sample during the month which produces blue colonies, regardless of the number. Adjust sample result accordingly.

If no samples during the month produce plates with blue colonies, verify 10 colonies from the culture positive. Count adjustments from the culture positive are not to be applied to sample results.

Reagents:

Lauryl Tryptose Broth (LTB): If possible, use a commercially available medium. Ref: SM 9221 B-2006. (2) (a).


Equipment and Supplies:


Inoculating equipment: Use wire loops made of 22- or 24-gauge nickel alloy or platinum-iridium for flame sterilization. Use loops at least 3 mm in diameter. Single-service hardwood or plastic applicators, 0.2 to 0.3 cm in diameter and at least 2.5 cm longer than the fermentation tube, also may be used. Sterilize wooden applicators by dry heat and plastic applicators by autoclave, while stored in glass or other non-toxic containers. Prepackaged disposable plastic loops also are available for ready use. Ref: SM 9030 B-2006. (18)

Incubar at 35 ± 0.5 °C: an air incubator with 60% relative humidity or a water bath incubator may be used. Ref: SM 9030 B-2006. (1).

Incubator at 44.5 ± 0.2 °C: a water bath is required. Ref: SM 9030 B-2006. (1).

Preparation of Media

1. Prepare LTB and EC Medium according to manufacturer’s instructions, or by following Standard Methods if commercially-prepared medium is not used.

2. Before sterilization, dispense – in sterilized fermentation tubes with an inverted vial (Durham tube) – sufficient medium to cover the inverted vial at least one-half to two-thirds after sterilization.

3. Loosely cover tubes with metal or heat-resistant plastic caps.

4. Autoclave at 121 °C for 12-15 minutes with the exhaust set to slow.

5. After autoclaving, ensure that the inverted vials are free of air bubbles and discard any that contain air bubbles.

6. Check and document pH of media after sterilization. LTB should be 6.8 ± 0.2 S.U. and EC Medium should be 6.9 ± 0.2 S.U. If not, adjust pH using 1N NaOH or 1N HCl that has been filtered and sterilized. If the pH
is more than 0.5 S.U. outside of the specified pH, discard and determine why (e.g., incorrect preparation or abnormal pH of reagent water).

7. Allow tubes to cool to room temperature and tighten caps on the tubes.
8. Follow manufacturer’s recommendations for storage and expiration of prepared media. If the media is refrigerated, allow to warm to room temperature (20°C) before use. Discard any tubes that contain air bubbles.

**Colonial Verification Procedure**

Verification testing takes 48-72 hours. Take this into consideration when preparing for analysis. An alternative procedure that may reduce the testing time to 24 hours is described in #9 below.

1. Immediately after performing the colony count for membrane filtration, using a random sample source if possible, inoculate the LTB using a sterilized inoculating instrument (e.g., a sterile 3- to 3.5-mm -diameter loop or sterile wooden applicator stick). This is performed by scraping each colony with the inoculating instrument, dipping it into the media and gently swirling it. The inoculating instrument must be sterile for each individual colony.

2. Inoculate ten LTB fermentation tubes with ten typical blue fecal coliform colonies from a single sample, sterilizing the inoculating equipment between each inoculation. It is also recommended that representative non-blue colonies also be inoculated in LTB fermentation tubes to determine false negatives.

3. Incubate the LTB fermentation tubes at 35 ± 0.5 °C for 24 ± 2 hours. If using a water bath, maintain a sufficient water depth to immerse tubes to the upper level of the medium.

4. Swirl each tube and examine it for growth and/or gas production. Document results.

5. If no growth and/or gas is evident, continue incubation and re-examine at the end of 48 ± 3 hours.

6. For any tubes that exhibit growth and/or gas production, inoculate the EC Medium by dipping the inoculating instrument into the LTB and transferring a small portion of the growth to the EC Medium.

7. Incubate the EC fermentation tubes within 30 minutes of inoculation at 44.5 ± 0.2 °C for 24 ± 2 hours. To maintain this temperature range, a water bath is required for incubation of EC Medium. Maintain a sufficient water depth to immerse tubes to the upper level of the medium.


9. It is permissible to inoculate tubes of LTB and EC Medium sequentially at the same time from a single colony to reduce the time of the analysis. This is performed by scraping the colony with the inoculating equipment, inserting it into the LTB and gently swirling it, and then inserting it into the EC Medium and gently swirling it. If both media are inoculated at the same time, but only the LTB tube produces growth and/or gas after the incubation period, fresh tubes of EC Medium must be inoculated from the LTB growth and incubated for an additional 24 ± 2 hours.

**Interpretation**

Growth and/or gas production in both the LTB and EC Mediums confirms that the colony is a fecal (thermotolerant) coliform. If any blue colonies are not confirmed to be fecal (thermotolerant) coliforms, adjust the colony count of the plate by the percentage of negative EC Medium fermentation tubes prior to reporting the results. For example, if one of ten EC tubes are negative, multiply colony count of the plate by 90% (0.90) and report the result rounded to whole numbers.

If any non-blue colonies that are tested produce growth and/or gas in the LTB, they may be fecal (thermotolerant) coliforms. It is recommended that this be confirmed by inoculating EC Medium and incubating for 24 hours.

Laboratories must choose another approved method for analysis if a low percentage of verification is found for a specific sample site.

It is recommended that the laboratory take notes about the size, shape and color of colonies that are verified, especially if there are any abnormalities. Pictures of the plates may also help the laboratory maintain this information.