IDEXX ENTEROLERT™ TEST METHOD FOR THE DETECTION OF ENTEROCOCCI IN WATER

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1. Scope and Application

1.1. This method is intended for use in the detection and confirmation of enterococci in water. Any positive sample for enterococci is an indication of contamination.

1.2. The minimum, non-zero number of bacterial counts detectable with this method is a function of the dilution scheme used when processing the sample.

1.3. The Enterolert method can be applied to drinking waters, recreational waters (marine and fresh), reuse waters and waste waters. Marine waters must be diluted at least tenfold with sterile water. It can be used as a Presence/Absence test or quantification with 5 X 20 mL, 10 X 10 mL, 15 tube serial dilution (MPN) or with the Quanti-Tray® system (19.1).

2. Summary of Method

2.1. The method is based on Defined Substrate Technology®. The product utilizes a nutrient indicator that produces a blue fluorescence when metabolized by enterococci. When the reagent is added to the sample and incubated, it can detect enterococci at 1 CFU/100 mL at 24 hours and up to 28 hours.

2.2. Enterolert is in *Standard Methods for the Examination of Water and Wastewater*, On-line and in the 22nd Edition, AWWA, APHA, WEF; section 9230 (19.3) and in ASTM Method 6503 (19.10).

3. Definitions

3.1. In this method, enterococci bacteria are bacteria which produce a blue fluorescent signal under a 6 watt, 365-366 nm UV light after incubation at 41 ± 0.5°C for 24 and up to 28 hours.

4. Safety

4.1. The analyst/technician must know and observe the normal safety procedures required in a microbiology laboratory preparing, using, and disposing of samples, reagents and materials, and while operating sterilizing equipment.

4.2. Mouth pipetting is prohibited.

5. Equipment and Supplies

5.1. Pipettes, sterile, T.D. bacteriological or Mohr, glass or plastic of appropriate volume and sterile loops.

5.2. Sterile vessels, glass or plastic (free from fluorescence) with or without sodium thiosulfate. Any water containing an oxidizing agent such as chlorine must be neutralized with sodium thiosulfate.

5.2.1. Vessels containing sodium thiosulfate must neutralize up to 5 mg/L of chlorine for drinking water samples and up to 15 mg/L for waste water effluents.

5.2.2. Vessels must be at least 120 mL or of larger capacity to hold 100 mL sample to allow for proper mixing of sample.

5.3. 51 Well Quanti-Tray or Quanti-Tray/2000 trays.

5.4. Quanti-Tray Sealer

5.5. Incubator maintained at 41 ± 0.5°C.

5.6. 6 Watt 365-366 nm UV light.

6. Reagents

6.1. Sterile, non-buffered, oxidant-free water for dilutions (19.1).

6.2. Store Enterolert at 2-30°C away from light. The expiration date is indicated on the package (12 months from the date of manufacture).

6.3. Sodium thiosulfate reagent *Standard Methods for the Examination of Water and Wastewater*, (19.3) or sterile vessels containing sodium thiosulfate to neutralize up to 15mg/L chlorine.

7. Sample Collection, Preservation and Storage

7.1. Sampling procedures as described in detail in the USEPA microbiology methods manual, Section II, A (19.2) and in Standard Methods for the Examination of Water and Wastewater (19.3).

7.1.1. Storage Temperature and Handling Conditions: Ice or refrigerate bacteriological samples at a temperature less than 10°C (2-10°C) during transit to the laboratory. Use insulated containers to assure proper maintenance of storage temperature. Ensure that sample vessels are not totally immersed in water during transit. Do not allow samples to freeze. If frozen, sample cannot be thawed and a new sample is required.

7.1.2. Holding Time Limitations: Examine samples as soon as possible after collection. For drinking water samples, do not exceed 30 hours hold time from collection to incubation. For non-potable water for compliance, do not exceed 8 hours from time of collection to incubation (19.4)

8. Quality Control

8.1. Quality control should be conducted on each lot of Enterolert or more often as regulations requires. One of the following quality control procedures is recommended for each lot of Enterolert when used for enterococci testing at 41 ± 0.5°C:

8.1.1. IDEXX-QC Enterococci: Consists 3 of each- *Enterococcus faecium*, *Escherichia coli*, and *Streptococcus bovis*.

1. See the package insert for instructions
2. Obtain the mean and range from the website; www.idexx.com.water under quality certificates

8.1.2. **ATCC**

A. For each of the American Type Culture Collection (ATCC) bacterial strains (*Enterococcus faecium* ATCC 35667, *Serratia marcescens* ATCC 43862, and *Aerococcus veridans* ATCC 10400), streak the culture onto labeled TSA or Blood Agar plates and incubate at 35 ± 2°C for 18–24 hours.

B. For each bacterial strain, touch a sterile 1 µL inoculating loop to a colony and use it to inoculate a labeled test tube containing 5 mL of sterile deionized water. Close cap and shake thoroughly.
C. For each bacterial strain, take a 1 µL loop from the test tube and use it to inoculate a labeled vessel containing 100 mL of sterile deionized water.

8.2. Follow Section 11. P/A Procedure or Section 12. Quanti-Tray Enumeration Procedure and Section 13.0 Interpretation and Calculations.

8.3. Sample bottle and Quanti-Tray sterility check per lot (19.9; see Section V, 5. 4.2)

8.3.1. At least one sample bottle/lot and one tray/lot are tested with Tryptic Soy Both (25 mL for the bottle and 100 mL for the tray) and incubated at 35±0.5°C. It is recommended that this be performed in a laminar flow hood. Aseptic technique must be used. If not available, aseptic technique must be maintained. Do not open bottle for long periods of time nor place the cap on the lab surface facing up. Open cap just enough to add the TSA to the bottle and close immediately.

8.3.2. Check samples at 24 and 48 hours for growth.

8.3.3. No growth should be observed.

8.3.4. If growth is observed, retest and if still positive, call IDEXX Water Technical Service (1-800-321-0207).

8.4. Monthly Sealer Check with food color or dye: (19.9; see Section V; 5.3.2.1.2)

8.4.1. Add 2-3 drops of food coloring dye or equivalent to 100 mL of water. Mix well.

8.4.2. Add this to the Quanti-Tray and seal the tray.

8.4.3. Observe the tray. There should be no dye observed outside the wells.

8.4.4. If dye is observed outside the well, repeat the testing. If it still occurs call IDEXX Water Technical Service (1-800-321-0207).

8.5. Media sterility check using sterile water per lot and vessels do not auto-fluoresce (19.9; Section V; 5.3.1.3)

8.5.1. Each new lot shall be checked for sterility. Select at least one vessel and one packet and add 100 mL of sterile DI water. Mix well and incubate up to18 and no longer than 22 hours at 41 ± 0.5°C

8.5.2. No fluorescence should be observed.

8.5.3. If fluorescence is observed, retest and if still positive, call IDEXX Water Technical Service (1-800-321-0207).

9. Calibration and Standardization

9.1. Check the temperature of the incubator at least twice per day (when in use) separated by at least 4 hours to insure it is within the stated limits. Record the date, temperature, time of reading and initial.

9.2. Check thermometers at least annually against NIST certified thermometer or one that meets the requirements of NIST Monograph SP 250-23 (19.3).

10. Corrective Action

10.1. Procedure to determine the cause of the failure to prevent this from reoccurring again by:

10.2. Defining the problem:

1. Identify corrective action and steps required to correct the problem.
2. Implement correction action.
3. Document corrective action.

10.3. Repeat testing to ensure that corrective action was successful.

10.4. Examples are:

10.4.1. Procedure followed for preparing the control and or diluent.

10.4.2. Incubation temperature within the required tolerance.

10.4.3. Verified the thermometer for the incubator or water bath was calibrated against NIST thermometer and corrections made if required (19.3).

10.4.4. Sample incubation within the required time period.

10.4.5. Test kit is within the expiration date.

10.4.6. Call and review problem encountered with Water Technical Service at 1-800-321-0207.

11. Procedure- Presence-Absence (P/A)

11.1. Carefully separate one blister pack from the strip taking care not to accidentally open the adjacent pack.

11.2. Ensure the powder is in the bottom of the blister pack.

11.3. Hold the blister pack face down (paper side up) at the top and towards the bottom and snap back at the scoreline forming a "V", with the opening facing into the open vessel.

11.4. Allow the powder to fall into the water sample contained in the sterile, non-fluorescent vessel.

11.5. Aseptically cap and seal the vessel.

11.6. Shake until dissolved.

11.7. Incubate for 24 hours and up to 28 hours at 41 ± 0.5°C.

11.8. Read the results at 24 hours and up to 28 hours.

11.9. Check vessel for blue fluorescence by placing a 6-watt, 365-366 nm UV light within five inches of the sample in a dark environment. Be sure the light is facing away from your eyes and towards the vessel. Alternatively, use a UV viewing cabinet.

11.10. If no blue fluorescence is observed, the test is negative.

11.11. If blue fluorescence is observed**,** the sample is positive for enterococci.

11.12. However, *if the results are ambiguous to the analyst based on the initial reading, incubate up to an additional four hours (but not to exceed 28 hours total) to allow the fluorescence to intensify*.

12. Procedure: Quantification

12.1. For accuracy and counting range, use the IDEXX Quanti-Tray System with either the 51 Well Quanti-Tray or the Quanti-Tray-2000 and follow the Presence-Absence procedure above (11.1-11.7) for adding the powder to the sample and incubation. Marine water samples require at least a 1:10 dilution.

Enterolert can be used with multiple tubes to yield a MPN; 5 tubes X 20 mL, 10 tubes X 10 mL, or 15 tube serial dilution. Consult Standard Methods for the Examination of Water and Wastewater for the appropriate MPN Tables

12.2. If a dilution is required, use sterile non buffered, oxidant free water for dilutions. Always add Enterolert to the final 100 mL diluted sample only.

12.3. Follow the package insert for the Quanti-Tray (19.4) along with the package insert for Enterolert (19.1) and/or see 11.1-11.6. Remove a sterile tray from the plastic bag (tear open the plastic bag at the bottom which has a black line around the bag) and remove the number of trays required for testing. Close the bag using tape or a clip. Label the back of the tray to identify the sample. Open the tray following the directions as outlined in the insert for Quanti-Tray (19.4). Pour the sample reagent mixture from the vessel into the tray avoiding contact with the foil tab. Seal the tray with the Quanti-Tray sealer.

12.4. Incubate at 41 ± 0.5°C for 24 hours and up to 28 hours.

13. Interpretation and Calculations

13.1. Follow the same interpretation directions from Section 11.9 -11.12 and count the number of positive wells. Refer to the appropriate Quanti-Tray MPN Table provided by IDEXX to determine the Most Probable Number (MPN) for fluorescent wells in the sample. Correct the MPN value for any dilution made. The fluorescence of positive wells may vary. Any blue fluorescence is a positive well.

14. Method Performance

14.1. Enterolert was found to be equivalent to the MF method. Correlation of 0.97 was found between Enterolert and MF enterococci method. A false positive and false negative rate of 5.1% and 0.4% was found (19.6)

15. Reporting Results

15.1. Report results as Presence or Absence for enterococci. For Quantification, report results as MPN/100 mL, refer to the Quanti-Tray MPN Table provided by IDEXX to determine the MPN. Correct the MPN value for any dilution made. The fluorescence of the positive well may vary in intensity.

15.2. Enterolert is approved by the US EPA for Recreational (19.7) and Waste Water (19.8)

16. Verification Procedure

16.1. Not applicable

17. Pollution Prevention

17.1. The solutions and reagents used in this method pose no threat to the environment when recycled and managed properly.

17.2. Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

18. Waste Management

18.1. It is the laboratory's responsibility to comply with all federal, state and local regulations governing waste management, particularly the biohazard and hazardous identification rules and land disposal regulations. Compliance with all sewage discharge permits and regulations is also required.

18.2. Samples, reference materials and equipment known or suspected to have viable bacteria attached or contained must be sterilized prior to disposal.

19. References

19.1. Enterolert Package Insert from IDEXX.

19.2. Bordner, R., J.A. Winter and P.V. Scarpino (eds.) Microbiological Methods for Monitoring the Environment, Water and Wastes, EPA-600/8-78-017. Office of Research and Development, USEPA. (December 1978)

19.3. Clesceri, L.S., A.E. Greenberg, A.D. Eaton (eds.). 1998 Standard Methods for the examination of Water and Wasterwater, 20th Edition, American Public Health Association, Washington, DC.

19.4. Federal Register/ Vol77, #97/ Friday, May 18th, 2012 page 29758

19.5. Quanti-Tray Package Insert from IDEXX.

19.6. Budnick, G et al; Evaluation of Enterolert for Enumeration of *E. coli* in Recreational Waters, AEM, Vol 62, No. 10, Oct.1966, p3881-3884

19.7. Federal Register / Vol. 68, No. 139 / Monday, July 21, 2003 / Rules and Regulations (Recreational Waters)

19.8. Federal Register / Vol. 72, No. 47 / Monday, March 12, 2007 / Rules and Regulations (Wastewater)

19.9. USEPA Manual for Certification of Laboratories Analyzing Drinking Water, Fifth Edition, Section V.

19.10. ASTM D6503 Standard Test Method for Enterococci in Water Using Enterolert