Standard Operating Procedure (SOP) 3.4.2.1

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Enterococcus Bacteria Analysis: Enterolert™ Method with Quanti-Trays®

Water contaminated with fecal matter may contain pathogens (bacteria and other micro-organisms that cause illness). Many pathogens are difficult measure in water samples. Certain bacteria, however, are relatively easy to measure in water samples and, if present, are used to measure the level of fecal contamination. These types of bacteria are referred to as indicator bacteria. Enterococcus bacteria are one such indicator of fecal contamination in water.

The general term enterococcus has been used for years to describe a group of gram-negative, coccoid shaped bacteria species, including *Streptococcus* species, found in the intestinal tracts (and therefore feces) of humans and other warm blooded animals. Recently, researchers have proposed the use of the term *Enterococcus* as a genus that includes these species of streptococcal bacteria. Therefore the species *Streptococcus faecium* and *Streptococcus faecalis* are now synonymously referred to as *Enterococcus faecium* and *Enterococcus faecalis.*

Enterolert™ reagent is used for the detection of enterococcus bacteria (enterococci) such as *E. faecium* and *E. faecalis* in fresh and marine water. This product is based on Defined Substrate Technology® (DST™) and utilizes a nutrient indicator that fluoresces when metabolized by enterococci. When the reagent is added to the sample and incubated, bacteria down to one MPN (most probable number) in a 100ml sample can be detected within 24 hours.

Equipment and Supplies

1) Disposable rubber gloves.

2) Sealed Whirlpack bags or sterile plastic bottles for sampling.

3) A clean ice chest and frozen plastic sealed “blue” ice.

4) A refrigerator maintained at a temperature of 4° C.

5) An incubator capable of maintaining a temperature of 41° C $\pm$ 0.5° C.

6) An IDEXX sealer.

7) A tightly capped or sealed vessel of distilled water.

8) Antibacterial solution (e.g., Lysol spray).

9) Sterile 10 ml pipettes and a pipette pump.

10) Quanti-trays and sterile 100 ml mixing bottles supplied by IDEXX Corp.

11) A 6 watt, 365nm, ultra-violet (UV) light.

12) Sterile Enterolert media, pre-packaged from IDEXX Corporation will be used. Media will be stored at 4-30°C away from light. Media will be used prior to the manufacturer's expiration date.

Safety Considerations

1) When sampling in potentially contaminated waters take all precautions to prevent coming into contact with the water. These precautions include wearing rubber gloves and washing with antibacterial soap following sampling and analysis.

2) Never sample waters after a known sewage spill or when the waters are officially closed by a Public Health Officer.

3) Never sample when river, surf or beach/shore conditions are hazardous.

4) Never sample in stream bank areas in which there is the potential for falling into the stream. Never sample during flood stage conditions.

5) Never sample alone on streams or storm drains.

6) Never sample from bridges or roads where there is the potential for being at risk from automobile traffic.

7) Always wash down all surfaces used in the processing of samples with anti-bacterial solutions (e.g., Lysol, etc.) once the Quanti-trays are in the incubator or after a spill of a sample.

8) All spent Quanti-trays containing live bacterial cultures (positive, yellow wells) must be autoclaved prior to disposal. Develop a partnership with a college, laboratory, public health agency or hospital that can accept and autoclave the spent Quanti-trays.

Field Sample Collection Procedure

1) Sterile sample bottles will be used (Whirl-Pak bags are acceptable) and will be labeled with the proper sample ID. The sampler will use aseptic technique, making certain that the bottle does not touch the benthic substrate (rock, sand, or mud on the bottom of the water body). Therefore, make an effort, within reason, to prevent sediment from entering the sample containers.

2) When samples are collected on a beach, it will be collected on an incoming wave. In all cases (beach or stream) the sampler must be downstream and away from the bottle, and the mouth of the bottle must face into the current.

3) After the sample is taken, the bottle will be immediately tipped to decant enough sample to ensure 1-2 inches of airspace in the sample bottle. The bottle will be tightly capped or twist closed (in the case of the Whirlpack) and promptly stored on ice (target temperature 4° C) in a clean ice chest.

4) Laboratory analysis should begin within 6 hours of sample collection and within two hours of receiving the sample at the lab.

Prevention of Contamination or Interferences in the Laboratory

1) Laboratory Sterile Technique: When performing bacterial analysis all laboratory personnel will wash hands prior to beginning tests and will wash hands thereafter whenever their hands become soiled with samples, etc. Use tight fitting sterile latex gloves when working with samples in the lab. All counters must be cleaned with a bactericide prior to performing tests. Sample bottles or Whirlpacks must be inverted (to mix) prior to opening in the lab. After opening the samples, sub samples for dilutions must be performed as soon as possible to minimize the potential for contamination. Only sterile pipettes must be used for sub sampling.

2) Sterile Dilution Water: Water used to prepare culture media and reagents will be sterile distilled water stored out of direct sunlight to prevent growth of algae. All marine water samples must be diluted by at least 1:10 with distilled sterile water.

3) False Positives: These are wells in which a different bacteria (other than enterococci) has grown and caused a biochemical reaction resulting in fluorescence. Even when all of the above precautions are followed there may still be false positive wells in the incubated quanti-trays. Do not record false positives as positive wells. However, you should make a note in your lab book or lab data sheet regarding the presence of the false positives for future reference.

Lab Procedures

1) Different types of water samples require different types of preparation as follows:
   a) For sterile (blank) water or relatively clean fresh water pour 100 ml of sterile water or sample directly into the sterile 100 ml mixing bottle (by filling to the 100 ml line) and add one package of the reagent. Cap and shake until dissolved.
   b) For fresh water that is suspected to contain contamination, pour 50 ml of sterile distilled water into the mixing bottle and add one package of the reagent. Cap and shake until dissolved. Then, after the foam subsides, using a sterile pipette add 10 ml of sample and top off with 40 ml (to the 100 ml line). Cap and shake again. This is a 1:10 dilution.
   c) For all marine or estuarine water samples (salinity greater than 5 ppt), pour 50 ml of sterile distilled water into the mixing bottle and add one package of the reagent. Cap and shake until dissolved. Then, using a sterile pipette add 10 ml of sample and top off with 40 ml (to the 100 ml line). Cap and shake again. This is a 1:10 dilution.

2) Make sure there is little or no foam left in the headspace of the mixing bottle prior to moving on to the next step.

3) Pour sample/reagent mixture from the mixing bottle into a quanti-tray and seal in the IDEXX Sealer.

4) Place the sealed tray in a 41° ± 0.5° C incubator for a minimum of 24 hours and a maximum of 28 hours (includes warming time). This is the incubation period.

Quality Control Elements

1) Duplicate Analyses: A minimum of 5 % of the samples on a given day should be sub sampled and run in duplicate. At least one duplicate should be run on every day in which the analyses are run. An attempt will be made to select samples that yield positive results (i.e., suspected of contamination) for the duplicate analyses.

2) Negative Blanks: One blank (sterile) water sample will be analyzed per batch of samples processed.

3) External Reference Samples: A positive control is a sample prepared in the lab to contain a known approximate concentration of enterococcus bacteria. An external reference sample is a positive control prepared and provided by a professional laboratory. The external reference sample is split. You should analyze the split external reference and compare your results to the professional lab. At least two external reference samples must be run per year.

Determining the Most Probable Number of Enterococci Cells Per 100ml of Sample

1) Following the incubation period observe and count the number of positive (fluorescent) wells. For enterococci look for blue fluorescence with a 6 watt, 365nm, UV light within 5 inches of the sample. Face light away from your eyes and towards the sample. . The fluorescence intensity of positive wells may vary.

2) Wells that fluoresce yellow or yellow-green are false positives.

3) Refer to the MPN table (provided by IDEXX) specific to the type of quanti-tray used (51 well or 97 well type of quanti-tray) to obtain a Most Probable Number per 100 ml of sample.

4) If a dilution was performed, after obtaining the initial MPN result from the table, multiply that result by the dilution level to obtain the final result (e.g., if a 1:10 dilution was employed, multiply the result from the MPN table by 10 to get the final result in MPN/100 ml).

5) If the sample is inadvertently incubated over 28 hours without observation, the following guidelines apply: Lack of fluorescence after 28 hours is a valid negative test. Fluorescence after 28 hours is an invalid result. In other words, only positive results obtained using the proper incubation period (24-28 hours) are valid.

Technical Assistance

For IDEXX technical assistance call 1-800-321-0207 or 207-856-0496.

References:

1. IDEXX Enterolert Manual
2. Quality Assurance Plan for the Southern California Bight 1998 Project, Microbiology Committee, Southern California Coastal Water Research Project