METHOD 9132

TOTAL COLIFORM: MEMBRANE-FILTER TECHNIQUE

1.0  SCOPE AND APPLICATION

1.1   This method is used to determine the presence of a member of a coliform group in wastewater and ground water.

1.2   The coliform group analyzed in this procedure includes all of the organisms that produce a colony with a golden-green metallic sheen within 24 hr of inoculation.

2.0  SUMMARY OF METHOD

2.1   A predetermined amount of sample is filtered through a membrane filter which retains the bacteria found in the sample.

2.2   In the two-step enrichment procedure, the filters containing bacteria are placed on an absorbent pad saturated with lauryl tryptose broth and incubated at 35°C $\pm$ 0.5°C for 2 hr.  The filters are then transferred to an absorbent pad saturated with M-Endo media or to a dish containing M-Endo agar and incubated for another 21 $\pm$ 1 hr at 35°C $\pm$ 0.5°C.  Sheen colonies are then counted under magnification and reported per 100 mL of original sample.

2.3   A more detailed treatment of this method is presented in Standard Methods for the Examination of Water and Wastewater and in Microbiological Methods for Monitoring the Environment (see References, Section 10.0).

3.0  INTERFERENCES

3.1   The presence of residual chlorine or other halogen can prevent the continuation of bacterial action.  To prevent this occurrence, sodium thiosulfate should be added.

3.2   Water samples high in copper, zinc, or other heavy metals can be toxic to bacteria.  Chelating agents such as ethylenediaminetetraacetic acid (EDTA) should only be added when heavy metals are suspected of being present.

3.3   Turbidity caused by the presence of algae or other interfering material may not permit testing of a sample volume sufficient to yield significant results.  Low coliform estimates may be caused by the presence of high numbers of noncoliforms or of toxic substances.

3.4   Samples containing large amounts of suspended solids will interfere with colony growth and with the subsequent counting of colonies on the filter membrane.  When this is the case, use Method 9131.

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4.0  APPARATUS AND MATERIALS

    4.1    Dilution bottles or tubes:

        4.1.1    Use bottles or tubes of resistant glass, preferably borosili-
cate glass, closed with glass stoppers or screw caps equipped with liners
that do not produce toxic or bacteriostatic compounds on sterilization.

        4.1.2    Do not use cotton plugs as closures.  Mark graduation levels
indelibly on side of dilution bottle or tube.  Plastic bottles of nontoxic
material and acceptable size may be substituted for glass, provided that
they can be sterilized properly.

    4.2    Pipets and graduated cylinders:

        4.2.1    Use pipets of any convenient size, provided that they deliver
the required volume accurately and quickly.  The error of calibration for
a given manufacturer's lot must not exceed 2.5%.  Use pipets having
graduations distinctly marked and with unbroken tips.  Bacteriological-
transfer pipets or pipets conforming to the APHA standards given in the
latest edition of Standard Methods for the Examination of Dairy Products may
be used.  Optimally, protect the mouth end of all pipets by a cotton plug
to eliminate hazards to the worker or possible sample contamination by
saliva.

        4.2.2    Use graduated cylinders meeting ASTM Standards (D-86 and
D216) and with accuracy limits established by the National Bureau of
Standards where appropriate.

    4.3    Containers for culture medium:

        4.3.1    Use clean borosilicate glass flasks presterilized to reduce
bacterial contamination.  Any size or shape of flask may be used, but
Erlenmeyer flasks with metal caps, metal foil covers, or screw caps provide
for adequate mixing of the medium and are convenient for storage.

    4.4    Culture dishes:

        4.4.1    Use Petri-type dishes, 60 by 15 mm, 50 x 12 mm, or other
appropriate size.  The bottoms of the dishes should be flat and large enough
so that the absorbent pads for the culture nutrient will lie flat.  Wrap
clean culture dishes before sterilization, singly or in convenient numbers,
in metal foil if sterilized by dry heat, or in suitable paper substitute
when autoclaved.  If glass Petri dishes are used, use borosilicate or
equivalent glass.  Because covers for such dishes are loose fitting, take
precautions to prevent possible loss of medium by evaporation, with
resultant change in medium concentration, and to maintain a humid
environment for optimal colony development.

        4.4.2    Disposable plastic dishes that are tight fitting and meet the
specifications noted above also may be used.  Suitable sterile plastic
dishes are available commercially.

4.5   Filtration units:

    4.5.1    The filter-holding assembly (constructed of glass, auto-
clavable plastic, porcelain, or any noncorrosive bacteriologically inert
metal) consists of a seamless funnel fastened by a locking device or held
in place by magnetic force or gravity.  The design should be such that the
membrane filter will be held securely on the porous plate of the receptacle
without mechanical damage and all fluid will pass through the membrane
during filtration.

    4.5.2    Separately wrap the two parts of the assembly in heavy
wrapping paper for sterilization by autoclaving and storage until use.
Alternatively, treat unwrapped parts by ultraviolet radiation before using
them.  Field units may be sanitized by igniting methyl alcohol or immersing
in boiling water for 5 min.  Do not ignite plastic parts.

    4.5.3    For filtration, mount receptacle of filter-holding assembly
in a 1-liter filtering flask with a side tube or other suitable device such
that a pressure differential can be exerted on the filter membrane.  Connect
flask to an electric vacuum pump, a filter pump operating on water pressure,
a hand aspirator, or other means of securing pressure differential.  Connect
an additional flask between filtering flask and vacuum source to trap carry-
over water.

4.6   Filter membranes:

    4.6.1    Use membrane filters with a rated pore diameter such that
there is complete retention of coliform bacteria (0.45 $\pm$ 0.02 um).  Use only
those filter membranes that have been found, through adequate quality
control testing and certification by the manufacturer, to exhibit full
retention of the organisms to be cultivated, stability in use, freedom from
chemical extractables inimical to the growth and development of bacteria,
a satisfactory speed of filtration, no significant influence on medium pH,
and no increase in number of confluent colonies or spreaders.  Preferably,
use membranes grid-marked in such a manner that bacterial growth is neither
inhibited nor stimulated along the grid lines.  Store membrane filters held
in stock in an environment without extremes of temperature and humidity.
Obtain no more than a year's supply at any one time.

    4.6.2    If presterilized membrane filters are to be used, use those
for which the manufacturer has certified that the sterilization technique
has neither induced toxicity nor altered the chemical or physical properties
of the membrane.  If the membranes are sterilized in the laboratory, remove
the paper separators -- but not the absorbent paper pads -- from the
packaged filters.  Divide filters into groups of 10 to 12, or other
convenient units, and place in 10-cm Petri dishes or wrap in heavy wrapping
paper.  Autoclave for 10 min at 121°C.  At the end of the sterilization
period, let the steam escape rapidly to minimize accumulation of water
condensation on filters.

4.7  Absorbent pads:

        4.7.1  Absorbent pads consist of disks of filter paper or other
material known to be of high quality and free of sulfites or other
substances that could inhibit bacterial growth.  Use pads approximately 48
mm in diameter and of sufficient thickness to absorb 1.8 to 2.2 mL of
medium.  Presterilized absorbent pads or pads subsequently sterilized in the
laboratory should release less than 1 mg total acidity (calculated as $CaCO_3$)
when titrated to the phenolphthalein end point, pH 8.3, using 0.02 N
NaOH.  Where there is evidence of absorbent pad toxicity, presoak pads in
Type II water at 121°C (in an autoclave) for 15 min, decant the water, and
repackage pads in a large Petri dish for sterilization and subsequent use.
Sterilize pads simultaneously with membrane filters available in resealable
Kraft envelopes or separately in other suitable containers.  Dry pads so
they are free of visible moisture before use.  See sterilization procedure
described above for membrane filters.

        4.7.2  As a substrate substitution for nutrient-saturated absorbent
pads, 1.5% agar may be added to the total coliform M-Endo broth medium.

    4.8  Forceps:

        4.8.1  Forceps should be round-tipped, without corrugations on the
inner sides of the tips.  Sterilize before use by dipping in 95% ethyl or
absolute methyl alcohol and flaming.

    4.9  Incubators

        4.9.1  Use incubators to provide a temperature of 35 $\pm$ 0.5°C and to
maintain a high level of humidity (approximately 90% relative humidity).

    4.10  Microscope and light source:

        4.10.1  Count membrane-filter colonies with a magnification of 10 to
15 diameters and a light source adjusted to give maximum sheen discernment.
Optimally, use a binocular wide-field dissecting microscope.  However, a
small fluorescent lamp with magnifier is acceptable.  Use cool-white
fluorescent lamps.  Do not use a microscope illuminator with optical system
for light concentration from an incandescent light source for coliform
colony identification on Endo-type media.


5.0  REAGENTS

    5.1  ASTM Type II water (ASTM D1193):  Water should be monitored for
impurities.

5.2 <u>M-Endo medium</u>:

   5.2.1 Components of the medium are:

   | | |
   |---|---|
   | Tryptose or polypeptone | 10.0 g |
   | Thiopeptone or thiotone | 5.0 g |
   | Casitone or trypticase | 5.0 g |
   | Yeast extract | 1.5 g |
   | Lactose | 12.5 g |
   | Sodium chloride, NaCl | 5.0 g |
   | Dipotassium hydrogen<br>   phosphate, $K_2HPO_4$ | 4.375 g |
   | Potassium dihydrogen<br>   phosphate, $KH_2PO_4$ | 1.375 g |
   | Sodium lauryl sulfate | 0.050 g |
   | Sodium desoxycholate | 0.10 g |
   | Sodium sulfite, $Na_2SO_3$ | 2.10 g |
   | Basic fuchsin | 1.05 g |
   | Distilled (Type II) water | 1 liter |

   5.2.2 Rehydrate in 1 liter Type II water containing 20 mL 95% ethanol. Heat to boiling in a water bath to avoid degradation of carbohydrates, promptly remove from heat, and cool to below 45°C. Do not sterilize by autoclaving. Final pH should be between 7.1 and 7.3.

   5.2.3 Store finished medium in the dark at 2 to 10°C and discard any unused medium after 96 hr. Medium is light sensitive.
      NOTE: This medium may be solidified by adding 1.2% to 1.5% agar before boiling.

   5.3 <u>Lauryl tryptose broth</u>: See Method 9131, Paragraph 5.3.


6.0 SAMPLE COLLECTION, PRESERVATION, AND HANDLING

   6.1 All samples must have been collected using a sampling plan that addresses the considerations discussed in Chapter Nine of this manual.

   6.2 Clean all glassware thoroughly with a suitable detergent and hot water, rinse with hot water to remove all traces of residual washing compound, and finally, rinse with distilled (Type II) water. If mechanical glassware washers are used, equip them with influent plumbing of stainless steel or other nontoxic material. Do not use copper piping to distribute Type II water. Use stainless steel or other nontoxic material for the rinse-water system.

   6.2.1 Sterilize glassware, except when in metal containers, for not less than 60 min at a temperature of 170°C, unless it is known from recording thermometers that oven temperatures are uniform, under which exceptional condition use 160°C. Heat glassware in metal containers to 170°C for not less than 2 hr.


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6.2.2    Sterilize sample bottles not made of plastic, as above, or in an autoclave at 121°C for 15 min.

6.2.3    For plastic bottles that distort on autoclaving, use low-temperature ethylene oxide gas sterilization.  If water containing residual chlorine and other halogens is to be collected, add sufficient $Na_2S_2O_3$ to clean sample bottle before sterilization to give a concentration of about 100 mg/L in the sample.  To a 120-mL bottle add 0.1 mL 10% solution of $Na_2S_2O_3$ (this will neutralize a sample containing about 15 mg/L residual chlorine).  Stopper bottle, cap, and sterilize by either dry or moist heat, as directed previously.

6.2.4    Collect water samples high in copper or zinc and wastewater samples high in heavy metals in sample bottles containing a chelating agent that will reduce metal toxicity.  This is particularly significant when such samples are in transit for 4 hr or more.  Use 372 mg/L of the tetrasodium salt of ethylenediaminetetraacetic acid (EDTA).  Adjust EDTA solution to pH 6.5 before use.  Add EDTA separately to sample bottle before bottle sterilization (0.3 mL 15% solution in a 120-mL bottle) or combine it with the $Na_2S_2O_3$ solution before addition.

6.3    When the sample is collected, leave ample air space in the bottle (at least 2.5 cm) to facilitate mixing by shaking, preparatory to examination.  Be careful to take samples that will be representative of the water being tested and avoid sample contamination at time of collection or in period before examination.

6.4    Keep sampling bottle closed until the moment it is to be filled. Remove stopper and hood or cap as a unit, taking care to avoid soiling.  During sampling, do not handle stopper or cap and neck of bottle, and protect them from contamination.  Hold bottle near base, fill it without rinsing, replace stopper or cap immediately, and secure hood around neck of bottle.

6.5    Start bacteriological examination of a water sample promptly after collection to avoid unpredictable changes.  If samples cannot be processed within 1 hr of collection, use an iced cooler for storage during transport to the laboratory.

6.6    Hold temperature of all stream pollution samples below 10°C during a maximum transport time of 6 hr.  Refrigerate these samples upon receipt in the laboratory and process within 2 hr.  When local conditions necessitate delays in delivery of samples longer than 6 hr, make field examinations using field laboratory facilities located at the site of collection or use delayed-incubation procedures.

## 7.0 PROCEDURES

### 7.1 Selection of sample size:

7.1.1 Size of sample will be governed by expected bacterial density, which in finished-water samples will be limited only by the degree of turbidity.

7.1.2 An ideal sample volume will yield growth of about 50 coliform colonies and not more then 200 colonies of all types. Examine finished waters by filtering duplicate portions of the same volume, such as 100 to 500 mL or more, or by filtering two diluted volumes. Examine other waters by filtering three different volumes, depending on the expected bacterial density. When less than 20 mL of sample (diluted or undiluted) is filtered, add a small amount of sterile dilution water to the funnel before filtration. This increase in water volume aids in uniform dispersion of the bacterial suspension over the entire effective filtering surface.

### 7.2 Filtration of sample:

7.2.1 Using sterile forceps, place a sterile filter over porous plate of receptacle, grid side up. Carefully place matched funnel unit over receptacle and lock it in place. Filter sample under partial vacuum. With filter still in place, rinse funnel by filtering three 20- to 30-mL portions of sterile dilution water. Unlock and remove funnel, immediately remove filter with sterile forceps, and place it on sterile pad or agar with a rolling motion to avoid entrapment of air.

7.2.2 Use sterile filtration units at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is considered to be interrupted when an interval of 30 min or longer elapses between sample filtrations. After such interruption, treat any further sample filtration as a new filtration series and sterilize all membrane-filter holders in use.

7.2.3 Decontaminate this equipment between successive filtrations by use of flowing steam, boiling water, or, if available, an ultraviolet sterilizer. When using the UV sterilization procedure, a 2-min exposure to UV radiation is sufficient and should kill 99.9% of all bacteria. Eye protection is recommended to protect against stray radiation from a non-light-tight sterilization cabinet. This UV equipment is not commercially available and is not required, although its use is recommended.

### 7.3 Two-step enrichment technique:

7.3.1 Place a sterile absorbent pad in the upper half of a sterile culture dish and pipet enough enrichment medium (1.8 to 2.0 mL lauryl tryptose broth) to saturate pad. Carefully remove any surplus liquid.

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Aseptically place filter through which the sample has been passed on pad. Incubate filter, without inverting dish, for 1.5 to 2 hr at 35 $\pm$ 0.5°C in an atmosphere of at least 90% relative humidity.

   7.3.2   Remove enrichment culture from incubator, lift filter from enrichment pad, and roll it onto the agar surface.  Incorrect filter placement is at once obvious, because patches of unstained membrane indicate entrapment of air.  Where such patches occur, carefully reseat filter on agar surface.  If the liquid medium is used, prepare final culture by removing enrichment culture from incubator and separating the dish halves. Place a fresh sterile pad in bottom half of dish and saturate it with 1.8 to 2.0 mL of final M-Endo medium.  Transfer filter, with same precautions as above, to new pad.  Discard used pad.  With either the agar or the liquid medium, invert dish and incubate for 20 to 22 hr at 35 $\pm$ 0.5°C.

7.4  <u>Counting</u>:

   7.4.1   The typical coliform colony has a pink to dark-red color with a metallic surface sheen.  The sheen area may vary in size from a small pinhead to complete coverage of the colony surface.  Count sheen colonies with the aid of a low-power (10 to 15 magnifications) binocular wide-field dissecting microscope or other optical device, with a cool-white fluorescent light source directed above and as nearly perpendicular as possible to the plane of the filter.  The total count of colonies (coliform and noncoliform) on Endo-type medium has no relation to the total number of bacteria present in the original sample and, so far as is known, no significance can be inferred or correlation made with the quality of the water sample.

7.5  <u>Calculation of coliform density</u>:

   7.5.1   Report coliform density as (total) coliforms/100 mL.  Compute the count, using membrane filters with 20 to 80 coliform colonies and not more than 200 colonies of all types per membrane, by the following equation:

$$\begin{array}{l} \text{(Total)} \\ \text{coliform colonies/100 mL} \end{array} = \frac{\text{coliform colonies counted x 100}}{\text{mL sample filtered}}$$

   7.5.2   **Water of drinking-water quality:**

   7.5.2.1 With water of good quality, the number of coliform colonies will be less than 20 per membrane.  In this event, count all coliform colonies and use the formula given above to obtain coliform density.

   7.5.2.2 If confluent growth occurs, that is, growth covering either the entire filtration area of the membrane or a portion thereof, and colonies are not discrete, report results as "confluent growth with or without coliforms."  If the total number of bacterial

colonies, coliforms plus noncoliforms, exceeds 200 per membrane, or if the colonies are too indistinct for accurate counting, report results as "too numerous to count" (TNTC). In either case, request a new sample and select more appropriate volumes to be filtered per membrane, remembering that the standard drinking-water portion is 100 mL. Thus, instead of filtering 100 mL per membrane, 50-mL portions may be filtered through each of two membranes, 25-mL portions may be filtered through each of four membranes, etc. Total the coliform counts observed on the membranes and report as number per 100 mL.

7.5.3 **Water of other than drinking-water quality:**

7.5.3.1 As with potable water samples, if no filter has a coliform count falling in the ideal range, total the coliform counts on all filters and report as number per 100 mL. For example, if duplicate 50-mL portions were examined and the two membranes had five and three coliform colonies, respectively, report the count as eight coliform colonies per 100 mL, i.e.,

$$\frac{(5 + 3) \times 100}{(50 + 50)}$$

7.5.3.2 Similarly, if 50-, 25-, and 10-mL portions were examined and the counts were 15, 6, and 1 coliform colonies, respectively, report the count as 25/100 mL, i.e.,

$$\frac{(15 + 6) \times 100}{(50 + 25 + 10)}$$

7.5.3.3 On the other hand, if 10-, 1.0-, and 0.1-mL portions were examined with counts of 40, 9, and 1 coliform colonies respectively, select only the 10-mL portion for calculating the coliform density because this filter had a coliform count falling in the ideal range. The result is 400/100 mL, i.e.,

$$\frac{(40 \times 100)}{10}$$

In this last example, if the membrane with 40 coliform colonies also had a total bacterial colony count greater than 200, report the coliform count as 400/100 mL.

7.5.3.4 Report confluent growth or membranes with colonies too numerous to count, as described in 7.5.2, above. Request a new sample and select more appropriate volumes for filtration.

7.5.4 **Statistical reliability of membrane filter results:** Although the statistical reliability of the membrane filter technique is greater than that of the MPN procedure, membrane counts really are not absolute numbers. Table 1 illustrates some 95% confidence limits.

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TABLE 1.   95% CONFIDENCE LIMITS FOR MEMBRANE-FILTER RESULTS
USING 100-mL SAMPLE

| Number of Coliform Colonies Counted | 95% Confidence Limits | |
| --- | --- | --- |
| | Lower | Upper |
| 1 | 0.05 | 3.0 |
| 2 | 0.35 | 4.7 |
| 3 | 0.81 | 6.3 |
| 4 | 1.4 | 7.7 |
| 5 | 2.0 | 9.2 |

8.0   QUALITY CONTROL

8.1   Extensive quality control procedures are provided in Part IV of U.S. EPA, 1978 (see Section 10.0, References).  These procedures should be adhered to at all times.

8.2   Samples must be maintained as closely as possible to original condition by careful handling and storage.  Sample sites and sampling frequency should provide data representative of characteristics and variability of the water quality at that site.  Samples should be analyzed immediately.  If this is not practical, they should be refrigerated at a temperature of 1-4°C and analyzed within 6 hr.

8.3   Quality control of culture media is critical to the validity of microbiological analysis.  Some important factors to consider are summarized below:

8.3.1   Order media to last for only 1 yr; always use oldest stock first.  Maintain an inventory of all media ordered, including a visual inspection record.

8.3.2   Hold unopened media for no longer than 2 yr.  Opened media containers should be discarded after 6 mo.

8.3.3   When preparing media, keep containers open as briefly as possible.  Prepare media in deionized or distilled (Type II) water of proven quality.  Check the pH of the media after solution and sterilization; it should be within 0.2 units of the stated value.  Discard and remake if it is not.

8.3.4   Autoclave media for the minimal time specified by the manufacturer, because the potential for damage increases with increased exposure to heat.  Remove sterile media from the autoclave as soon as pressure is zero.  Effectiveness of the sterilization should be checked weekly, using strips or ampuls of <u>Bacillus</u> <u>stearothemophelus.</u>

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8.3.5    Agar plates should be kept slightly open for 15 min after pouring or removal from refrigeration to evaporate free moisture.  Plates must be free of lumps, uneven surfaces, pock marks, or bubbles, which can prevent good contact between the agar and medium.

8.3.6    Quality control checks of prepared media should include the incubation of 5% of each batch of medium for 2 days at 35°C to inspect for growth and positive/negative checks with pure culture.

8.4    <u>Analytical quality control procedures should include</u>:

8.4.1    Duplicate analytical runs on at least 10% of all known positive samples analyzed.

8.4.2    At least one positive control sample should be run each month for each parameter tested.

8.4.3    At least one negative (sterile) control should be run with each series of samples using buffered water and the medium batch used at the beginning of the test series and following every tenth sample.  When sterile controls indicate contamination, new samples should be obtained and analyzed.

8.4.4    The Type II water used should be periodically checked for contamination.

8.5    <u>Quality control specifications for membrane filters</u>:

8.5.1    Membrane filters can be purchased sterile or packaged for sterilization.  They can be sterilized by autoclaving, ethylene oxide, or irradiation.  Membrane manufacturers should certify that their membranes meet stated specifications on sterility, retention, recovery, pore size, flow rate, pH, total acidity, phosphate, and other extractables.

8.5.2    Membrane performance should be tested to ensure proper results.  Each lot ordered should be inspected for proper shape, grid lines, diffusability, and correct colony development.  Membranes containing sizable areas with no colony development are questionable.
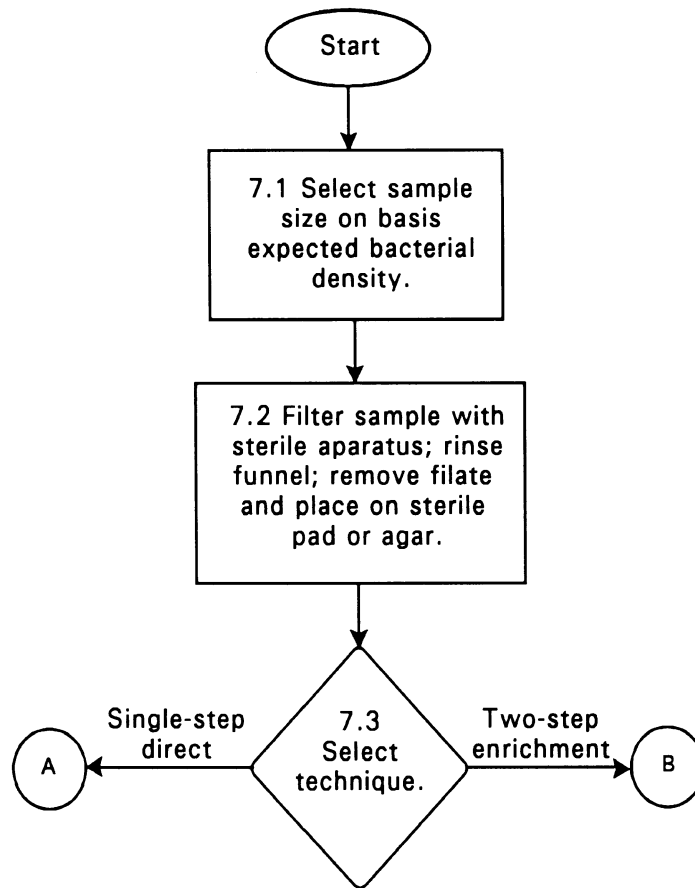

9.0  METHOD PERFORMANCE

9.1  No data provided.


10.0  REFERENCES

1.    Standard Methods for the Examination of Water and Wastewater, 15th ed.

2.    Bordner, R.H., et al., Microbiological Methods for Monitoring the Environment, Environmental Monitoring and Support Laboratory, U.S. EPA, Cincinnati, OH, EPA-600/8-78-017, 1978.

# METHOD 9132
## TOTAL COLIFORM; MEMBRANE FILTER TECHNIQUE

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                    ┌─────────┐
                    │  Start  │
                    └────┬────┘
                         │
                         ▼
            ┌────────────────────────┐
            │   7.1 Select sample    │
            │       size on basis    │
            │   expected bacterial   │
            │        density.        │
            └───────────┬────────────┘
                        │
                        ▼
            ┌────────────────────────┐
            │  7.2 Filter sample with│
            │  sterile aparatus; rinse│
            │  funnel; remove filate │
            │  and place on sterile  │
            │      pad or agar.      │
            └───────────┬────────────┘
                        │
                        ▼
                      ◇─────◇
  Single-step        │  7.3  │      Two-step
    direct           │ Select│    enrichment
  (A) ◄──────────────│techniq│──────────────► (B)
                      ◇─────◇
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