



Heterotrophic Bacteria

Methods 8241 and 8242

Pour Plate and
Membrane Filter
Methods

Plate Count Agar*, m-HPC, m-TGE, m-TGE with TTC, and m-TSB/USP

Scope and Application: For water and wastewater

* This method meets or exceeds the specification criteria stated in *Standard Methods for the Examination of Water and Wastewater*, 19th edition, Method 9215 B. Pour Plate Method.

Introduction

The standard plate count attempts to provide a standardized means of determining the density of aerobic and facultatively anaerobic heterotrophic bacteria in water. Bacteria occur singly or in pairs, chains, clusters or packets, and no single method, growth medium, or set of physical conditions can satisfy the physiological requirements of all bacteria in a water sample. However, the heterotrophic plate count is a good measure of water treatment plant efficiency, aftergrowth in transmission lines, and the general bacterial composition of source water.

Technique is Important

Good laboratory technique is essential when accuracy is important, particularly in microbiological laboratory procedures. Care in sample collection and preservation, a clean laboratory or work surface, proper sterilization and inoculation practices, and close temperature control help assure reliable results.

Preparing the Work Area

To save time, start the incubator before preparing the other materials. Set the incubator for the temperature required in the procedure (usually 35 ± 0.5 °C).

Disinfect the work bench with a germicidal cloth, dilute bleach solution, bactericidal spray, or dilute iodine solution. Wash your hands thoroughly with soap and water.

Mark each pour plate, membrane filtration petri dish, or other sample container with the sample number, dilution, date, and any other necessary information. Take care not to contaminate the inside of the sample container in any way.

Preparing Sample Containers

Take care to prevent contamination when conducting bacterial tests. All materials used for containing or transferring samples must be sterile. To collect samples, use any of the following: sterilized plastic bags, sterilized disposable bottles, autoclavable glass bottles, or autoclavable plastic bottles.

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Sterilized plastic bags or disposable bottles: Presterilized plastic bags and bottles are available with or without dechlorinating agent. The bottles are available with a 100-mL fill-to line.

Note: Dechlorinating reagent should be used with potable or chlorinated water samples. It is not necessary for unchlorinated or nonpotable water samples. However, dechlorinating reagent will not interfere with unchlorinated samples so, for simplicity, plastic bags containing dechlorinating reagent may be used for all samples.

Autoclavable glass or plastic bottles: Glass or plastic bottles (125-mL size) may be used instead of sterilized plastic bags or disposable bottles. These containers should be prepared as follows:

1. Wash in hot water with detergent.
2. Thoroughly rinse with hot tap water, followed by a distilled water rinse to make sure that all detergent is removed.
3. If dechlorinating agent is needed (for chlorinated, potable water), add the contents of one Dechlorinating Reagent Powder Pillow for each 125-mL of container volume. (A 250-mL sample container will require two powder pillows.)
4. Steam sterilize glass and autoclavable plastic containers at 121 °C for 15 minutes. Glass sample containers may be sterilized by hot air at 170 °C for one hour.
5. Store sterile containers, tightly capped, in a clean environment until needed.

Preparing Test Equipment

Use high-quality laboratory equipment and ready-to-use media to save time and minimize errors. Hach's prepared media helps eliminate contamination due to technique.

Preparing the Materials

Note: Disinfect the work bench or work area with a germicidal cloth, dilute bleach solution or dilute iodine solution. Wash hands thoroughly with soap and water.

Using Presterilized Equipment And Media

Bacteriological testing requires sterile materials, a disinfected work area and proper handling techniques, or contamination may give false results. To simplify technique and minimize the possibility of contamination, Hach offers membrane filters, disposable pipets, petri dishes with and without absorbent pads, inoculating loops, buffered dilution water, sampling bags and 2-mL prepared growth media. All have been presterilized. Hach offers presterilized and disposable pipets, petri dishes, with and without absorbent pads, inoculating loops, 99-mL bottles of buffered dilution water, sampling bags, and prepared growth media. When using these materials, an autoclave is unnecessary because only the filter funnel and forceps require sterilization. The funnel can be sanitized by immersion in boiling water for 5 minutes prior to use. (An optional, disposable sterile filter unit is also available.) The forceps can be sterilized by dipping them in alcohol and flaming.

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Using Conventional Equipment and Materials

When numerous samples must be run on a routine basis, the analyst may prefer using an autoclave for conventional, non-disposable materials. Wash sample bottles, pipets, petri dishes, filter holder with stopper and graduated cylinder (if needed) with hot water and detergent.

6. Rinse several times with tap water and then with demineralized water. Dry thoroughly.
7. Prepare all equipment for autoclaving.
 - Loosely thread caps on sample bottles and cover caps and bottle necks with metal foil or paper.
 - Cover the openings of graduated cylinders with metal foil or paper.
 - Insert the filter funnel base into an autoclavable rubber stopper that will fit the filter flask.
 - Wrap the two parts of the filter funnel assembly separately in heavy wrapping paper and seal with masking tape.
 - Wrap petri dishes (borosilicate glass) in paper or place in aluminum or stainless cans.
8. Sterilize equipment in an autoclave at 121 °C for 15 minutes. Borosilicate glass items may be sterilized with dry heat at 170 °C for a minimum of 1 hour.

Using Field Filtration Apparatus for Membrane Filtration

1. Flame sterilize the top surface of the stainless steel Field Vacuum Support.
2. Attach the syringe tip to the vacuum support tubing.
3. Using sterile forceps, place a membrane filter, grid side up, onto the center of the vacuum support.
4. To sterilize forceps, dip forceps in alcohol and flame in an Alcohol or Bunsen Burner. Let forceps cool before use.
5. Open a package of funnels (start at the bottom of the package). Remove a funnel (base first) from the package.
6. Place the funnel onto the vacuum support. Do not touch the inside of the funnel. Push evenly on the funnel's upper rim to snap it onto the vacuum support.
7. Pour the sample into the funnel.
8. See specific procedures for the sample volume required.
9. Pull on the syringe plunger to draw the sample through the filter apparatus.
10. Remove the funnel.
11. Press the lever on the vacuum support stem to lift the membrane filter from the vacuum support surface.
12. Use sterile forceps to remove the membrane filter.
13. Place the membrane filter into a prepared petri dish and incubate according to the appropriate procedure.

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14. Disconnect the syringe tip from the vacuum support tubing. Dispose of the liquid in the syringe.

Using an Autoclavable Filter Assembly for Membrane Filtration

Note: Disinfect the work bench or work area with a germicidal cloth, dilute bleach solution or dilute iodine solution. Wash hands thoroughly with soap and water.

1. After sterilization, remove the filter funnel assembly from the wrapping paper.
2. Do not contaminate the funnel by touching the inner surfaces that will be exposed to the sample.
3. Insert the funnel with rubber stopper into the filtering flask or filter funnel manifold and connect to the water trap and aspirator with rubber tubing.
4. Using sterile forceps, place a sterile membrane filter on the filter base and attach the filter funnel top.
5. Filter a small quantity of sterile Buffered Dilution Water through the funnel to assure a good seal on the filter and connections before filtering the sample.

Collecting and Preserving Samples

General Guidelines

Use proper sampling procedures to insure that seasonal variances are detected and that results are representative of the sample source. Using a sterile container, collect a sufficient volume of sample (usually 100 mL) for the guidelines to be met. The World Health Organization guidelines prescribe 200 mL per sample, while *Standard Methods for the Examination of Water and Wastewater* prescribes 100 mL per sample. Maintain at least 2.5 cm (approximately 1 inch) of air space to allow adequate space for mixing the sample prior to analysis.

Avoid sample contamination during collection. Carefully open each sample container just prior to collection, and close immediately following collection. Do not lay the lid or cap down and avoid touching the mouth or the inside of the container. Do not rinse the container.

No dechlorination is necessary if the sample is added directly to the medium on site. Otherwise, samples should be treated to destroy chlorine residual and immediately transported for analysis after collection. Sodium thiosulfate, sterilized within the collection container, is commonly used to destroy chlorine residual.

Failure to properly collect and transport samples will cause inaccurate results.

Analyze as soon as possible after collection. Allow no more than 6 hours to elapse between collection and examination for nonpotable water samples and 30 hours for potable water samples. For best results maintain the sample at or below 10 °C, but do not freeze. Failure to properly collect and transport samples will cause inaccurate results.

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Collecting samples from faucets, spigots, hydrants or pumps:

Collect representative samples by allowing the water to run from a faucet, spigot, hydrant, or pump at a moderate rate, without splashing, for two to three minutes before sampling. Do not adjust the rate of flow while the sample is being collected. Valves, spigots, and faucets that swivel or leak should be avoided. Remove aerators or screens attached to valves, spigots, and faucets before collecting samples.

Collecting samples from rivers, lakes and reservoirs:

When sampling a river, lake or reservoir, fill the sample container below the water surface. Do not sample near the edge or bank. Remove the cap, grasp the sample container near the bottom, and plunge the container, mouth down, into the water. (This technique excludes any surface scum.) Fill the container by positioning the mouth into the current, or, in non flowing water, by slightly tilting the bottle and allowing it to fill slowly.

Disposing of Completed Tests

Active bacterial cultures grown during incubation must be disposed of safely. This may be accomplished in one of the following two ways:

Bleach – Used test containers may be sterilized by using a 10% bleach solution. Add approximately 12 mL of bleach to each test container. Allow 10 to 15 minutes contact time with the bleach. Pour the liquid down the drain, then dispose of the test containers in the normal garbage.

Autoclave – Place used test containers into a contaminated items bag or a biohazard bag and seal tightly to prevent their leaking into the autoclave. Autoclave at 121 °C for 15 minutes at 15 pounds pressure. Place the bag of sterilized test containers in a separate garbage bag and tie tightly; dispose of with the normal garbage.

Introduction

The Pour Plate Method, also known as the standard plate count, is simple to perform and is commonly used to determine heterotrophic bacteria density. This method does, however, have disadvantages that limit recovery of the maximum number of organisms. Tempered medium at 44 to 46 °C may cause heat shock to stressed bacteria and the nutritionally rich medium may decrease recovery of starved bacteria.

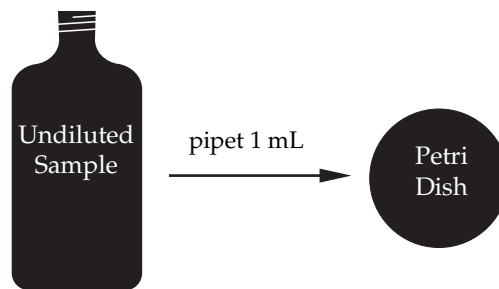
Diluting the Sample

The pour plate method requires use of 1 mL, 0.1 mL, and 0.01 mL or 0.001 mL of sample. The difficulty measuring and working with the two smaller volumes, 0.01 and 0.001 mL, require the use of sample dilutions. These dilutions are prepared by pipetting 1 mL of undiluted sample into 99 mL of Buffered Dilution Water. diluting the sample allows 1mL of diluted sample to be used instead of 0.01 mL of undiluted sample, and 0.1 mL of diluted sample instead of 0.001 mL of undiluted sample. See *Figure 1 Sample Dilution* on page 6.

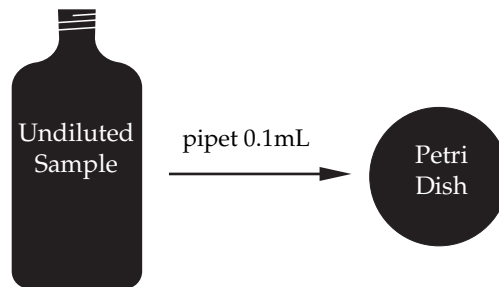
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Figure 1 Sample Dilution

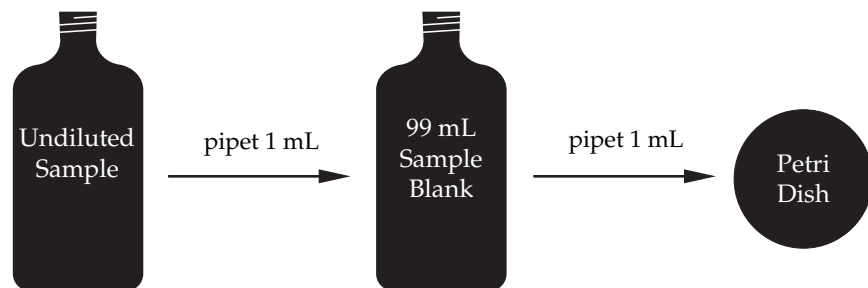
To use 1-mL sample volume
Dilution Factor 1



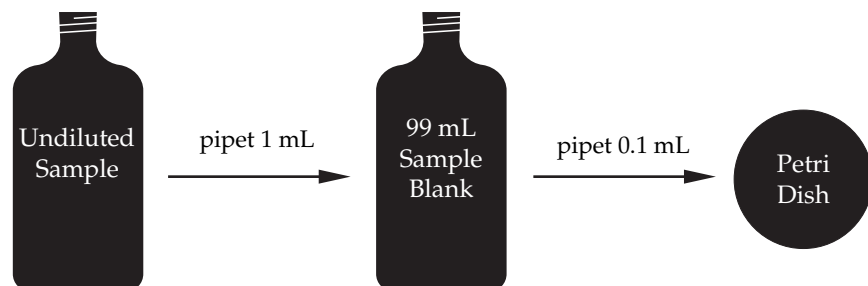
To use 0.1-mL sample volume
Dilution Factor 10



To use 0.01-mL sample volume
Dilution Factor 100



To use 0.001-mL sample volume
Dilution Factor 1000



Note: Shake the sample container vigorously before performing dilutions (approximately 25 times).

Note: Use a sterile pipet for each transfer of undiluted sample into a petri dish or into buffered dilution water. Use a different sterile pipet to transfer diluted sample into the petri dish. When removing sample, do not insert pipets more than 2.5 cm below the surface of sample or dilution. Do not prepare dilutions in direct sunlight.

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Selecting Sample Volumes/Dilutions

Select the sample volumes or dilutions to be used so that the total number of colonies on a plate will be between 30 and 300. For most potable water samples, plates suitable for counting will be obtained by plating 1 mL of undiluted sample, 0.1 mL of undiluted sample and 1 mL of diluted sample (which equals 0.01 mL of undiluted sample). In examining sewage or turbid water, do not measure a 0.1-mL inoculum of the original undiluted sample, but do prepare an appropriate dilution.

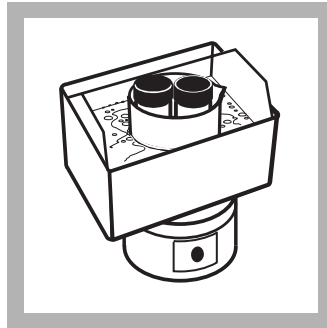
Limit the number of samples to be plated at any one time, so that no more than 20 minutes (preferable 10 minutes) elapse between the dilution of the first sample and the pouring of the last plate.



1. Melt the sterile solid agar medium by placing a tube of Plate Count Agar in a beaker of boiling water. (Each tube contains enough medium for two plates.)

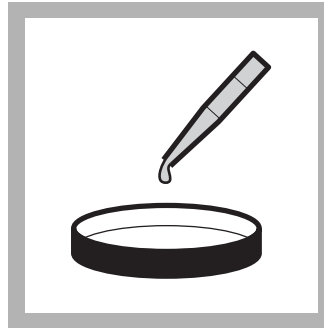
Note: Avoid prolonged exposure to unnecessarily high temperatures during and after melting.

Note: When the medium is melted in two or more batches, use all of each batch in order of melting, provided the contents remain fully melted. Discard any melted agar that contains precipitate.



2. Keep the melted medium in a water bath, between 44 and 46 °C, until used.

Note: Do not depend on the sense of touch to indicate the proper temperature when pouring the agar. To make sure that the medium is at the proper temperature, also place a thermometer in the water bath. To avoid contamination, the thermometer should be in a separate container of melted agar or water, not in the agar tubes to be plated.



3. Pipet the appropriate amount of undiluted or diluted sample (1 mL or 0.1 mL) into the sterile petri dish. Prepare at least two plates for each different volume of undiluted or diluted sample used.

Note: Thoroughly mix all undiluted and diluted samples by rapidly making about 25 complete up-and-down (or back-and-forth) movements. Or, use a mechanical shaker to shake samples or dilutions for 15 seconds.

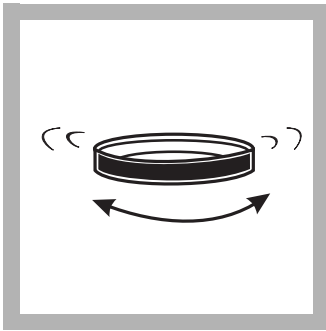
Note: Lift the lid of the petri dish just high enough to insert the pipet. Hold the pipet at a 45° angle, with the tip just touching the bottom of the dish. Allow enough time (at least 2-4 seconds) for the pipet to drain.



4. Pour at least 10 to 12 mL of liquefied medium (1/2 of the contents of Plate Count Agar tube) into the dish by gently lifting the cover just high enough to pour.

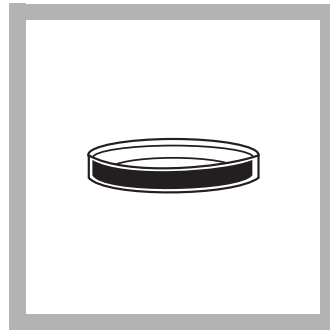
Note: Avoid spilling the medium on the outside of the container or on the inside of the dish lid when pouring.

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5. Mix the melted medium thoroughly with the sample in the petri dish by rotating the dish in opposite directions or by rotating and tilting.

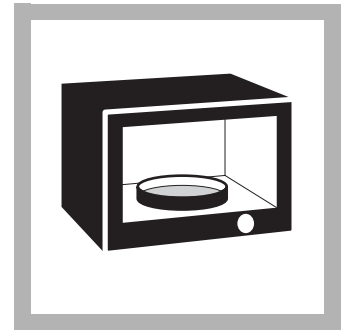
Note: Do not invert the petri dish to mix.



6. Place the plates on a level surface and let them solidify. This generally takes 10 minutes.



7. Invert the plates, place them in a plastic bag, and seal the bag. Place the bag in an incubator that has been prewarmed to 35 °C.



8. Incubate the plates for 48 ± 3 hours at 35 ± 0.5 °C.

Note: During incubation, maintain humidity within the incubator so that plates will not have moisture weight loss greater than 15%. A pan of water placed at the bottom of the incubator may be sufficient. For incubation in non-humidified incubators, make certain that the plastic bags are tightly sealed.



9. Using a Quebec Colony Counter, count all colonies on the plates promptly after incubation. See the following section on "Interpreting and Reporting Results."

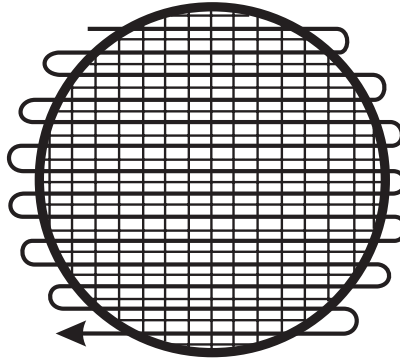
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Interpreting and Reporting Results

Count all colonies on selected plates promptly after incubation. If count must be delayed temporarily, store plates at 5 to 10 °C for no more than 24 hours, but avoid routine delays.

Quebec Colony Counters feature a built-in grid to simplify counting. The easiest way to count colonies is to follow a back and forth pattern, moving down the grid. (See *Figure 2*).

Figure 2 Colony Counting Technique



Report all counts as colony-forming units (CFU)/mL. Include in the report the method used, the incubation temperature and time, and the medium.

For example: 75 CFU/mL, pour plate method, 35 °C/48 hours, plate count agar.

Generally, results are obtained by averaging the number of colonies on all plates from the same undiluted or diluted sample volume, and multiplying by a dilution (described below). In this case, results should be rounded to two significant digits to avoid creating false precision. For three-digit results, raise the middle digit if the last digit is 5 or greater. Retain the middle digit if the last digit is 4 or smaller. The last digit will be zero.

For example, 143 would become 140, 255 would become 260. Two digit numbers require no rounding.

Be familiar with the following terms before counting and reporting results:

Average Number of Colonies/Plate — The average number of colonies per plate is derived by dividing the total number of colonies on all plates that were inoculated with the same sample volume or dilution volume, and dividing that sum by the number of plates used.

For example, if two plates were each inoculated with 1 mL of diluted sample, and there were 89 colonies on one plate and 103 on the other, then the average number of colonies/plate would be:

$$\frac{89 \text{ colonies} + 103 \text{ colonies}}{2 \text{ plates}} = 96 \text{ colonies}$$

Colony-Forming Units (CFU)/mL — This is the unit used for reporting bacterial density. To derive the number of CFU/mL, multiply the **average number of colonies/plate** by the **dilution factor** of the incubated sample.

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Note: In some instances where a large number of colonies are observed, the average number of colonies/plate is obtained by adding colonies counted only in a specified number of squares on each plate.

Dilution Factor — The dilution factor is the reciprocal of the volume of original, undiluted sample plated, and is used to standardize the results according to the sample volume.

For example, if 1 mL of original sample was used, the dilution factor is 1. If 0.1 mL of original sample was used, the dilution factor is 10. The dilution factor for 1 mL of diluted sample (0.01 mL of original sample) is 100, and the dilution factor for 0.1 mL of diluted sample (0.001 mL of original sample) is 1000.

Representative Colony Distribution — When counting colonies in a specified number of squares (as seen through the colony counter), count those squares that appear to have an average number of colonies. Avoid counting squares that have many less or many more colonies than most of the other squares on the plate.

Spreaders — Spreaders are colonies of bacteria which grow in such a way that they appear to be “spread” across the plate. (See *Figure 3*).

Figure 3 Spreader Growth



It is preferable when counting and recording results, to consider plates having between 30 and 300 colonies. However, this is not always the case, so when counting and recording colonies, choose the situation that best describes your results.

If spreaders are encountered on the plates selected, count colonies on representative portions only when the colonies are well distributed in spreader-free areas, and the area covered by the spreaders does not exceed one-half of the plate area.

When spreading colonies must be counted, count each of the following types as one colony:

1. A chain of colonies that appears to be caused by disintegration of a bacterial clump as agar and sample were mixed.
2. A spreader that develops as a film of growth between the agar and the bottom of the petri dish.
3. A colony that forms in a film of water at the edge or over the agar surface.

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Count as **individual** colonies the similar-appearing colonies growing in close proximity but not touching, provided that the distance between them is at least equal to the diameter of the smallest colony.

Also count as **individual** colonies those colonies which are touching, but are different in appearance, such as morphology or color.

To obtain results, multiply the **average number of colonies/plate** by the **dilution factor**. Report counts as CFU/mL.

If plates have excessive spreader growth, report as “spreaders” (Spr). When plates are uncountable because of missed dilution, accidental dropping, or contamination, or the control plates indicate that the medium or other material or labware was contaminated, report as “laboratory accident” (LA).

No colonies — If plates from all dilutions of any sample have no colonies, report the count as less than one (<1) times the dilution factor for the largest volume of original sample used.

For example, if no colonies develop using 0.1 mL of original sample, report the count as less than 10 (<10) estimated CFU/mL.

Less than 30 colonies/plate — Ordinarily, no more than 1.0 mL of sample is plated. Therefore, when the total number of colonies developing from 1.0 mL is less than 30, record the number of colonies as CFU/mL.

30 to 300 colonies/plate — Compute bacterial count per mL by multiplying the **average number of colonies/plate** by the **dilution factor**. Report counts as CFU/mL.

For example: 0.1 mL of undiluted sample was used to inoculate two plates. After incubation, the plates had colony counts of 115 and 145. The CFU/mL value is computed as follows:

$$\frac{115 \text{ colonies} + 145 \text{ colonies}}{2 \text{ plates}} \times 10 \text{ (dilution factor)} = 1300 \text{ CFU/mL}$$

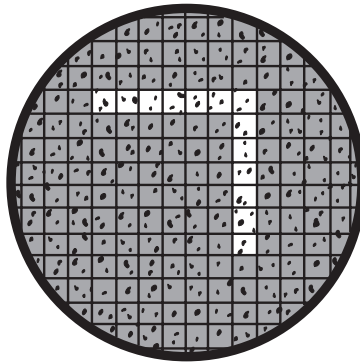
Greater than 300 colonies/plate — If no plate has 30 to 300 colonies, and one or more plates have more than 300 colonies, use the plates having a count closest to 300 colonies. Compute the count by multiplying the **average number of colonies/plate** by the **dilution factor**, and report as estimated CFU/mL.

Far more than 300 colonies/plate — If there are greater than 300 colonies/plate, *do not* report the result as “too numerous to count” (TNTC). Instead, follow these guidelines for reporting:

Less than 10 colonies/cm² — If there are fewer than 10 colonies per cm² (one “square” as seen through the colony counter), then count colonies in 13 squares having **representative colony distribution**. If possible, select seven consecutive squares horizontally across the plate, and six consecutive squares vertically, being careful not to count a square more than once (See *Figure 4*). Add the number of colonies in each square. Multiply this sum by 4.38 when the plate area is 57 cm² (disposable plastic plates). Multiply the sum by 5 when the plate area is 65 cm² (glass plates). To determine colony-forming units (CFU)/mL, compute the **average number of colonies/plate** and multiply the result by the dilution factor (See next page). Report as estimated CFU/mL.

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Figure 4 Colony Counting Technique
For plates with far more than 300 colonies (less than 10 colonies/cm²)



Note: One thousand is the dilution factor for 0.1 mL of diluted sample. This is the sample volume that should be used when bacterial counts are this high.

More than 10 colonies/cm² When there are more than 10 colonies per cm² (one “square” as seen through the colony counter), count colonies in four squares having **representative colony distribution**. Add the number of colonies in these four squares, and divide the sum by 4, to get the average number of colonies/square. Multiply this number by 57 when the plate area is 57 cm² (disposable plastic plates). Multiply this number by 65 when the plate area is 65 cm² (glass plates). To determine CFU/mL, compute the **average number of colonies/plate** and multiply the result by 1000 (see “Note” below). Report as estimated CFU/mL.

Avoiding Errors

Avoid inaccuracies in counting due to damaged or dirty optics that impair vision, or due to failure to recognize colonies. Be careful not to contaminate plates due to improper handling. Laboratory workers who cannot duplicate their own counts on the same plate within 5%, and counts of other analysts within 10%, should discover the cause and correct such disagreements.

REQUIRED MEDIA AND REAGENTS

Description	Unit	Cat No.
Dechlorinating Reagent Powder Pillows	100/pkg	14363-69
Dilution Water, Buffered, sterile, 99-mL	25/pkg	14305-98
Plate Count Agar Tubes	20/pkg	24067-20

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REQUIRED APPARATUS

Description	Unit	Cat No.
Bags, Whirl-Pak with dechlorinating agent, sterile, 180-mL.....	100/pkg.....	20753-33
Bags, Whirl-Pak without dechlorinating agent, sterile, 205-mL.....	100/pkg.....	22331-99
Bags, Whirl-Pak without dechlorinating agent, sterile, 710-mL.....	10/pkg.....	14372-97
Beaker, 250-mL.....	each.....	500-46
Bottle, sample, glass, with cap, 118-mL.....	3/pkg.....	21631-03
Clamp, Test Tube.....	each.....	634-00
Colony Counter, Quebec, 110 VAC, 60 Hz.....	each.....	22521-00
Colony Counter, Quebec, 220 VAC, 50 Hz.....	each.....	22521-02
Counter, hand tally.....	each.....	14696-00
Dish, Petri, 100 x 15 mm, sterile, disposable.....	20/pkg.....	21789-01
Dish, Petri, 100 x 15 mm, sterile, disposable.....	500/pkg.....	21789-00
Germicidal Cloths.....	50/pkg.....	24632-00
Hot Plate, 120 VAC, 50/60 Hz.....	each.....	23443-00
Hot Plate, 240 VAC, 50/60 Hz.....	each.....	23443-02
Incubator, Culture, Low-Profile, 120 VAC, 50/60 Hz.....	each.....	26192-00
Incubator, Culture, Low-Profile, 220 VAC, 50/60 Hz.....	each.....	26192-02
Pipets, Serological, 1 mL, sterile, disposable.....	25/pkg.....	2098-98
Pipets, Serological, 10-11 mL, sterile, disposable.....	25/pkg.....	2097-98
Thermometer -10 to 110 °C.....	each.....	1877-01

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Membrane Filter Procedure

The Membrane Filter (MF) Heterotrophic Plate Count Method* is a fast, simple way to estimate bacterial populations in water. Since no single medium can satisfy the growth requirements of all bacteria, several types of media are offered for detecting heterotrophic bacteria in water. The m-HPC medium, available in both the broth and agar formats, is a high-nutrient medium used to enumerate heterotrophs in treated potable water samples. The m-TGE broth, originally developed for use with dairy products, is now commonly used to determine bacterial counts in water by membrane filtration. The m-TGE broth with TTC contains a redox dye, triphenyltetrazolium chloride, which colors the colonies red, thus enhancing their visibility. The m-TSB/USP broth is a general purpose medium which was designed to conform with the formula specified in the USEPA's Code of Federal Regulations (21 CFR) for sterility testing of pharmaceutical products.

In the initial step, an appropriate sample volume is passed through a membrane filter with a pore size small enough (0.45 microns) to retain the bacteria present. The filter is placed either on an absorbent pad (in a petri dish) saturated with a culture medium or on an agar medium that is selective for heterotrophic bacteria growth. The petri dish containing the filter and pad is incubated, upside down, for 24 to 48 hours, depending on the medium used, at the appropriate temperature. After incubation, the colonies which have developed are identified and counted by using a low-power microscope. The MF method is especially useful for testing drinking water because large volumes of sample can be analyzed in a short time.

Diluting the Sample

The volume of sample to be filtered will vary with the sample type. Select a maximum sample size to give 20 to 200 colony-forming units (CFU) per filter.

Generally, for finished, potable water, the volume to be filtered will be 100 mL. For samples which are suspected to have higher heterotrophic bacteria counts, use a smaller sample volume. Some sample types will require a very small volume to obtain the optimum 20 to 200 CFU. Because it is almost impossible to measure these small volumes accurately, a series of dilutions should be made. The following procedure describes one method of preparing a series of dilutions.

Dilution Technique

1. Wash hands.
2. Open a bottle of sterile, Buffered Dilution Water.
3. Shake the sample collection container vigorously, approximately 25 times.
4. Using a sterile transfer pipet, pipet the required amount of sample into the sterile Buffered Dilution Water.
5. Recap the buffer dilution water bottle and shake vigorously 25 times.

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Dilution Series

A. If 10-mL sample is required:

- Transfer 11 mL of sample into 99 mL of sterile buffered dilution water. Filter 100 mL of the dilution to obtain the 10-mL sample.

B. If 1-mL sample is required:

- Transfer 11 mL of the 10-mL dilution from sample A into 99 mL of sterile buffered dilution water. Filter 100 mL of the dilution to obtain the 1-mL sample.

C. If 0.1-mL sample is required:

- Transfer 11 mL of the 1-mL dilution from sample B into 99 mL of sterile buffered dilution water. Filter 100 mL of the dilution to obtain the 0.1-mL sample.

D. If 0.01-mL sample is required:

- Transfer 11 mL of the 0.1-mL dilution from sample C into 99 mL of sterile buffered dilution water. Filter 100 mL of the dilution to obtain the 0.01-mL sample.

E. If 0.001-mL sample is required:

- Transfer 11 mL of the 0.01-mL dilution from sample C into 99 mL of sterile buffered dilution water. Filter 100 mL of the dilution to obtain the 0.001-mL sample.

F. If 0.0001-mL sample is required:

- Transfer 11 mL of the 0.001-mL dilution from sample D into 99 mL of sterile buffered dilution water. Filter 100 mL of the dilution to obtain the 0.0001-mL sample.



1. Use sterilized forceps to place a sterile, absorbent pad in a sterile petri dish. Replace the lid on the dish.

Note: Do not touch the pad or the inside of the petri dish.

Note: To sterilize the forceps, dip them in alcohol and flame in an alcohol or Bunsen burner. Let the forceps cool before use.

Note: Alternatively, a prepared m-HPC agar plate may be used.

Note: For ease of use, petri dishes containing pads are available.



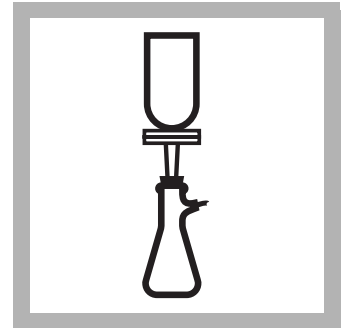
2. Invert ampules two or three times to mix broth. Open an ampule of m-HPC, m-TGE with TTC, or m-TSB/USP Broth, using an ampule breaker if necessary. Pour the contents evenly over the absorbent pad. Replace the petri dish lid.

Note: For broth prepared from dehydrated medium, pipet approximately 2.0 mL of broth onto the pad using a sterile pipet. Drain excess medium from the petri dish and replace the lid.



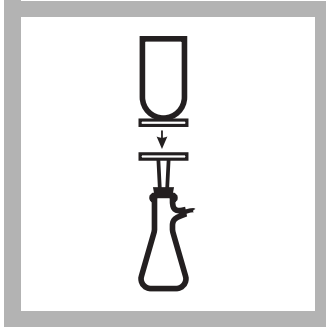
3. Set up the Membrane Filter Assembly as described under *Preparing the Materials* on page 2. Using sterile forceps, place a membrane filter, grid side up, in the assembly.

Note: Alternatively, a sterile, disposable filter unit may be used.

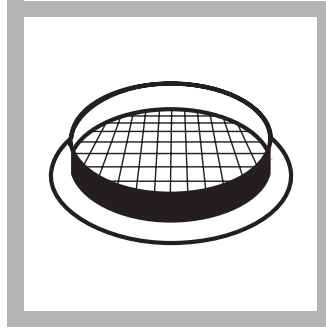


4. Shake the sample vigorously to mix. Filter the appropriate volume through the sterile 47 mm, 0.45 μ m, gridded membrane filter. Apply vacuum and filter the sample. Rinse the funnel walls three times with 20 to 30 mL of sterile buffered dilution water.

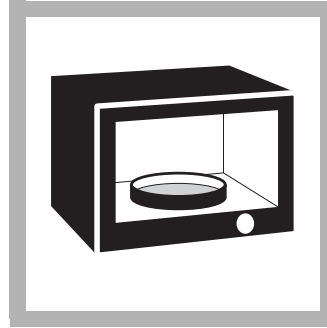
Heterotrophic Bacteria



5. Turn off the vacuum and lift off the funnel top. Remove the membrane filter, using sterile forceps. Still using the forceps, transfer the filter immediately to the previously prepared petri dish.



6. With a slight rolling motion, place the filter, grid side up, on the absorbent pad. Check for trapped air under the filter and make sure the filter touches the entire petri dish lid. Replace the petri dish lid.



7. Label the petri dish with the sample number, dilution and date. Invert the petri dish and incubate at 35 ± 0.5 °C for 48 hours for m-HPC, or 24 hours for m-TGE, m-TGE with TTC, or m-TSB/USP.



8. Remove the dish from the incubator. Count colonies on membrane filters using a 10 - 15X stereo binocular microscope.

Note: Bacterial colonies grown on m-HPC, m-TGE, or m-TSB/USP medium appear clear to cream. Colonies grown on m-TGE medium with TTC indicator appear red to aid visibility.

Counting, Computing, and Reporting Results

Optimal colony density per filter is 20 to 200. Report all colonies counted as colony forming-units (CFU)/mL. Include in the report the method used, the incubation temperature and time, and the medium.

For example: 98 CFU/L, mL, 35 °C, 24 hours, m-TGE broth.

1 to 2, or fewer colonies per square — Count all of the colonies on the filter, and divide the results by the volume of original sample used.

For example: if there are 122 colonies on the filter, and the volume of original sample used was 10 mL, compute results as follows:

$$\frac{122 \text{ colonies}}{10 \text{ mL sample}} = 12.2 \text{ CFU/mL}$$

3 to 10 colonies per square — Count all colonies in 10 representative squares and divide by 10 to obtain an average number of colonies per square. Multiply this number by 100 and divide by the volume of original sample used.

For example: if you calculated an average of 8 colonies per square, and the volume of original sample used was 0.1 mL, compute results as follows:

$$\frac{8 \text{ colonies/square} \times 100}{0.1 \text{ mL sample}} = 8000 \text{ CFU/mL}$$

Heterotrophic Bacteria

10 to 20 colonies per square — Count all colonies in 5 representative squares and divide by 5 to obtain an average number of colonies per square. Multiply this number by 100 and divide by the volume of original sample used.

For example: if there are an average of 17 colonies per square, and the volume of original sample used was 0.1 mL, compute results as follows:

$$\frac{17 \text{ colonies/square} \times 100}{0.1 \text{ mL sample}} = 17,000 \text{ CFU/mL}$$

More than 20 colonies per square — If there are more than 20 colonies per square, record the count as > 2000 divided by the volume of original sample used.

For example: if the original volume of sample used were 0.01 mL, results would be > 2000/0.01 or > 200,000 CFU/mL.

Report averaged counts as estimated CFU/mL. Make estimated counts only when there are discrete, separated colonies without spreaders.

REQUIRED MEDIA AND REAGENTS

Description	Unit	Cat No.
Dilution Water, Buffered, sterile, 99-mL.....	each.....	14305-72
Dilution Water, Buffered, sterile, 99-mL.....	25/pkg.....	14305-98
m-HPC Agar Plates	15/pkg.....	28114-15
m-HPC Broth Ampules, plastic, 2-mL.....	50/pkg.....	28124-50
m-TSB/USP Broth Ampules, plastic, 2-mL	50/pkg.....	28126-50
m-TGE PourRite™* Ampules, glass, 2-mL.....	20/pkg.....	23738-20
m-TGE with TTC PourRite™ Ampules, 2-mL	20/pkg.....	24284-20

*PourRite is a Hach Company trademark.

REQUIRED APPARATUS

Description	Unit	Cat No.
Absorbent Pads with dispenser, sterile, Gelman	1000.pk	14918-00
Alcohol Burner	each	20877-42
Aspirator	each	2131-02
Bags, Whirl-Pak with dechlorinating agent, sterile, 180-mL	100/pkg	20753-33
Counter, hand tally	each	14696-00
Filter Holder, magnetic coupling.....	each	13529-00
Filtering Flask, 500-mL	each	546-49
Forceps	each	21411-00
Germicidal Cloth.....	50/pkg	24632-00
Graduated cylinder, 100-mL	each	508-42
Illuminator for microscope, 110 VAC, 50/60 Hz.....	each	23175-00
Incubator, Culture, 120 VAC	each	26192-00
Incubator, Culture, 240 VAC	each	26192-02
Membrane filters, 0.45- μ m, gridded, sterile, Gelman	200/pkg	13530-01
Membrane Filters, 0.45- μ m, gridded, sterile, Millipore	200/pkg	28176-00
Microscope, Stereo Binocular.....	each	23174-00
Petri Dish, polystyrene, sterile, disposable, without pad	100/pkg	14852-99
Petri Dish, polystyrene, sterile, disposable, w/pad, Gelman	100/pkg	14717-99
Petri Dish, polystyrene, sterile, disposable, w/pad, Millipore	100/pkg	28177-00
Pump, vacuum, 110/115 VAC.....	each	28248-00
Pump, vacuum, 220/230 VAC, North American Plug.....	each	28248-01
Pump, vacuum, 220/230 VAC, Continental European Plug.....	each	28248-01
Rubber Stopper, one hole, No. 7	6/pkg	2119-07
Rubber Tubing, 3.6-m	each	560-19
Pipets, Serological, 10–11 mL, sterile, disposable	25/pkg	2097-98



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