Heterotrophic Bacteria

DOC316.53.01229

Plate Count Agar[1]

Heterotrophic Plate Count (HPC)

Method 8241

Pour Plate Method

Scope and application: For water and wastewater.

[1] Adapted from *Standard Methods for the Examination of Water and Wastewater*.

Test preparation

Before starting

Set the temperature of the incubator to 35 ± 0.5 °C (95 ± 0.9 °F). Let the incubator temperature become stable, then add the samples.
Wash hands thoroughly with soap and water.
Use a germicidal cloth, bactericidal spray, weak bleach solution or weak iodine solution to clean the work area.
Make sure that all of the materials that come in contact with samples are sterile.
Prepare a minimum of two petri dishes (plates) for each sample volume.
Each tube of plate count agar contains sufficient agar to prepare two plates.
Put a beaker with water on a hot plate to melt the tubes of agar.
Put a thermometer in the water bath to make sure that the temperature is correct. To prevent contamination, do not put the thermometer in an agar tube that is used for analysis.
If the agar is melted in groups of tubes, use all of the tubes in the group that was melted first. If the agar does not stay melted or shows a precipitate, discard the agar.
Analyze a small number of samples at a time. Keep the time from the first sample dilution to the last plate preparation within 10 minutes.

Items to collect

Description	Quantity
Plate count agar tube	1
Hot plate and 250-mL beaker	1
Thermometer	1
Sterile buffered dilution water	1
Pipet, 1 mL and/or 0.1 mL	1
Petri dish, 100 mm	2
Plastic bag for petri dish	1
Incubator	1
Colony counter	1

Refer to Consumables and replacement items on page 7 for order information.

Sample collection

- Use a sterile glass or plastic container such as a Whirl-Pak bag that contains sterilized sodium thiosulfate. The sodium thiosulfate is not necessary if the sample does not contain a residual disinfectant.
- Open the sample containers immediately before collection and close immediately after collection. Do not put the lid or cap down. Do not touch the lip or inner surfaces of the container. Do not rinse the containers before use.
- To collect a potable water sample from a faucet, spigot, hydrant or pump, let the water flow at a moderate rate for 2–3 minutes. Remove the screens or aerators. Do not use faucets or spigots that have a bad seal or that show a leak between components.
- To collect a non-potable sample from a river, lake or reservoir, hold the container below the water surface, then remove the cap. As an alternative, remove the cap and push the container, mouth down, below the water surface to prevent the collection of surface scum. Put the mouth of the container into the current. Fully fill the container below the water surface.
- Collect a minimum of 100 mL of sample. Keep a minimum of 2.5 cm (1 inch) of air space in the container.
- Write the sample information on the container and start the analysis as soon as possible.
- If immediate analysis is not possible, keep the sample at or below 10 °C (50 °F) for a maximum of 8 hours. Do not let the sample freeze.

Sample volumes and dilution

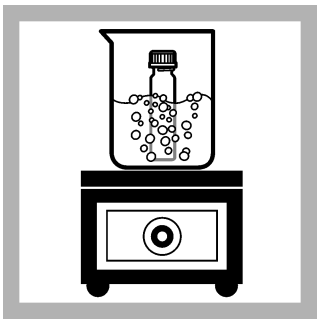
Use a sample volume that gives a result of 30 to 300 colonies on the agar plate. For samples with a low level of bacteria such as finished, potable water, use 1 mL of sample (dilution factor = 1). For samples with more bacteria, use 0.1 mL of undiluted sample (dilution factor = 10) or 1 mL of a 100x sample dilution (dilution factor = 100). For turbid water or water with high bacteria levels, use 1 mL and/or 0.1 mL of a 100x sample dilution. Use the steps that follow to make a 100x sample dilution.

1. Wash hands thoroughly with soap and water.
2. Invert the sample container for 30 seconds (approximately 25 times).
3. Open a bottle of sterile buffered dilution water.
4. Use a sterile pipet to add 1 mL of sample into the dilution water bottle. Refer to Figure 1.
5. Put the cap on the dilution water bottle and invert for 30 seconds (25 times). This is a 100x dilution (sample is diluted by a factor of 100).
6. Use a sterile pipet to add 1 mL or 0.1 mL of the 100x dilution to the petri dish. Refer to Figure 1 and Figure 2.

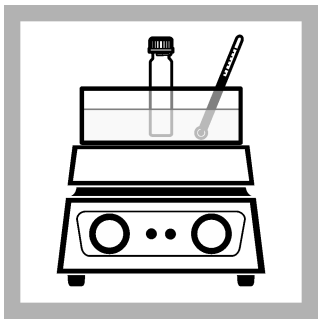
Figure 1 1 mL of 100x sample dilution, dilution factor = 100

Figure 2 0.1 mL of 100x sample dilution, dilution factor = 1000

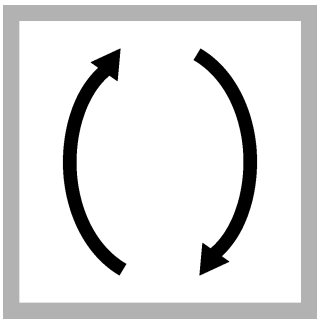
Pour plate test procedure



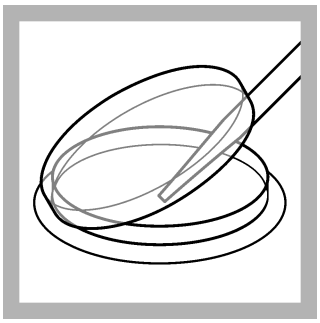
1. Put a tube of the sterile agar in a beaker of boiling water. Loosen the cap to make it easier to pour after the agar has melted.



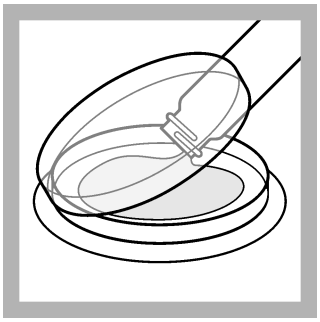
2. Keep the melted agar in a water bath at 44–46 °C (111–115 °F). Use a thermometer to monitor the temperature.



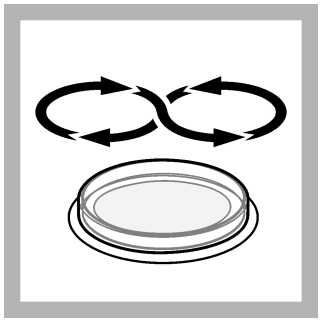
3. Invert the sample or the diluted sample for 30 seconds (25 times) to make sure that the sample is mixed well.



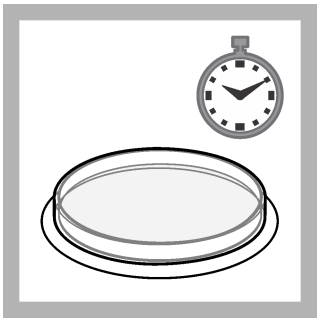
4. Use a pipet to add the sample (or dilution) to a sterile petri dish. Touch the pipet tip to the bottom of the petri dish and hold at a 45° angle as the pipet drains. Wait 2–4 seconds for the pipet to drain.



5. Pour half (10 to 12 mL) of the melted agar from one tube into the petri dish. Do not spill the agar. Close the lid.



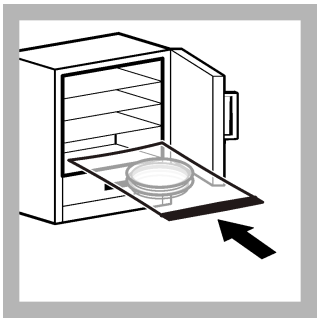
6. Move the petri dish in a figure-eight motion on a flat surface to mix the melted agar with the sample. Do not invert the petri dish.



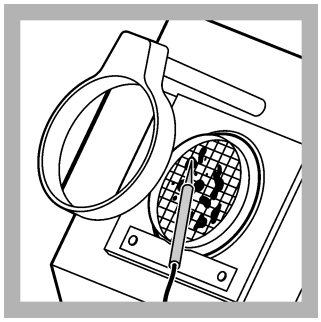
7. Put the petri dish on a level surface. Wait approximately 10 minutes for the agar to become solid.

8. Invert the petri dish.

9. Put the inverted petri dish in a plastic bag and seal the bag.



10. Put the bag with the inverted petri dish in an incubator at 35 (± 0.5) °C (95 (± 0.9) °F) for 48 hours. Make sure that the plastic bag is sealed tightly so that the agar stays moist, or put a container of water in the incubator.



11. Remove the petri dish from the incubator. Use a colony counter to count the number of bacteria colonies in the agar. Refer to Interpret and report the results on page 5.

Colony count guidelines

Count the colonies on the agar plate soon after the specified incubation time. If immediate analysis is not possible, keep the plates at 5–10 °C (41–50 °F) for a maximum of 24 hours. Use the guidelines that follow to help count the bacteria colonies.

- Keep the optics on the colony counter clean.
- Be careful not to contaminate the agar plates.
- Use the average colony count during the colony count determination. To determine the average colony count, add the count from each plate of the same dilution, then divide by the number of plates.
- Use two significant digits to report the CFU/mL.
- The colony counter has a grid to help count the colonies. The easiest way to count the colonies is to use a left-to-right pattern as shown in Figure 3.
- When it is necessary to count the colonies in a specified number of squares, use the squares that are representative of the colony distribution on the plate.
- Colonies can grow together to form large irregular shapes known as spreaders. If spreaders occur, count the colonies in spreader-free areas when less than one-half of the plate area has spreaders. Refer to Figure 4.
- When it is necessary to count the spreaders, count the spreader types that follow as one colony:
  - A chain of colonies that can occur from a clump of bacteria that breaks apart when the agar and sample are mixed.
  - A spreader that shows as a film of growth between the agar and the bottom of the petri dish.
  - A colony that forms in a film of water at the edge or over the agar surface.
- Count colonies that touch each other as individual colonies when they are different in shape or color.
- Count colonies that are near to each other and look similar as individual colonies when the distance between them is at minimum the diameter of the smallest colony.

Figure 3 Colony count technique

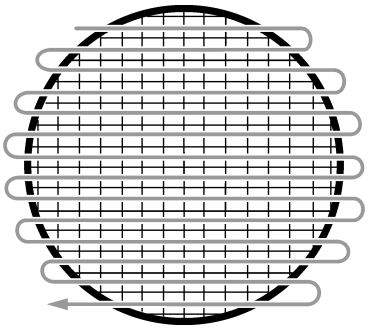
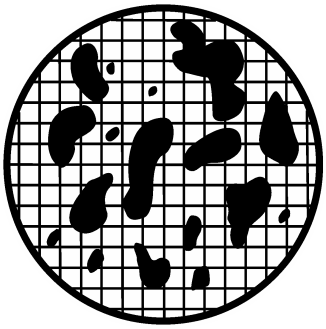


Figure 4 Spreader colonies in plate count agar



Interpret and report the results

Use the steps that follow to determine the result as colony-forming units per mL of sample (CFU/mL).

1. Use the colony counter to look at the colonies on the agar plate. Make an estimate of the number of colonies in each square of the grid.

   Note: *Make estimated counts only when there are isolated colonies without spreaders. When there are plates with 30 to 300 colonies in each plate, use only those plates to determine the count.*

2. Determine the CFU/mL for the estimated colony count as follows:

Colony count	CFU/mL determination
No colonies	Report the result as less than one (<1) multiplied by the dilution factor. Refer to Table 1. Use the smallest dilution. *Example: The plates for all sample dilutions showed no colonies. The sample volume was 0.1 mL. The result is <10 estimated CFU/mL.*
Less than 30 colonies per plate	When the sample volume is 1.0 mL and the total number of colonies on the plate is less than 30, report the number of colonies as CFU/mL. *Example: The sample volume was 1.0 mL. 8 colonies were counted. The result is 8 CFU/mL.*
30 to 300 colonies per plate	1. Count the total number of colonies on each plate. 2. Determine the average number of colonies per plate. 3. Multiply the average number per plate by the dilution factor. Refer to Table 1. *Example: The sample volume was 0.1 mL on each of two plates. The colony count on each plate was 115 and 145. The result is (115 + 145) ÷ 2 × 10 = 1300 CFU/mL.*

Colony count	CFU/mL determination
More than 300 colonies per plate	If all of the plates have more than 300 colonies, use only the plates that have a count nearest to 300. **1.** Count the total number of colonies on each plate that contain the same sample volume. **2.** Determine the average number of colonies per plate. **3.** Multiply the average number per plate by the dilution factor. Refer to Table 1.
Much more than 300 colonies per plate	If the colony count is much more than 300 colonies per plate, refer to Very high colony counts on page 6.

3. Report the result as CFU/mL. Include in the report the method used, the incubation temperature, time and the nutritional medium. *Example: 75 CFU/mL, pour plate method, 35 °C (95 °F), 48 hours, plate count agar.*

Table 1 Dilution factors

Sample or dilution	Volume added to petri dish	Dilution factor
Sample	1 mL	1
Sample	0.1 mL	10
Dilution (100x)	1 mL	100
Dilution (100x)	0.1 mL	1000

Very high colony counts

If the colony count is much more than 300 colonies per plate, do not report the result as "too numerous to count" (TNTC). Use the steps that follow to determine the CFU/mL.

1. Look through the colony counter to determine if there are more or less than 10 colonies per cm^2 (square) on the plate.
2. Determine the number of colonies per plate as follows:

Option	Description
Less than 10 colonies per cm^2	**1.** Select 13 squares that are representative of the colony distribution. If possible, select seven consecutive squares horizontally across the plate and six consecutive squares vertically. Refer to Figure 5. **2.** Count the total number of colonies in the 13 squares. **3.** Multiply the number of colonies from the 13 squares by 4.38 for 57-cm^2 plates (plastic disposable) or by 5 for 65-cm^2 plates (glass). This value is the number of colonies per plate.
More than 10 colonies per cm^2	**1.** Select four squares that are representative of the colony distribution. **2.** Count the total number of colonies in the 4 squares. **3.** Divide the total number of colonies in the 4 squares by 4 to get the average number of colonies per square. **4.** Multiply the average number of colonies per square by 57 for 57-cm^2 plates (plastic disposable) or by 65 for 65-cm^2 plates (glass). This value is the number of colonies per plate.

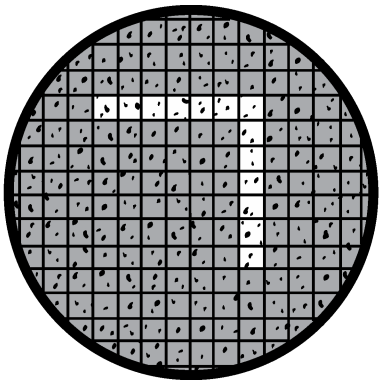
3. Determine the number of colonies per plate for the remaining plates.
4. Divide the number of colonies per plate by the number of plates that were used to get the average number of colonies per plate.

5. Multiply the average number of colonies per plate by the dilution factor. Refer to Table 1 on page 6.

   Note: *Use 0.1 mL of diluted sample when the colony counts are much more than 300 colonies per plate.*

6. Report the result as estimated CFU/mL. Include in the report the method used, the incubation temperature, time and the nutritional medium. *Example: 570,000 estimated CFU/mL, pour plate method, 35 °C (95 °F), 48 hours, plate count agar.*

Figure 5 Count technique for < 10 colonies/cm^2



Summary of method

The HPC (heterotrophic plate count) method is used to make an estimate of the number of aerobic and facultatively anaerobic heterotrophic bacteria in water. The concentration of heterotrophic bacteria in water gives information about the quality of the water and how much bacteria is removed during treatment. Different nutritional broths and agars are available to supply the necessary nutrients to bacteria in different types of water.

The pour plate procedure, also known as the standard plate count, is an easy procedure and is commonly used to determine heterotrophic bacteria density. The sample mixes with a nutritional agar at a high temperature. The agar cools and becomes solid. During incubation, the bacteria in the sample grow and form colonies in the agar. After incubation, the agar is examined with a colony counter for bacteria colonies. The heat of the melted agar can cause heat shock to stressed bacteria, and the nutritionally rich agar can decrease the recovery of starved bacteria.

The heterotrophic plate count is a good way to measure the efficiency of water treatment plants, growth in distribution lines and the general bacterial composition of source water. No single method, growth medium or set of physical conditions can satisfy the physiological requirements of all bacteria in a water sample. The colonies that grow in the agar are smaller and less likely to grow into each other than colonies that are grown on membrane filters.

Consumables and replacement items

Required reagents

Description	Quantity/test	Unit	Item no.
Plate count agar tubes	1	20/pkg	2406720
Dilution water, buffered, 99 mL, sterile[1]	1	25/pkg	1430598

[1] Buffered dilution water is prepared with magnesium chloride and potassium dihydrogen phosphate.

Required apparatus

Description	Unit	Item no.
Beaker, 250 mL, glass	each	50046H
Clamp, test tube	each	63400
Colony counter, Quebec, 110 VAC, 60 Hz	each	2252100
Colony counter, Quebec, 220 VAC, 50 Hz	each	2252102
Hot plate, 7 x 7 inch, digital, 115 VAC	each	2881500
Petri dish, 100 x 15 mm, sterile, disposable	20/pkg	2178901
Pipet, serological, 1 mL, sterile, disposable, individually wrapped	50/pkg	2092835
Pipet, serological, 10–11 mL, sterile, disposable	25/pkg	209798
Pipet filler, safety bulb	each	1465100
Thermometer, –20 to 110 °C (–4 to 230 °F), non-mercury	each	2635702

Incubators

Description	Unit	Item no.
Laboratory incubator, culture, 110 VAC	each	2619200
Laboratory incubator, culture, 230 VAC	each	2619202
Portable incubator with 12 VDC power socket	each	2569900
AC power supply for portable incubator, 110–240 VAC	each	2968100
Battery pack, rechargeable, for portable incubator 12 VDC	each	2580300
Portable incubator rack, general purpose/petri dish	each	2580502

Sample collection

Description	Unit	Item no.
Sampling bags, Whirl-Pak® with dechlorinating reagent, 177 mL	100/pkg	2075333
Sampling bags, Whirl-Pak without dechlorinating reagent, 207 mL	100/pkg	2233199
Sampling bottles, sterilized, with dechlorinating agent, 100-mL sample	100/pkg	8888006
Sampling bottles, sterilized, without dechlorinating reagent, 100-mL sample	12/pkg	2495012
Sampling bottles, sterilized, without dechlorinating reagent, 100-mL sample	50/pkg	2495050
Sample transport kit, includes 100 sample bags with dechlorinating agent, refrigerant pack, rack and 9-L cooler	each	2568700

Optional reagents and apparatus

Description	Unit	Item no.
Pipet, TenSette®, 1.0–10.0 mL	each	1970010
Pipet tips, TenSette, 1.0–10.0 mL, sterile, individually wrapped	200/pkg	2558996
Pipet, TenSette®, 0.1–1.0 mL	each	1970001
Pipet tips, TenSette, 0.1–1.0 mL, non-sterile	50/pkg	2185696

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12/2015, Edition 8