9215 HETEROTROPHIC PLATE COUNT*

9215 A. Introduction

1. Applications

The heterotrophic plate count (HPC), formerly known as the standard plate count, is a procedure for estimating the number of live culturable heterotrophic bacteria in water and measuring changes during water treatment and distribution or in swimming pools. Colonies may arise from pairs, chains, clusters, or single cells, all of which are included in the term "colony-forming units" (CFU). The final count also depends on interaction among the developing colonies. Choose the procedure and medium that comply with the application of the information. To compare data, use the same procedure and medium. Four different methods and five different media are described.

2. Selection of Method

a. Pour plate method: The pour plate method (9215B) is simple to perform and can accommodate volumes of sample or diluted sample ranging from 0.1 to 2.0 mL. The colonies produced are relatively small and compact, showing less tendency to encroach on each other than those produced by surface growth. On the other hand, submerged colonies often are slower growing and are difficult to transfer. A thermostatically controlled water bath is essential for tempering the agar. Heat shock to bacteria from the transient exposure of the sample to 45 to 46°C agar may occur.

b. Spread plate method: The spread plate method (9215C) causes no heat shock and all colonies are on the agar surface where they can be distinguished readily from particles and bubbles. Colonies can be transferred quickly, and colony morphology easily can be discerned and compared to published descriptions. However, this method is limited by the small volume of sample or diluted sample that can be absorbed by the agar: 0.1 to 0.5 mL, depending on the degree to which the prepoured plates have been dried. To use this procedure, maintain a supply of suitable predried, absorbent agar plates.

c. Membrane filter method: The membrane filter method (9215D) permits testing of large volumes of low-turbidity water and is the method of choice for low-count waters (<1 to 10 CFU/mL). This method produces no heat shock but adds the expense of the membrane filter. Further disadvantages include the smaller display area, the need to detect colonies by reflected light against a white background if colored filters or contrast stains are not used, possible damage to cells by excessive filtration pressures, and possible variations in membrane filter quality (see Section 9020B.4*k*).

d. Enzyme substrate method for heterotrophic bacteria: The enzyme substrate method (9215E) can be used with samples having a wide range of bacterial concentrations. The method† uses a substrate-based medium in which the substrates are hydrolyzed by microbial enzymes causing the release of 4-methylumbelliferone maximally after 48 h of incubation at 35°C. 4-Methylumbelliferone fluoresces when exposed to long-wavelength (365-nm) ultraviolet light. The number of fluorescing wells corresponds to a most probable number (MPN) of bacteria in the original sample. The test may be used for the analysis of drinking water and source water samples. Individual colonies cannot be directly recovered for subsequent analysis. This method produces no heat shock to the bacteria in the sample. The enzyme substrate test is comparable to the pour plate method.

3. Work Area

Provide a level table or bench top with ample area in a clean, draft-free, well-lighted room or within a horizontal-flow laminar hood. Use table and bench tops having nonporous surfaces and disinfect before any analysis is made.

4. Samples

Collect water as directed in Section 9060A. Initiate analysis as soon as possible after collection to minimize changes in bacterial population. The recommended maximum elapsed time between collection and analysis of sample is 8 h (maximum transit time 6 h, maximum processing time 2 h). When analysis cannot begin within 8 h, maintain sample at a temperature below 4°C but do not freeze. Maximum elapsed time between collection and analysis must not exceed 24 h.

5. Sample Preparation

Mark each plate with sample number, dilution, date, and any other necessary information before examination. Prepare at least two replicate plates for each volume of sample or dilution examined. For the pour or spread plate methods use sterile glass (65 cm^2) or presterilized disposable plastic (57 cm^2) petri dishes.

Thoroughly mix all samples or dilutions by rapidly making about 25 complete up-and-down (or back-and-forth) movements. Optionally, use a mechanical shaker to shake samples or dilutions for 15 s.

6. Media

Compare new lots of media with current lot in use according to Section 9020B.5*j*.

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* Approved by Standard Methods Committee, 2004.
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† SimPlate® for HPC, Idexx Laboratories, Westbrook, ME.

a. Plate count agar (tryptone glucose yeast agar): Use for pour and spread plate methods. This high-nutrient agar, widely used in the past, may give lower counts than R2A or NWRI agar.

Tryptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar	15.0 g
Reagent-grade water	1 L

pH should be 7.0 ± 0.2 after autoclaving at 121°C for 15 min.

b. m-HPC agar:‡ Use this high-nutrient medium only for the membrane filter method

Peptone	20.0 g
Gelatin	25.0 g
Glycerol	10.0 mL
Agar	15.0 g
Reagent-grade water	1 L

For medium produced from basic ingredients, adjust to pH 7.2 with 1*N* NaOH. Heat slowly to dissolve thoroughly, add glycerol and sterilize at 121°C for 15 min. Commercially prepared medium should not require post-sterilization pH adjustment; see Section 9020B.5*j*1). Final pH is 7.1 ± 0.2.

c. R2A agar: Use for pour plate, spread plate, and membrane filter methods. This low-nutrient agar gives higher counts than high-nutrient formulations.

Yeast extract	0.5 g
Proteose peptone No. 3 or polypeptone	0.5 g
Casamino acids	0.5 g
Glucose	0.5 g
Soluble starch	0.5 g
Dipotassium hydrogen phosphate, K_2HPO_4	0.3 g
Magnesium sulfate heptahydrate, $MgSO_4 \cdot 7H_2O$	0.05 g
Sodium pyruvate	0.3 g
Agar	15.0 g
Reagent-grade water	1 L

For medium produced from basic ingredients, adjust to pH 7.2 with solid K_2HPO_4 or KH_2HPO_4 before adding agar. Heat to dissolve agar and sterilize at 121°C for 15 min. Commercially prepared medium should not require post-sterilization pH adjustment; see Section 9020B.5*j*1). Final pH is 7.2 ± 0.2.

d. NWRI agar (HPCA): Use for pour plate, spread plate, and membrane filter methods. This low-nutrient medium is likely to produce higher colony counts than high-nutrient media. It is not currently available in dehydrated form and requires preparation from the basic ingredients; this makes its usage less desirable.

Peptone	3.0 g
Soluble casein	0.5 g
K_2HPO_4	0.2 g
$MgSO_4$	0.05 g
$FeCl_3$	0.001 g
Agar	15.0 g
Reagent-grade water	1 L

Final pH is 7.2 ± 0.2 after autoclaving at 121°C for 15 min.

e. Enzyme substrate medium: See 9215E.3.

‡ Formerly called m-SPC agar.

7. Incubation

For compliance monitoring purposes under U.S. EPA's Surface Water Treatment Rule (40 CFR 141.74) provision on heterotrophic bacteria, incubate pour plates at 35°C for 48 h. Otherwise, select from among recommended times and temperatures for monitoring changes in water quality. The highest counts typically will be obtained from 5- to 7-d incubation at a temperature of 20 to 28°C.

During incubation, maintain humidity within the incubator so the agar plates will have no moisture weight loss greater than 15%. This is especially important if prolonged incubation is used. A pan of water placed at the bottom of the incubator may be sufficient, but note that to prevent rusting or oxidation of the incubator, the inside walls and shelving should be of high-grade stainless steel or anodized aluminum. For long incubation in nonhumidified incubators, seal plates in plastic bags.

8. Counting and Recording

a. Pour and spread plates: Count all colonies on selected plates promptly after incubation. If counting must be delayed temporarily, store plates at 5 to 10°C for no more than 24 h, but avoid this as routine practice. Record results of sterility controls on the report for each lot of samples.

For compliance samples, count colonies manually using a dark-field colony counter, such as a Quebec colony counter. If such equipment is not available, for samples not for compliance purposes, use other equipment, provided such equipment gives equivalent magnification. Automatic plate counting instruments are available. These generally use a television scanner coupled to a magnifying lens and an electronics package. Their use is acceptable if evaluation in parallel with manual counting gives comparable results.

In preparing plates, pipet sample volumes that will yield from 30 to 300 colonies/plate. The aim is to have at least one dilution giving colony counts between these limits, except as provided below.

Ordinarily, do not pipet more than 2.0 mL of sample; however, when the total number of colonies developing from 2.0 mL is less than 30, disregard this rule and record result observed. With this exception, consider only plates having 30 to 300 colonies in determining the plate count. Compute bacterial count per milliliter by the following equation:

$$\text{CFU/mL} = \frac{\text{colonies counted}}{\text{actual volume of sample plated, mL}}$$

If there is no plate with 30 to 300 colonies, and one or more plates have more than 300 colonies, use the plate(s) having a count nearest 300 colonies. Compute the count as above and report as estimated CFU per milliliter.

If plates from all dilutions of any sample have no colonies, report the count as less than one (<1) divided by the corresponding largest sample volume used. For example, if no colonies develop from the 0.01-mL sample volume, report the count as less than 100 (<100) CFU/mL.

If the number of colonies per plate exceeds 300, do not report results as "too numerous to count" (TNTC). If there are fewer

than 10 colonies/cm^2, count colonies in 13 squares (of the colony counter) 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. Compute estimated colonies per plate as follows: When the plate is 65 cm^2 (the typical area of a glass plate), multiply the sum of the number of colonies in 13 representative square centimeters by 5; when the plate is 57 cm^2 (the typical area of a plastic plate), multiply the sum of the number of colonies in 19 representative square centimeters by 3. (NOTE: The nominal diameter of both disposable and nondisposable glass plates is 100 mm. However, the internal diameter of disposable plates is nearer to 85 mm and that of nondisposable plates is near to 90 mm.) When bacterial counts on crowded plates are greater than 100 colonies/cm^2, report results as greater than (>) 6500 divided by the smallest sample volume plated for glass plates or (>) 5700 divided by the smallest sample volume plated for plastic plates. Report as estimated colony-forming units per milliliter.

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

When spreading colonies must be counted, count each of the following types as one: a chain of colonies that appears to be caused by disintegration of a bacterial clump as agar and sample were mixed; a spreader that develops as a film or growth between the agar and bottom of the petri dish; and a colony that forms in a film of water at the edge or over the agar surface. The last two types largely develop because of an accumulation of moisture at the point from which the spreader originates. They frequently cover more than half the plate and interfere with obtaining a reliable plate count.

Count as individual colonies 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. Count impinging colonies that differ in appearance, such as morphology or color, as individual colonies.

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

b. Membrane filter method: Count colonies on membrane filter using a stereoscopic microscope at 10 to 15× magnification. Preferably slant petri dish at a 45° angle on microscope stage and adjust light source vertical to the colonies. Optimal colony density per filter is 20 to 200. If colonies are small and there is no crowding, a higher limit is acceptable.

Count all colonies on the membrane when there are ≤2 colonies per square. For 3 to 10 colonies per square count 10 squares and obtain an average count per square. For 10 to 20 colonies per square count 5 squares and obtain an average count per square. Multiply average count per square by 100 and divide by the sample volume to give colonies per 100 mL. If there are more than 20 colonies per square, record the count as >2000 divided by the sample volume. Report averaged counts as estimated colony-forming units. Make estimated counts only when there are discrete, separated colonies without spreaders.

c. Enzyme substrate method: See 9215E.6.

9. Computing and Reporting Counts

The term "colony-forming unit(s)" (CFU) is descriptive of the methods used; therefore, report all counts as colony-forming units. Include in the report the method used, the incubation temperature and time, and the medium. For example: CFU/mL, pour plate method, 35°C/48 h, plate count agar.

To compute the heterotrophic plate count for pour plate, spread plate, and membrane filter methods, CFU/mL, divide the total number of colonies or average number (if duplicate plates of the same dilution) per plate by the sample volume. For the enzyme substrate method, use the MPN obtained from the MPN tables, adjusted for sample dilution. Record sample volumes used and number of colonies on each plate counted or estimated.

When colonies on duplicate plates and/or consecutive dilutions are counted and results are averaged before being recorded, round off counts to two significant figures only when converting to colony-forming units.

Avoid creating fictitious precision and accuracy when computing colony-forming units by recording only the first two left-hand digits. Raise the second digit to the next higher number when the third digit from the left is 5, 6, 7, 8, or 9; use zeros for each successive digit toward the right from the second digit. For example, report a count of 142 as 140 and a count of 155 as 160, but report a count of 35 as 35.

10. Analytical Bias

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

9215 B. Pour Plate Method

1. Samples and Sample Preparation

See 9215A.4 and 5.

2. Sample Dilution

Prepare water used for dilution blanks as directed in Section 9050C.

a. Selecting dilutions: If possible (e.g., on the basis of historical information), select the dilutions so at least one plate in a series will contain 30 to 300 CFU (Figure 9215:1). For example, where a heterotrophic plate count as high as 3000 is suspected, prepare plates with 10^{-2} dilution.

For most potable water samples, plates suitable for counting will be obtained by plating 1 mL and 0.1 mL undiluted sample and 1 mL of the 10^{-2} dilution.

b. Measuring sample portions: Use a sterile pipet for initial and subsequent transfers from each container. If pipet becomes contaminated before transfers are completed, replace with a sterile pipet. Use a separate pipet for transfers from each different dilution. Do not prepare dilutions and pour plates in direct sunlight. Use caution when removing sterile pipets from the container; to avoid contamination, do not drag pipet tip across exposed ends of pipets in the pipet container or across lips and necks of dilution bottles. When removing sample, do not insert pipets more than 2 to 3 cm below the surface of the same or dilution.

c. Measuring dilutions: When discharging sample portions, hold pipet at an angle of about 45° with tip touching bottom of petri dish or inside neck of dilution bottle. Lift cover of petri dish just high enough to insert pipet. Allow 2 to 4 s for liquid to drain from 1-mL graduation mark to tip of pipet. If pipet is not a blow-out type, touch tip of pipet *once* against a dry spot on petri dish bottom. Less preferably, use a cotton-plugged blow-out type pipet and a pipet bulb and gently blow out remaining volume of sample dilution. When 0.1-mL quantities are measured, let diluted sample drain from chosen reference graduation until 0.1 mL has been delivered. Remove pipet without touching it to dish. Pipet 1 mL, 0.1 mL, or other suitable volume into a sterile petri dish before adding melted, tempered culture medium. Use decimal dilutions in preparing sample volumes of less than 0.1 mL. In examining sewage or turbid water, do not measure a 0.1-mL inoculum of original sample, but prepare an appropriate dilution. Prepare at least two replicate plates for each volume of sample or dilution examined. After depositing test portions for each series of plates, pour culture medium and mix carefully. Do not let more than 20 min elapse between starting pipetting and pouring plates.

3. Plating

a. Melting medium: Melt sterile solid agar medium in boiling water or by exposure to flowing steam in a partially closed container, but avoid prolonged exposure to unnecessarily high temperatures during and after melting. Do not resterilize plating medium. If the medium is melted in two or more batches, use all of each batch in order of melting, provided that the contents remain fully melted. Discard melted agar that contains precipitate.

Maintain melted medium in a water bath between 44 and 46°C until used, preferably no longer than 3 h. In a separate container place a thermometer in water or medium that has been exposed to the same heating and cooling as the plating medium. Do not depend on the sense of touch to indicate proper medium temperature when pouring agar.

Use plate count agar, R2A agar, or NWRI agar as specified in 9215A.6.

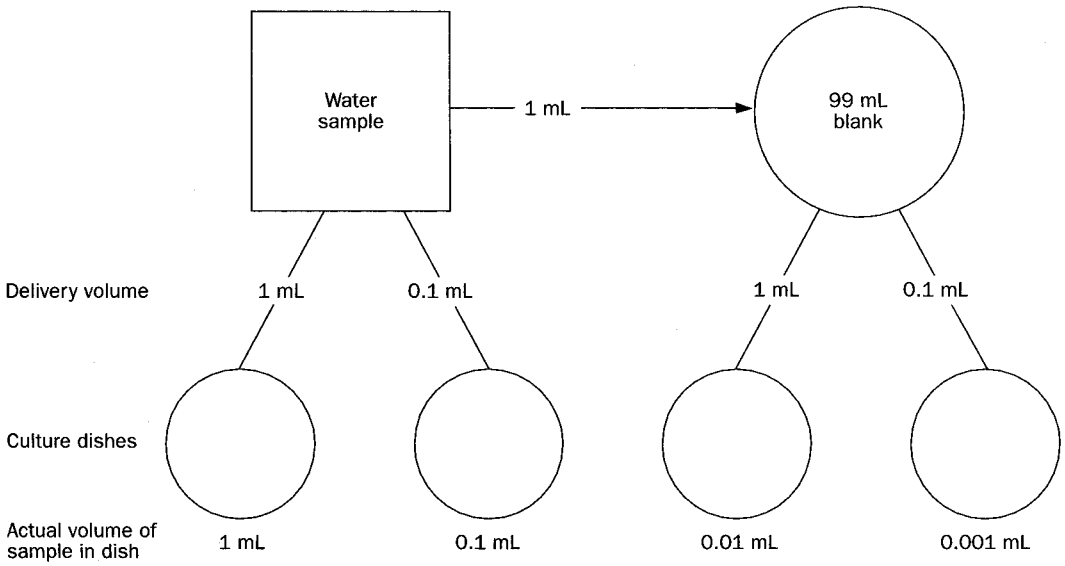

Figure 9215:1. Preparation of dilutions

b. Pouring plates: Limit the number of samples to be plated in any one series so no more than 20 min (preferably 10 min) elapse between dilution of the first sample and pouring of the last plate in the series. Pour at least 10 to 12 mL liquefied medium maintained at 44 to 46°C in each dish by gently lifting cover just high enough to pour. Carefully avoid spilling medium on outside of dish lid when pouring. When pouring agar from flasks or tubes that have been held in a water bath, wipe with clean paper towel and flame before pouring. As each plate is poured mix melted medium thoroughly with test portions in petri dish, taking care not to splash mixture over the edge, by rotating the dish first in one direction and then in the opposite direction, or by rotating and tilting. Let plates solidify (within 10 min) on a level surface. After medium solidifies, invert plates and place in incubator.

c. Sterility controls: Check sterility of medium and dilution water blanks by pouring control plates for each series of samples. Prepare additional controls to determine contamination of plates, pipets, and room air.

4. Incubation

See 9215A.7.

5. Counting, Recording, Computing, and Reporting

See 9215A.8 and 9.

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9215 C. Spread Plate Method

1. Samples and Sample Preparations

See Sections 9215A.4 and 5.

2. Laboratory Apparatus

a. Glass rods: Bend 4-mm-diam, fire-polished glass rods, 200 mm in length, 45° about 40 mm from one end. Sterilize before using.

b. Pipet: 1.0-mL glass or plastic pipets.

c. Turntable (optional).*

d. Incubator or drying oven, set at 42°C, or laminar-flow hood.

* Fisher Scientific, hand operated, No. 08-758 or Lab-Line, motor driven, No. 1580, or equivalent.

3. Media

See 9215A.6*a*, *c*, and *d*. If R2A agar is used, best results are obtained at 28°C with 5 to 7 d incubation; if NWRI agar is used, incubate at 20°C for 7 d.

4. Preparation of Plates

Pour 15 mL of the desired medium into sterile 100- × 15-mm or 90- × 15-mm petri dishes; let agar solidify. Predry plates inverted so there is a 2- to 3-g water loss overnight with lids on. See Figure 9215:2, Table 9215:I, or Figure 9215:3. Use predried plates immediately after drying or store for 2 weeks in sealed plastic bags at 4°C. For predrying and using plates the same day, pour 25 mL agar into petri dish and dry in a laminar-flow hood at room temperature (24 to 26°C) with the lid off to obtain the desired 2- to 3-g weight loss. See Figure 9215:3.

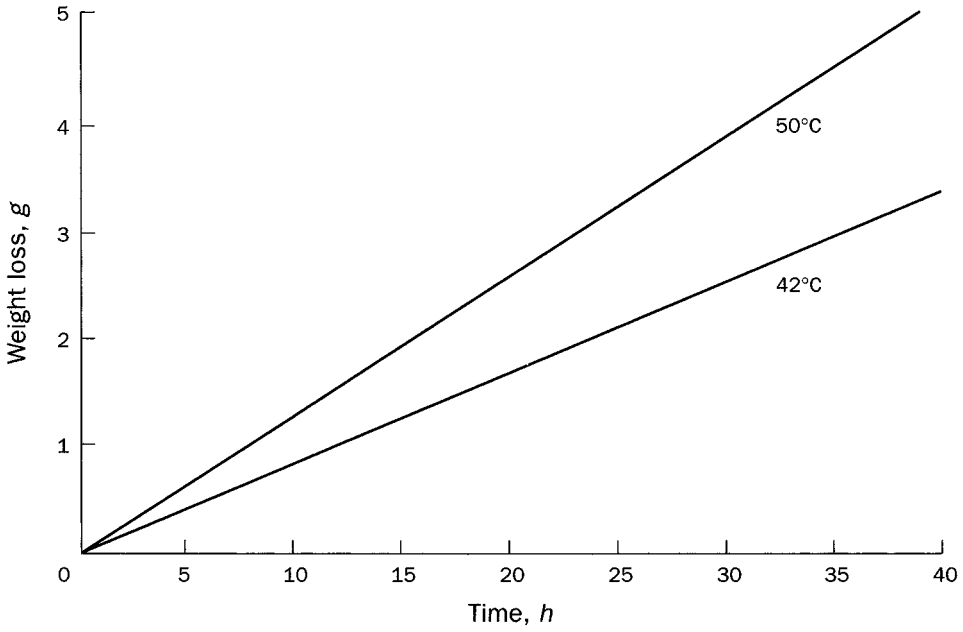

Figure 9215:2. Drying weight loss of 15-mL agar plates stored separately, inverted with lids on. SOURCE: Unpublished data. Water Purification Lab., Chicago Dep. Water.

5. Procedure

Prepare sample dilutions as directed in 9215B.2.

a. Glass rod: Pipet 0.1 or 0.5 mL sample onto surface of a predried agar plate. Using a sterile bent glass rod, distribute inoculum over surface of the medium by rotating plate by hand or on a turntable. Let inoculum be absorbed completely into the medium before incubating.

b. Pipet: Pipet desired sample volume (0.1, 0.5 mL) onto the surface of the predried agar plate while plate is being rotated on a turntable. Slowly release sample from pipet while making one to-and-fro motion, starting at center of plate and stopping 0.5 cm from plate edge before returning to center. Lightly touch the pipet to plate surface. Let inoculum absorb completely into medium before inverting and incubating.

6. Incubation

See 9215A.7.

7. Counting, Recording, Computing, and Reporting

See 9215A.8 and 9.

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TABLE 9215:I. EFFECT OF TEMPERATURE OF DRYING ON WEIGHT LOSS OF 15-mL AGAR PLATES STORED SEPARATELY*

Temp.	Time for Plates to Lose 1 to 4 g of Water (Avg. for 5 Plates) *h*							
	Plates Inverted with Lids on				Plates Inverted with Lids Removed			
°C	1 g	2 g	3 g	4 g	1 g	2 g	3 g	4 g
24	32	64	95	125	3.7	7.0	10.5	14.0
37	17	35	51	67	1.7	3.5	5.3	7.0
50	6	12	18	24	0.7	1.3	1.9	2.7
60	4	8	12	16	—	—	—	—

* Referenced in Canada Centre for Inland Waters Manual, Burlington, Ont.

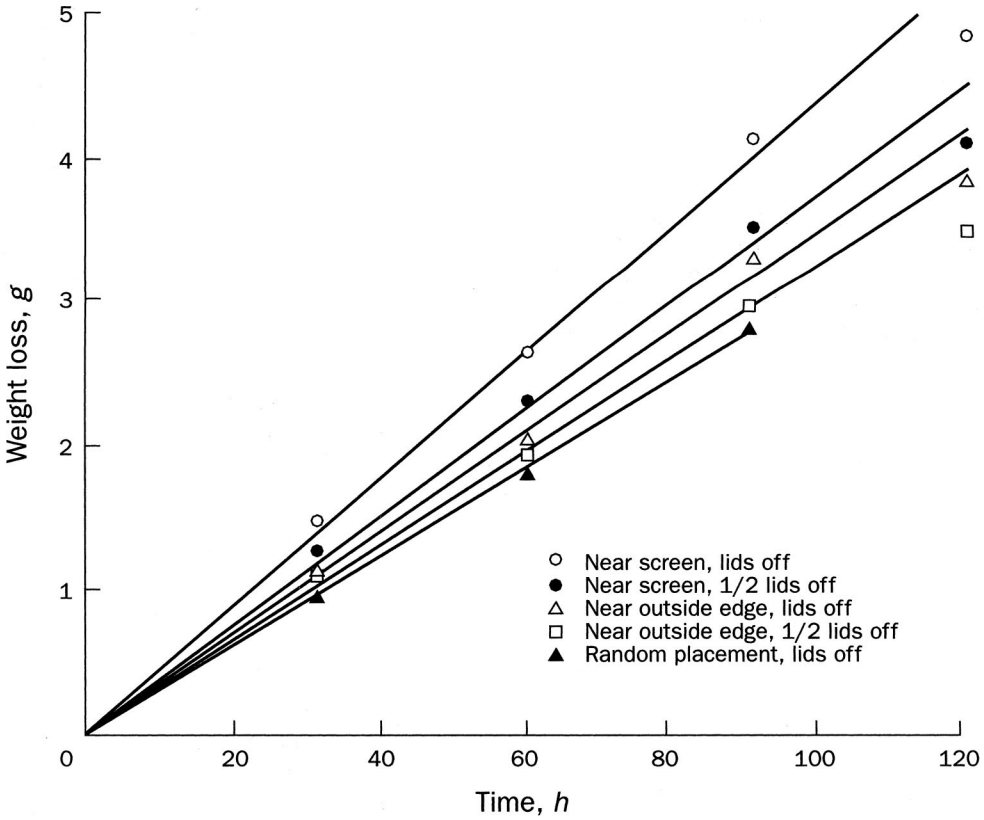

Figure 9215:3. Weight loss of 25-mL agar plates (100 × 15 mm) dried separately in a laminar-flow hood at room temperature (24 to 26°C), relative humidity (30 to 33%), and air velocity 0.6 m/s. SOURCE: Unpublished data. Alberta Environmental Centre, Vegreville, Alta.

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9215 D. Membrane Filter Method

1. Samples and Sample Preparation

See 9215A.4 and 5.

2. Laboratory Apparatus

See Section 9222B.1.

3. Media

See 9215A.6. Use m-HPC agar or, alternatively, R2A or NWRI agar.

4. Preparation of Plates

Dispense 5-mL portions of sterile medium* into 50- × 9-mm petri dishes. Let solidify at room temperature. Prepared plates may be stored inverted in a plastic box or tight container in a refrigerator for no longer than 2 weeks.

* m-HPC agar may not be sterile.

5. Sample Size

The volume to be filtered will vary with the sample. Select a maximum sample size to give 20 to 200 CFU per filter.

6. Procedure

Filter appropriate volume through a sterile 47-mm, 0.45-μm-pore-diam, gridded membrane filter, under partial vacuum. Rinse funnel with three 20- to 30-mL portions of sterile dilution water. Place filter on agar in petri dish.

7. Incubation

Place dishes in close-fitting box or plastic bag containing moistened paper towels. Incubate at 35 ± 0.5°C for 48 h if using m-HPC agar, or longer if using R2A medium, or at 20 to 28°C for 5 to 7 d if using NWRI or R2A agar. Duplicate plates may be incubated for other time and temperature conditions as desired.

8. Counting, Recording, Computing, and Reporting

See 9215A.8 and 9. Report as CFU/mL, membrane filter method, time, and medium.

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9215 E. Enzyme Substrate Method

1. Samples and Sample Preparation

See Section 9215A.4 and 5.

2. Laboratory Apparatus

a. Pipets, 0.1-, 1.0-, and 10-mL, sterile, graduated, glass or plastic.

b. Incubator set at 35 ± 0.5°C.

c. Ultraviolet light source, long wavelength, 6-W, 365 nm.

d. Sample plates.*

3. Medium

The formulation is available commercially† in sterile medium vessels for 100-mL multi-dose procedures or is available in test tubes for 10-mL unit-dose procedures. Store medium between 2 and 25°C. The product has a shelf life of up to 12 months from date of manufacture.

Test each lot for sterility by following the inoculation procedure (¶ 5 below), using 10 mL rehydrated medium without sample. Incubate for 48 h at 35°C. No wells should fluoresce after incubation.

4. Sample Diluent

Use either sterile deionized water, distilled water, buffered water, or 0.1% peptone.

5. Procedure

Rehydrate medium by filling medium vessel to 100-mL mark with sterile diluent, re-capping, and shaking until medium has dissolved. Aseptically pipet 1.0 mL sample and 9 mL of rehydrated medium onto the center of the sample plate. Alternatively, aseptically pipet 0.1 mL sample and then 9.9 mL rehydrated medium onto center of the sample plate. NOTE: Final volume of sample plus medium must be 10 ± 0.2 mL. Cover plate with lid and gently swirl to distribute mixture into wells. Tip plate 90° to 120° to drain excess mixture into the absorbent pad. Invert plate and incubate for 48 h (range of 45 to 72 h) at 35°C. If a sample is suspected of having a count greater than 738 CFU/mL, dilute by adding 1 mL sample to a sterile vessel containing 99 mL sterile diluent. Make additional dilutions as required to keep counts below 738 CFU/mL in final dilution.

6. Counting, Recording, Computing, and Reporting

After incubation, examine plate for fluorescent wells. Count number of fluorescent wells by holding a 6-W, 365-nm UV light about 13 cm above plate. Preferably use UV-filtering laboratory safety glasses during counting. Count number of wells exhibiting blue fluorescence.

Use MPN chart (provided by the medium manufacturer) to calculate MPN/mL. Adjust MPN to reflect sample volume and/or dilution made to yield a corrected MPN value. Record as MPN/mL.

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* SimPlates®, IDEXX Laboratories, Westbrook, ME, or equivalent.

† IDEXX Laboratories, Westbrook, ME.