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QUALITIES CONTROL METHOD - 1

Count of Aerobic Microorganisms
& Spore Count

Description:

The total plate count method is used to determine the number of aerobic and facultative anaerobic bacteria present in a sample. Aerobic bacteria metabolically require oxygen while facultative anaerobic bacteria are able to metabolically utilize oxygen when it is available. A trained laboratory technician should be employed to utilize this procedure.

Notes About this Procedure

Most laboratory cultures and environmental samples contain thousands to billions of cells per ml or gram. The sample must be diluted before it is plated. Serial dilution is the method to sequentially dilute a culture through a series of sterile dilution blanks. Sampling error usually occurs because of an unequal distribution of cells in the culture or dilution fluid; the goal is to distribute the cells evenly by thorough mixing and then to obtain a representative sample in the pipette. Technical error is most often due to some inaccuracy in preparing dilution blanks or in pipetting technique. Error can be minimized by precise measurements in preparing blanks and by accurate pipetting technique, but is impossible to avoid all error. Serial dilution and plating are at best an estimate of the number of live organisms present in the sample. The 95% confidence limit for pour plates containing between 15 and 300 colonies is ± 12% to ± 37%[1]. In practice, even greater variation may be found especially among results obtained by different microbiologists. See also published precision parameters from collaborative AOAC studies[2].

Equipment:

4 dilution bottles per sample prepared as follows:

Use Weber Scientific DB (#3127-25) dilution bottle with 99 ml in a 125 ml bottle. Alternatively use bottles that hold at least 125 ml, with each containing 99 ml of BOD phosphate (with magnesium sulfate) dilution water that has been sterilized.

2 dilution tubes per sample prepared as follows:

Using an Eppendorf Repeater Pipettor with Biopur Sterile 50 ml Combitip, with setting of 5 and then of 4, add 9 ml from Weber Scientific DB #3127-55 dilution bottles, to each 12 ml sterile disposable test tube with snap or screw caps. For salt-tolerant strains, you cansubstitute BBL Phosphate Buffered Saline (PBS) tubes, catalog #297486. Valerie keeps these in stock in her personal research laboratory.

1 dilution test tube per sample (for spore count) prepared as detailed in 1.3 below (can substitute PSB tube for salt-tolerant *Bacillus*)

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1 wrist-action shaker capable of agitating 50-60 times per minute (*Burrell* Wrist-Action shaker preferred by AMC)
(If shaker is not available, dilution bottles must be shaken 50 times by hand before transfer or plating)

1 autoclave or steam sterilizer

1 Vortex mixer (*Fisher* Vortex Genie 2 preferred by AMC)

1 low speed blender (*Waring* 7 speed model recommended with sterile 250 ml stainless steel "semi-micro" jar)

3 sterile petri dishes per sample, per dilution to be tested (total of 18 plates for most products)

2 adjustable air displacement pipettors (100 µl to 1000 µl) or 2 fixed pipettors, one at 100 µl and one at 1000 µl.

Sterile 1 ml (1000 µl) pipet tips (preferably aerosol resistant, known as ART or filter tip)

Sterile 0.1 ml (100 µl) pipet tips (preferably aerosol resistant, known as ART or filter tip)

Eppendorf Repeater Pipettor with Biopur Sterile 50 ml Combitip

A 10 ml sterile measuring cylinder or a 10 or 12 cc sterile disposable syringe

A 100 ml measuring cylinder

1 Analytical chemical balance (accurate to 0.0001 g)

1 *BioWorld* inoculating turntable(optional)

1 Quebec Darkfield microorganism colony counter

1 *Bel-Art* Products hand-held colony counter (to use with Quebec counter) - with black or red ink
Note: If your Quebec counter is an electronic version with a probe, you do not need this

Steri-Wrap® II (green, not blue) We order 20" x 20" size and cut to needed dimensions.

Rapid-Flo double gauze faced milk filter disks from *Filter Fabrics Incorporated*

Individually wrapped isopropyl alcohol swabs

Reusable glass bacterial spreaders or sterile disposable spreaders

A sterile scalpel to enlarge tip of pipettor, for a dry product only

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Solutions:

Stock buffer solution - Dissolve 34 grams monobasic potassium phosphate (KH_2PO_4 - certified pure) in 500 ml distilled water. Adjust with 1N NaOH to pH of 7.2. Dilute with distilled water to 1 liter (as recommended in *Standard Methods for Examination of Water and Sewage*).

Phosphate dilution water - Add 1.25 ml stock buffer solution to one (1) liter of aerated distilled water. Or use Weber #3126-73 magnesium chloride, phosphate buffer solution for the 99 ml (1:100 dilution) or the #3126-63 phosphate buffer for the 90 ml (1:10 dilution) 1-800-328-8378. Weber Scientific in Hamilton, NJ

Media - Prepared sterile culture plates may be obtained from Hardy Diagnostics or Culture Media & Supplies, for Tryptic Soy Agar, Plate Count Agar or Standard Methods agar. If the bacterial product is listed by us as HIGHLY salt tolerant or if the formulation lists the following *Bacillus* strains: **AMH 100, AMH 102, AMH 103, AMH 104, AMH 105, AMH 106, AMH 107, AMH 108, AMH 109, AMH 114, AMH 115, AMH 116, AMH 118** and/or **AMH 119**, either use QC-22 (TSA) or else supplement QC-24 (PCA) or QC-16 (SMA) with 0.5% NaCl, to prevent under-reporting of calculated total count.

Procedure:

It is often important to know how many live microorganisms are present in a culture. Microbes are alive (viable) if they are capable of forming colonies on a suitable solid medium. Therefore, a viable number is that number of cells capable of division on a solid medium. It is possible for an organism to be alive and not be capable of division, especially if the essential nutrients for division are missing or the cells are injured. Ideally one organism forms one colony, but cells may stick together to form chains and clumps and they too will form a colony. It is for reasons such as these that microbiologists refer to colony-forming units (CFU) rather than to viable numbers when describing numbers.

1 BASIC LABORATORY PRECAUTIONS

The following information is provided to minimize the errors described above and improve reproducibility.

1.1 Observe good aseptic technique

1.1.1 Disinfect bench top work area before and after use with germicide.

1.1.2 Wipe outside of air-displacement pipetter before use with individually wrapped isopropyl alcohol swabs.

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1.1.3 Flame lips of glass tubes and bottles before and after use.

1.1.4 Avoid touching the sides of the dilution bottles and tubes with the pipetter.

1.1.5 Raise lids of petri dishes only high enough to dispense a sample onto agar, except when spreading.

1.1.6 Keep lids on sterile pipet tip racks when not in use.

1.1.7 Use individually wrapped, disposable spreaders or clean a reusable glass or steel spreader as follows: Clean glass spreaders after each use by soaking and scrubbing each in cleaning solution. Rinse each spreader and wipe the surface of each spreader. Wrap several spreaders in Steri-Wrap II or equivalent (or autoclave bags that are first heat sealed) and sterilize spreaders by autoclaving at 121°C (249°F) for at least 30 minutes.

1.2 Pipettor Use

1.2.1 Confirm pipet calibrations before each use. Adjust 1000 µl pipets so that successive deliveries of deionized water dispensed with the same tip on an analytical balance each weigh 1.00 ± 0.01g. Adjust 100 µl pipet so that 3 to 10 successive deliveries of deionized water dispensed with the same tip on an analytical balance each weigh 0.100± 0.001g.

1.2.2 Do not wash pipet tips out except in the case of viscous liquids. Viscous liquids should preferably be measured by weight (i.e., use 1 gram of a sample instead of 1 ml).

1.2.3 Do not insert the pipet tip into the liquid of the next dilution when dispensing to avoid excess sample carry over from the outside of the tip.

1.2.4 Use a separate tip for preparing each dilution.

1.2.5 Use the same tip to dispense 0.1 ml (100 µl) of the same dilution onto the agar surface of each triplicate. The same tip may be used for plating multiple dilutions of the same product, as long as the more dilute solutions are plated before less dilute solutions.

1.2.6 Avoid pipetting foam. Allow most air bubbles to rise to the surface, then insert the pipet tip close to the bottom of the vessel to obtain the sample. Mix samples by vortexing whenever possible.

1.2.7 Use sterile Aerosol Resistant Tips (ART) whenever possible to prevent the internal contamination of the pipettor and minimize cross contamination.

1.3 Total and spore counts

1.3.1 Use same dilution series for total and spore count.

1.3.2 Perform total count before spore count. The heat treatment kills vegetative cells and only heat resistant spores capable of surviving the 80°C (176°F) remain.

1.3.3 Perform spore count heat shock only in dilution tubes and not bottles. If the platable dilution is in a dilution bottle, approximately 10 ml from the dilution bottle

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should be aseptically transferred to a sterile, empty dilution tube to perform the heat shock. The temperature of 10 ml in a test tube will easily reach 80°C (176°F) in a very short time, whereas the temperature of 99 ml in a dilution bottle may not reach 80°C (176°F) even after 10 minutes.

1.3.4 Petri dishes are normally stored refrigerated. Allow the plates to warm to room temperature and the surface to partially dry before use.

1.3.5 Use Standard Methods Agar for non-selective total and spore counts. Use other plating media as required for selective counts.

1.3.6 Use sterile phosphate buffer (Standard Methods for Water and Wastewater) as the diluent (9 ± 0.2 ml and 99 ± 2.0 ml quantities) or use Weber Phosphate dilution bottle #3127-25 for the 99 ml volume and dispense 9 ml using the Eppendorf Repeater pipettor with sterile 50 ml Combi-Tip (use setting 5 and then 4 to obtain 9 ml in the test tube). Alternatively, you may use Weber #3127-55 for 90 ml ± 2 ml for the same dilution as 9 ml in a test tube. Use other dilution volumes as needed.

1.3.7 Use triplicate plates at each platable dilution to improve accuracy.

1.3.8 Ideally, samples should be diluted so that 25 to 250 colonies per plate are obtained.

1.4 Mixing

1.4.1 Mix dilution bottles by shaking vigorously 25 times in about a 30 cm arc in approximately 7 seconds.

1.4.2 Mix dilution tubes by vortexing for about 10 seconds on a vortex mixer at a setting of 7 or above.

1.4.3 Mix original liquid samples containing surfactants by vortexing and not shaking to minimize foam production.

1.4.4 Use low speed blending (in a Waring blender with sterile semi-micro SS jar), sonication, glass beads, bead beating, surfactant diluent and/or other techniques as needed to break up clumps.

2 STANDARD PLATE COUNT PROCEDURE (TOTAL COUNT)

2.1 Blend ultra-filtered liquid spore intermediates 45 seconds. Modify blending as needed to optimize count. Skip this step if dry product or other liquid products.

2.2 Measure 1± .01g (dry or viscous liquid samples) or 1± 0.01 ml (liquid samples) of sample and transfer to 99 ml sterile phosphate buffer (= 10^{-2} dilution).

2.3 If testing a **dry product**, rehydrate for at least 30 minutes at room temperature while shaking at 100-200 rpm. Transfer contents of first dilution bottle to sanitized 360 ml blender jar and blend on low speed. Blend dry spore intermediates for 90 seconds, but blend all other dry products for 15 seconds. **Skip this step for liquid product samples**. Modify blending as needed to optimize count.

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2.4 Transfer 1 ml to bottle containing 99 ml of sterile phosphate buffer and to a tube containing 9 ml of sterile phosphate buffer, using a 1000 µl pipetter (if dry product use a pipet tip with the tip enlarged so it will not clog). If test tubes with 9 ml of sterile buffer are not available, instead transfer 10 ml to bottles containing 90 ml of Weber #3127-55 sterile phosphate buffer, by using a sterile, individually wrapped 10 ml pipette with appropriate suction bulb or Pipet-Aid. For salt tolerant strains, BBL Phosphate buffered saline (PSB) tubes can be substituted for a standard phosphate buffer, for salt-tolerant strains, since these are stocked in Alken-Murray's research laboratory.

2.5 Mix thoroughly.

2.6 Prepare additional 1:10 or 1:100 dilutions as needed so that ultimately 25 to 250 colonies per plate are obtained.

2.7 Transfer in triplicate 0.1 ml from the appropriate plating dilutions to the surface of each petri dish containing appropriate plating medium using a 100 µl pipettor.

2.8 Spread the 0.1 ml over the agar surface of each plate using a pre-sterilized spreader by placing the petri dish on the inoculating turntable, placing the spreader on the agar and spinning the turntable until the sample has been uniformly distributed on the surface of the agar. Only several revolutions are required and spreading to dryness is no longer deemed necessary. Use the same spreader on all three plates of the same dilution, for the same product. To spread several dilutions, use one spreader starting with the more dilute sample first and proceeding to the less dilute. **Note that the transfer of 0.1 ml to the plate constitutes an additional 10x dilution.**

2.9 Incubate plates inverted at 35± 2° C (95°F) for 18-24 hours. Additional incubation time is sometimes required, especially for some Gram-negative strains. Certain products with a known lower heat tolerance, may be incubated instead at 30± 2° C.

3 STANDARD PLATE COUNT PROCEDURE (SPORE COUNT)

3.1 Prepare dilutions as described above.

3.2 Complete total count first (if required), then heat the appropriate platable dilutions in test tubes @ 80°C (176°F) for 10 minutes.

3.3 Allow samples to cool to room temperature.

3.4 Plate the samples as described above.

4 COUNTING COLONIES FOR STANDARD PLATE COUNT[3]

4.1 When computing the count, report only the first two significant digits to avoid creating a fictitious impression of precision and accuracy. Round off calculated results to 2 significant figures by raising the second digit to the next highest number when the third digit is 6, 7, 8 or 9, and by rounding down when the third digit is 1, 2, 3 or 4. When the third digit is 5, round up if the second digit is odd and round down when the second digit is even. Use zeroes for each successive digit toward the right from the second digit or scientific notation.

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Calculated	Actual	Scientific notation
12,700	13,000	1.3×10^4
12,400	12,000	1.2×10^4
15,500	16,000	1.6×10^4
14,500	14,000	1.4×10^4

4.2 Plates with 25 to 250 colonies:

Calculate the count as follows: $N = \Sigma C /[(1 \times n1) + (0.1 \times n2)]d$,
Where N = number of colonies per ml or gram of product,
ΣC = sum of all colonies on all plates counted
n1 = number of plates in lower dilution counted,
n2 = number of plates in next higher dilution counted
d = dilution from which the first counts were obtained.
Example
10^{-7}= 232, 244, 220 and 10^{-8} = 33, 28, 30
$N = (232+244+220+33+28+30)/[(1x 3)+(0.1 \times 3)]10^{-7} = 2.4 \times 10^9$

4.3 When counts of triplicate plates fall both within and outside the 25 to 250 colony range, use only those counts that fall within this range.

4.4 If all plates have fewer than 25 or greater than 250 colonies, the sample should be retested if possible. If replating is not feasible, use the following guidelines to estimate the count.

4.5 All plates with fewer than 25 colonies:

When plates from two dilutions yield fewer than 25 colonies each, record the actual count and record the count as less than 25 times d/1, where d/1 is the reciprocal of the dilution factor from which the first counts were obtained.

4.6 All plates with more than 250 colonies:

When plates from two dilutions yield more than 250 colonies each (but fewer than 100 per square centimeter), estimate counts from the plates nearest 250 and multiply by the reciprocal of the dilution (d/1).

4.7 All plates with more than an average of 100 colonies per square centimeter:

Estimate the count as greater than 100 times the highest dilution plated times the area of the plate.

4.8 Questionable petri dish counts that appear to be outliers may be statistically eliminated using following formula:

$$\frac{\text{Questionable colony count - average}}{\text{result standard deviation (n-1 weighting)}}$$

For three petri dish counts, if the result is greater than 1.148, 1.153 or 1.155 the outlier can be rejected at 90, 95 or 99% significance levels, [4]respectively.

4.9 Record the results in the appropriate QC database log. Dry products are usually reported to two significant figures times 10^9 CFU/ gram and liquid products are usually reported to two significant figures times 10^7 through 10^9 CFU/ ml, the latter found only for *Bacillus* manufacturing concentrates.

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References:

1. ISO procedure 4833:1991 (E), "Microbiology - General guidance for the enumeration of microorganism - colony count technique at 30°C"
2. AOAC Official Methods of Analysis and BioControl Systems, Including instruct., copyright 2006
3. American Public Health Association, copyright 1993, Standard Methods for the Examination of Dairy Products, 16th ed. APHA, Washington, D.C.
4. American Public Health Association, American Waterworks Association and Water Environment Federation, copyright 2003, Standard Methods for the Examination of Water and Wastewater, 21st ed., APHA, AWWA & WEF, Washington, D.C.