

STANDARD OPERATING PROCEDURE

For

SM 9215B

Heterotrophic Plate Count - Pour Plate Procedure

SOP #: SM 9215B

SOP REVISION #: 1.7 DATE: November 2010

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LIST OF REVISIONS

Rev. #	Date	Description of Revision	Page #
0	February 2001	None	
1.0	February 2003	Numerous edits/updates	Throughout document
1.2	September 2005	Table 1 added – QC elements, acceptance criteria, and corrective actions	10
1.3	December 2006	Replaced old DEP Logo with state seal + MassDEP	Title page & header
1.4	May 2007	Section 9.3 & Table 1 – “Negative Control” changed to “Method Blank” Section 9.5 added – Required to monitor incubator humidity Section 9.6 added – Required comparative colony counts among 2 or more analysts	6 & 10 7 7
1.5	May 2008	Section 11 – Added note that method must temporarily be performed in a biological safety cabinet due to airborne contamination caused by building construction Minor revisions	7 Throughout document
1.6	January 2010	Section 8.4: $\leq 10^{\circ}\text{C}$ changed to $1 - 8^{\circ}\text{C}$ Section 9.2 added Sections 9.2 through 9.6 renumbered to 9.3 through 9.7 Section 12.7 – “Calculation or Precision QD Criterion/Range of Logs” added Reference 16.2 added	6 6 6 & 7 8 & 9 9
1.7	November 2010	Section 11 – Removed note dealing with background contamination during construction activities Section 12.7 – Renumbered to Section 12.8 and revised New Section 12.7 – Amended how range of logs is calculated	



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TABLE 1. QUALITY CONTROL ELEMENTS, ACCEPTANCE LIMITS, AND CORRECTIVE ACTIONS FOR THE ANALYSIS OF HETEROTROPHIC PLATE COUNT BY SM 9215B POUR PLATE PROCEDURE	10
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1.0 SCOPE & APPLICATION

- 1.1 The heterotrophic plate count (HPC) pour plate procedure is utilized to estimate the number of live heterotrophic bacteria in water.

2.0 SUMMARY OF METHOD

- 2.1 A volume of sample (0.1 mL or 1.0 mL) is aseptically pipetted into a Petri dish to which tempered plate count agar (PCA) is added and then gently mixed, allowed to cool, and incubated for 48 hours at $35^{\circ}\pm 0.5^{\circ}\text{C}$. All colonies are counted following incubation.

3.0 DEFINITIONS

- 3.1 When using the pour plate procedure, heterotrophic bacteria are considered to be any bacteria that grow on PCA within 48 hours at $35 \pm 0.5^{\circ}\text{C}$.

4.0 INTERFERENCES

- 4.1 This method is subject to interferences from the environment in which analyses are performed. Care must be taken to ensure elevated counts do not occur due to poor laboratory technique or exposure of plates and samples to sources of contamination such as airflow from an open window or air conditioning vent.

5.0 SAFETY

- 5.1 Samples may contain organisms that are pathogenic to humans. All precautions are to be taken to minimize exposure. All personnel are to wear lab coats, safety glasses, and protective gloves while working in the laboratory.

6.0 EQUIPMENT AND SUPPLIES

- 6.1 Colony counter: Use the Quebec dark-field colony counter or a similar counter with an equivalent magnification (1.5 x).
- 6.2 Incubator: Use an incubator that is capable of maintaining a temperature of $35^{\circ}\pm 0.5^{\circ}\text{C}$.
- 6.3 Petri dishes: Use disposable plastic 100- x 15-mm Petri dishes that are free of bubbles and scratches.
- 6.4 Pipettes: Use sterile pipettes of the appropriate size having graduations clearly marked. Make sure that the tips are not cracked or distorted in any way. The error of calibration must not exceed 2.5% for each lot of pipettes.

7.0 Medium

- 7.1 Preparation of Plate Count Agar (i.e., PCA – tryptone glucose yeast extract agar): Use commercial dehydrated media. Follow manufacturer's directions on the bottle for re-hydration. Store opened supplies of dehydrated media in a desiccator for up to one year.

Tryptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g



Agar	15.0 g
Reagent-grade water	1 L

- 7.1.1 In a 2-L Erlenmeyer flask, add 23.5 grams of PCA dehydrated commercial product to 1 L of reagent water, mix thoroughly, and heat using a hot plate with constant stirring to dissolve. Dispense into test tubes or screw-capped flasks. Cover the test tubes/flasks and sterilize for 15 minutes at 121°C. The final pH should be 7.0 ± 0.2 .
- 7.1.2 Make sure to temper the agar in a 44-46°C water bath prior to use. Ensure agar has reached the proper temperature by the use of a temperature blank. Agar may not be tempered for longer than 3 hours.
- 7.1.3 If not for immediate use, tighten the caps once the medium has cooled, make sure that the dates of preparation and expiration are noted on the medium, and refrigerate. Screw-capped medium may be stored in the refrigerator for 3 months. Agar must be melted in the microwave and then tempered as above, prior to use. Once melted, agar must be used or discarded.

8.0 SAMPLE COLLECTION, PRESERVATION AND STORAGE

- 8.1 Samples must be aseptically collected in a sterile disposable plastic bottle leaving at least 1" (2.5 cm) of headspace to allow for sufficient mixing of the sample prior to analysis. If the sample is chlorinated, make sure that the appropriate concentration of sodium thiosulfate is added (see 6.1)
- 8.2 Remove all attachments from the water tap (screens, etc.). If tap cleanliness is in question, apply a solution of sodium hypochlorite (100 mg NaOCl/L) to faucet. Open tap and let run to waste for 2-3 minutes. Reduce the water flow to allow for filling of the bottle without splashing.
- 8.3 Keep sample bottle closed until it is to be filled; fill the bottle without rinsing; replace cap immediately; and secure the top with the attached plastic "lock".
- 8.4 Samples must be kept at 1 - 8°C from the time of collection to the time of analysis.
- 8.5 Drinking water source samples must be analyzed within a maximum of 8 hours from collection (i.e., maximum of 6 hours in the field and 2 hours in the laboratory). To the extent possible, all other samples shall also be analyzed within a maximum of 8 hours from collection (i.e., maximum of 6 hours in the field and 2 hours in the laboratory) but never longer than 24 hours after collection.

Note: 40 CFR Part 141.74 requires that drinking water source samples be analyzed within 8 hours of collection.

- 8.6 All samples must be accompanied by a sample tracking sheet/chain-of-custody form filled out by the collector.

9.0 QUALITY CONTROL

- 9.1 Test each new lot of medium against a previously acceptable lot for satisfactory performance.
- 9.2 Test each new batch of media against a positive control (*E. coli*) and test media sterility with a blank control.



- 9.3 Run each dilution in duplicate and report average count.
- 9.4 With each batch of 20 or fewer samples, run a method blank plate containing only PCA to ensure the sterility of the batch of media. This plate must be poured after the samples, exposing the empty plate to air for a minimum amount of time.
- 9.5 With each batch of samples, prepare an air blank by exposing an empty Petri dish to the same conditions as the Petri dishes used for samples. Add PCA to this plate at the same time the agar is added to the samples.
- 9.6 With each batch of samples, monitor the humidity of the incubator as follows. Weigh the method blank plate (containing medium) before and after incubation to ensure that the percent humidity loss is less than 15%. Record the data in the WES Microbiology Laboratory's Monthly QC Form.
- 9.7 A colony count comparison between at least 2 analysts must be performed on an HPC plate each month that samples are received by the laboratory for HPC testing. The colony counts must be within 10% and results documented in the WES Microbiology Laboratory's Monthly QC Form.

10.0 CALIBRATION AND STANDARDIZATION

Not applicable. Refer to Laboratory QA Plan for the calibration and standardization of laboratory equipment used for this analysis.

11.0 PROCEDURE

- 11.1 Select a volume that will yield between 30-300 colonies on a plate (typically plate 0.1 and 1.0 mL in order to fall within this range).
- 11.2 Label the bottom of each Petri dish with the: 1) LIMS sample number, and 2) dilution/volume filtered.
- 11.3 Shake sample at least 25 times. Aseptically pipet the sample into the bottom of a sterile Petri dish.
- 11.4 Using an auto-pipette, add 10-12 mL of the tempered PCA to the dish and gently swirl to mix the sample with the medium.

Note: Do not let more than 20 minutes elapse between starting pipetting and pouring plates.

- 11.5 When solidified, invert the plate, seal in a plastic Ziploc bag, and incubate at $35 \pm 0.5^{\circ}\text{C}$ for 48 hours. Stack plates no more than four high in the incubator.

12.0 DATA ANALYSIS AND CALCULATIONS

- 12.1 With the aid of the Quebec colony counter, count all colonies on plates containing 30-300 colonies and compute bacterial count per milliliter as follows:

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



Report the average number of colonies on duplicate plates of the same dilution.

- 12.2 If no plates contain 30-300 colonies, and one or more plates have more than 300 colonies, use the plate with the count closest to 300 and compute the bacterial density according to Section 12.1 above.
- 12.3 If plates from all dilutions have no colonies, report the count as less than one (< 1) divided by the corresponding largest sample volume used. For example, if no colonies are on the plate from the 1.0 mL sample volume, report the count as < 1 estimated CFU/mL.
- 12.4 If the number of colonies per plate far exceeds 300, do not report as too numerous to count (TNTC). Proceed as follows:
- 12.4.1 Fewer than 10 colonies/cm²: Count colonies in 13 squares of the colony counter having representative distribution. Preferably, select seven consecutive squares horizontally across the plate and six consecutive squares vertically. Be careful not to count a square more than once. Multiply the sum of the numbers of colonies in the 13 squares by 5 to compute the estimated colonies per plate when the plate area is 65 cm².
- 12.4.2 More than 10 colonies/cm²: Count colonies in four representative squares, take an average count per square centimeter, and multiply by a factor of 57 (65 if using glass Petri dishes).
- 12.4.3 More than 100 colonies/cm²: Report result as > 5700 (> 6500 for glass Petri dishes) divided by the smallest sample volume plated – report as estimated CFU/mL.
- 12.5 Spreading colonies: If spreaders exist on the plate, count colonies on representative portions of the plate only when colonies are well distributed in spreader-free areas and less than half of the plate is covered by spreaders.
- 12.5.1 When spreaders must be counted, count the following as one colony: 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 of growth between the agar and the bottom of the Petri dish; or a colony that forms in a film of water at the edge or over the agar surface.
- 12.5.2 When plates are covered excessively by spreaders, report as “spreaders”.
- 12.6 Count as individual colonies similar appearing colonies growing next to each other but not touching *if* the distance between the two colonies is at least equal to the diameter of the smallest colony. If colonies are overlapping and are different in appearance (color, morphology), count the colonies individually.
- 12.7 Ten percent of the samples in a batch are used to calculate the duplicate range of logs. WES uses the first sample of every set of 10 in a batch as the duplicate to calculate the range of logs (e.g., if a batch has 15 samples, sample # 1 and # 11 are used to calculate the duplicate range of logs).
- 12.8 Calculation of Precision QC Criterion: For raw potable water source samples or potable water samples where enough volume is collected and enumeration is requested, determine the range of logs for the duplicate colony counts as follows (*Standard Methods for the Examination of Water and Wastewater*, 1998, Page 9-10):



Range of Logs for a Duplicate Set = $\text{Log}(\text{Count } 1) - \text{Log}(\text{Count } 2)$

If either result of a duplicate set is < 1 , add 1 to both values before calculating the logarithms as follows:

Range of Logs for a Duplicate Set = $\text{Log}[(\text{Count } 1) + 1] - \text{Log}[(\text{Count } 2) + 1]$

Precision QC Criterion = $3.27 \times (\text{Mean Range of Logs for 15 Most Recent Duplicate Sets})$

13.0 METHOD PERFORMANCE

- 13.1 The detection limit of this method is one colony-forming unit per unit volume plated.

14.0 POLLUTION PREVENTION

- 14.1 Refer to the WES Environmental Management System (EMS) policy and SOPs regarding pollution prevention.
- 14.2 The quantity of media and reagents purchased should be based on expected usage during its shelf life. Actual media and reagent preparation volumes should reflect anticipated usage and stability.

15.0 WASTE MANAGEMENT

- 15.1 WES laboratories fully comply with all applicable federal, state, and local environmental regulations. WES is also committed to protecting the air, water, and land by minimizing and controlling all chemical releases from fume hoods, biological safety cabinets, and bench operations. Refer to the WES EMS policy and SOPs regarding waste management.
- 15.2 All sample plates are placed in autoclave bags and autoclaved at 121°C for a minimum of 30 minutes. Decontaminated plastic Petri plates are separated from agar for recycling; agar waste is disposed to the WES dumpster.

16.0 REFERENCES

- 16.1 *Standard Methods for the Examination of Water and Wastewater*, 20th Edition. 1998. American Public Health Association, American Water Works Association, and Water Environment Federation, Washington, DC.
- 16.2 *Standard Methods for the Examination of Water and Wastewater*, 21st Edition. 2004. American Public Health Association, American Water Works Association, and Water Environment Federation, Washington, DC.



17.0 TABLES

TABLE 1. Quality Control Elements, Acceptance Limits, and Corrective Actions for the Analysis of Heterotrophic Plate Count by SM 9215B Pour Plate Procedure

QC Elements	Frequency	Acceptance Criteria	Corrective Action
Sample storage	Every sample	Samples must be analyzed within a maximum of 8 hours from collection (i.e., maximum of 6 hours in the field + 2 hours in the laboratory) and are stored at $\leq 10^{\circ}\text{C}$ from time of collection to time of analysis	Qualify data (H or J) as estimated value (exceeded holding time or holding temperature, respectively) and contact sample collector to obtain new sample
Air blank	With each batch of 20 or fewer samples ($\geq 5\%$)	< 2 HPC colonies	Qualify data (B) as estimated value (HPC colonies present in sample, and in air blank – laboratory contamination) and contact sample collector to obtain new sample as suspect
Method blank (uninoculated PCA plate)	With each batch of 20 or fewer samples ($\geq 5\%$)	< 2 HPC colonies	Qualify data (B) as estimated value (HPC colonies present in sample, and in the negative control plate – laboratory contamination) and contact sample collector to obtain new sample