New York State Department of Health - Wadsworth Center
Laboratory of Environmental Biology
NYS ELAP Laboratory ID 10765

Division of Environmental Health Sciences
Albany, New York

NYS DOH LEB-605

Aerobic Bacteria and Mold Plate Counts for Medical Marijuana Testing

Contents

1.0. Scope and Application

1.1. This protocol is used to estimate the number of aerobic heterotrophic bacteria and molds in samples of medical marijuana products. Plate counts are determined for all samples submitted for analysis in the Medical Marijuana Testing Program.

2.0. Summary of the Method

2.1. Sample aliquots prepared according to NYS DOH LEB-603 are spread-plated onto Trypticase Soy agar and incubated for 72 hours at 30-35°C for aerobic bacteria enumeration. Colonies are enumerated with the aid of a Quebec colony counter and the number of CFU/dose is reported.

2.2. Sample aliquots prepared according to NYS DOH LEB-603 are spread-plated onto Sabouraud Dextrose agar and incubated for 3-7 days at 20-25°C for mold enumeration. Colonies are enumerated with the aid of a Quebec colony counter and the number of CFU/dose is reported.

3.0. Definitions

3.1. For the purposes of this test, aerobic bacteria are defined as aerobic or facultatively anaerobic bacteria capable of growth on Trypticase Soy agar within 72 hours at 30-35°C.

3.2. PBST stands for Phosphate Buffered Saline, pH 7.2, containing 0.1% Tween® 80.

3.3. Polysorbate 80 (Tween® 80) is a nonionic surfactant and emulsifier.

3.4. Diluted sample aliquot is one measured dose of sample diluted 1:10 in PBST in accordance with NYS DOH LEB-603.

3.5. SDA stands for Sabouraud Dextrose Agar

3.6. TSA stands for Trypticase Soy Agar

3.7. APC stands for Aerobic Plate Count

3.8. MPC stands for Mold Plate Count

3.9. CFU stands for colony forming unit. Because a colony theoretically arises from a single microorganism or spore, the number of CFUs reflects the number of culturable microorganisms in the sample.

4.0. Health and Safety Warnings

4.1. Microbiological analyses involve the culturing of potentially pathogenic organisms.

4.1.1. All microbiologically contaminated materials, including media, shall be autoclaved after use.

4.1.2. Laboratory equipment and benches shall be disinfected using either Envirocide®, 10% bleach, or a minimum concentration of 70% ethanol before and after use.

4.1.3. Mouth pipetting is prohibited.

4.1.4. Contaminated glassware and plastic ware shall be decontaminated prior to washing.

4.1.5. All accidents, particularly those which may result in infection, shall be reported according to laboratory-specific policies and procedures.

4.1.6. Laboratory safety procedures shall be followed at all times. Regulations required by federal, state and local government agencies shall be implemented and followed.

5.0. Shipping Conditions, Receiving, Preservation and Storage

5.1. Sample shipping conditions

5.1.1. The medical marijuana products from the Registered Organizations (ROs) are shipped as per manufacturer's specifications and must adhere to all regulatory requirements.

5.2. Sample Receipt

5.2.1. Medical marijuana products from the RO are received, verified and documented ensuring that method, regulatory and Accreditation Body requirements are met.

5.3. Method holding times

5.3.1. None.

5.4. Preservation

5.4.1. Samples diluted in PBST that are not required for analyses are stored refrigerated until it has been determined that they are not needed for additional microbiological evaluation.

5.5. Storage

5.5.1. If storage is required prior to analysis, samples are refrigerated within a box having double locks in a locked refrigerator.

5.5.2. Once analysis is complete, remaining doses are maintained at room temperature within a box having double locks, in a locked room, until destroyed.

6.0. Interferences

6.1. The presence of spreading colonies or confluent growth can interfere with accurate colony enumeration.

6.2. Some components of medical marijuana products, e.g., ethanol, may inhibit the growth of microorganisms.

7.0. Apparatus and Materials

7.1. Equipment

7.1.1. Incubator, set at 20.0-25.0°C

7.1.2. Incubator, set at 30.0-35.0°C

7.1.3. Automatic pipettes and sterile aerosol-resistant micropipette tips.

7.1.4. Pipette-aid

7.1.5. Pipettes, sterile – 25mL, 10mL, and 5mL

7.1.6. Disposable sterile inoculum spreader, or equivalent

7.1.7. Quebec colony counter

7.1.8. Hand tally

7.1.9. Biosafety cabinet with HEPA filter

7.2. Reagents and Chemicals.

7.2.1. Phosphate Buffered Saline with 0.1% Tween 80 (PBST) pH 7.2, 10mL and 100mL aliquots in tubes/bottles. Ensure that the formulation is in agreement with that specified by USP.

7.2.2. Sabouraud Dextrose Agar plates, 15 x 150 mm. Ensure that the formulation is in agreement with that specified by USP.

7.2.3. Trypticase Soy Agar plates, 15 x 100 mm. Ensure that the formulation is in agreement with that specified by USP.

7.2.4. Disinfectants such as Envirocide®, Fisher Scientific cat. no. 19898220. 70% ethanol and/or Clorox.

7.2.5. Diluted sample aliquots, matrix spikes, and positive and negative controls of medical marijuana, prepared according to NYS DOH LEB-603.

7.3. Forms

7.3.1. Medical Marijuana Aerobic Bacteria and Mold Plate Count Results Sheet (e.g., LEB-RS-605A).

7.3.2. Analyst APC Count Comparison Results Sheet (e.g., LEB-QARS-605A).

7.3.3. Analyst MPC Count Comparison Results Sheet (e.g., LEB-QARS-605B).

8.0. Quality Control/Assurance

8.1. Method Detection Limits

8.1.1. Method Detection Limits are product specific.

8.2. Calibration and Standardization

8.2.1. Incubator temperatures shall be observed and recorded twice daily, separated by at least 4 hours.

8.2.1.1. Temperature of the 20.0-25.0°C incubator is recorded on the Incubator Temperature Record.

8.2.1.1.1. If the incubator temperature does not stay within 20.0-25.0°C, laboratory-specific corrective actions are followed. Analytical results may be invalidated if the incubator temperature exceeds 25.0°C, at the discretion of the Laboratory.

8.2.1.2. Temperature of the 30.0-35.0°C walk-in is recorded on the Walk-In Temperature Record.

8.2.1.2.1. If the incubator temperature does not stay within 30.0-35.0°C, laboratory-specific corrective actions are followed. Analytical results may be invalidated if the incubator temperature exceeds 35.0°C, at the discretion of the Laboratory.

8.2.2. Temperatures of the cold room and refrigerator are observed and recorded twice daily separated by at least 4 hours on either the Cold Room or Refrigerator Temperature Records.

8.2.2.1. If the cold room or refrigerator does not stay within 1.0-8.0°C, laboratory-specific corrective actions are followed.

8.2.2.2. The optimum temperature range for a cold room or refrigerator is 1.0-4.0°C.

8.2.2.3. If the cold room or refrigerator was in a defrost cycle at the time that the temperature was recorded, and the temperature does not reach 8.0°C, re-testing of media is not required.

8.2.2.4. Media may be re-tested for quality, depending on the number of degrees and the amount of time that the cold room temperature was out of compliance, at the discretion of the Laboratory.

8.2.3. Max/min temperatures are recorded when twice-daily temperature measurements are not possible, such as on holidays and weekends.

8.2.4. Thermometers must be calibrated against a NIST-certified thermometer as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.2.5. Sterility of disposable loops, spreaders, and spatulas as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.2.6. The volumetric accuracy of automatic pipettors and serological pipettes as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.2.7. The intensity and efficacy of the UV light in the biosafety cabinet is measured quarterly as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.2.8. Biosafety cabinets are certified annually as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.3. Quality Control

8.3.1. Comparative recovery and sterility between lots of TSA and SDA will be determined as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.3.2. As an additional control, perform air density tests on days during which medical marijuana samples are analyzed.

8.3.2.1. Remove the lids from TSA and SDA plates and expose the agar surface to ambient air for 15 minutes.

8.3.2.1.1. Use a plate of the same size as that used for sample analysis that day.

8.3.2.2. Record exposure time (e.g., LEB-RS-605A).

8.3.2.3. Replace the lid and incubate in parallel with aerobic plate count analyses for 72 ± 3 hours at 30.0-35.0°C and mold plate count analyses for 3-7 days at 20.0-25.0°C.

8.3.2.4. Count the number of colonies and record the number of CFUs (e.g., LEB-RS-605A).

8.3.2.5. Should the number of surface colonies exceed 10, laboratory-specific corrective actions are followed.

8.3.2.5.1. If high numbers of surface colonies would interfere with accurate determination of sample APCs and/or MPCs, the results will be qualified and reported as a QC failure.

8.3.2.5.2. The analysis for APC and MPC will be repeated if product is available.

8.3.3. Analyst APC and MPC counts are compared monthly and recorded on (e.g., LEB-QARS-605A and LEB-QARS-605B).

8.3.3.1. If there are two (2) or more analysts in the lab, each analyst counts the same set of duplicate plates.

8.3.3.1.1. Counts must be within ten percent (10%) difference to be acceptable.

8.3.3.1.2. Should inter-analyst counts be more than 10%, counts are repeated.

8.3.3.1.2.1. If counts are still greater than 10%, analyst(s) responsible shall be re-trained.

8.3.3.2. In a laboratory with only one (1) analyst, the same set of duplicate plates must be counted twice by the analyst, with no more than a five percent (5%) difference between the counts.

8.3.3.3. Percent difference is calculated as follows:

$$\frac{(highest\ count - \ lowest\ count)}{(highest\ count + lowest\ count)/2} \times 100$$

8.3.4. Agar plates can be used for up to 2 weeks after preparation date if stored refrigerated in plastic bags and in the dark.

8.3.4.1. Agar plates can be used after 2 weeks storage if ongoing QC demonstrates no loss in selectivity or growth promotion.

8.3.5. The use test for deionized water is performed annually, when cartridges are changed, or repairs are made to the deionized water systems as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.4. Corrective/Preventive Actions

8.4.1. The laboratory will initiate non-conformances and/or corrective/preventive actions in accordance with laboratory-specific procedures and as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

9.0. Procedure

9.1. General

9.1.1. Aseptic technique is used for all procedures.

9.1.1.1. Aseptic technique can be found in a general microbiology textbook or on-line.

9.1.1.2. Contact the Laboratory Director, designee or supervisor with questions.

9.1.2. All work surfaces are disinfected prior to preparation of spiking solutions, sample pooling/batching, sample preparation, and sample analyses.

9.1.3. Plating is performed in a biosafety cabinet with a HEPA filter.

9.2. Preparation of Positive Controls, Negative Controls, and Matrix Spikes.

9.2.1. Prepare a negative control by plating 0.1mL of PBST onto one TSA and one SDA plate.

9.2.1.1. Neither spiking solutions nor sample are added to negative controls.

9.2.2. Prepare an APC positive control by splitting an aliquot of the *E. coli* BioBalls® or laboratory prepared spiking solution (prepared according to NYS DOH LEB-603) and plating on two TSA plates.

9.2.2.1. The aliquot of the *E. coli* spike should not contain more than 100 CFUs.

9.2.3. Prepare an MPC positive control by splitting an aliquot of the *A. brasiliensis* BioBalls® spiking solution (prepared according to NYS DOH LEB-603) and plating on two SDA plates.

9.2.3.1. The aliquot of *A. brasiliensis* should not contain more than 100 CFUs.

9.2.4. Prepare a matrix spike for aerobic plate counts by adding an aliquot of the *E. coli* BioBalls® or laboratory prepared spiking solution (prepared according to NYS DOH LEB-603) to one tube containing a diluted sample aliquot.

9.2.4.1. If the diluted sample aliquot volume exceeds 1mL, add the matrix spike to 1 mL of the diluted sample aliquot and analyze this volume.

9.2.4.2. If the diluted sample aliquot is 1mL or less, add the matrix spike to the entire volume of the diluted sample aliquot and analyze this volume.

9.2.4.3. The aliquot of *E. coli* should contain no more than 100 CFUs.

9.2.4.4. Vortex to mix.

9.2.4.5. Plate the matrix spike between two TSA plates

9.2.5. Follow step 9.2.4. using *A. brasiliensis* BioBalls® spiking solution (prepared according to NYS DOH LEB-603) and SDA plates.

9.2.5.1. The aliquot of the *A. brasiliensis* spike should contain no more than 100 CFUs.

9.3. Sample Set-Up

9.3.1. Remove enough TSA and SDA plates from the cold room to accommodate the diluted sample aliquot, the associated matrix spike, positive controls, and negative controls prepared according to section 9.2.

and warm to room temperature while drying in the biohazard hood if necessary.

9.3.1.1. Up to 0.5 mL can be plated onto one 15 x 100 mm plate.

9.3.1.2. Up to 1mL can be plated onto one 15 x 150 mm plate.

9.3.2. Plating Volumes

9.3.2.1. Mold Plate Counts

9.3.2.1.1. The entire diluted sample aliquot is plated onto SDA plates unless the volume is greater than 10mLs.

9.3.2.1.2. If the diluted sample aliquot volume is greater than 10mLs, one fifth of the volume is plated onto SDA plates.

9.3.2.1.2.1. For example, if the diluted sample aliquot is 50mL, plate 10mL total volume onto SDA plates.

9.3.2.2. Aerobic Bacteria Plate Counts

9.3.2.2.1. The first three times a product type is received and every twentieth time it is received, the entire diluted sample aliquot is plated onto TSA plates unless the diluted sample aliquot volume is greater than 10mLs.

9.3.2.2.1.1. If the diluted sample aliquot volume is greater than 10mLs, one tenth of the diluted sample aliquot is plated onto TSA plates.

9.3.2.2.1.1.1. For example, if the diluted sample aliquot is 50mL, plate 5mL total volume onto TSA plates.

9.3.2.2.2. After a product has been evaluated three times for APC with acceptable results (APC ≤10 CFU), one tenth of the diluted sample aliquot may be plated onto TSA plates.

9.3.2.2.2.1. For example, if the diluted sample aliquot is 2mL, plate 0.1mL on two TSA plates (total volume plated is 0.2mL).

9.3.2.2.2.2. If the diluted sample aliquot is less than 1mL then the entire diluted aliquot is plated on TSA plates.

9.3.3. Label TSA and SDA plates with the sample accession number.

9.3.4. Using an automatic micropipettor and sterile tips or pipette aid and sterile pipettes, pipette up to 0.5mL and 1mL aliquots of diluted sample onto each TSA and SDA plate, respectively.

9.3.5. Use a sterile spreader to spread the sample aliquot over the entire agar surface.

9.3.6. Repeat 9.3.3-9.3.4 for the associated matrix spike, positive control and negative control samples prepared according to section 9.2.

9.3.7. After samples have dried, invert the plates and incubate covered for 72 ± 3 hours at 30.0-35.0°C for TSA plates and 3-7 days at 20.0-25.0°C for SDA plates.
9.3.7.1. Stack plates no more than four high.

9.4. Sample Analysis

9.4.1. Count colonies on all plates inoculated with diluted sample aliquot using a Quebec colony counter and hand tally, if required.
9.4.1.1. Record the total number of colonies (e.g., LEB-RS-605A).
9.4.1.2. If colony numbers are too numerous to count or overgrowth of certain mold species inhibits enumeration, dilute an archived aliquot if available and repeat analysis.
9.4.1.3. Ten-fold serial dilutions may be prepared in PBST upon receipt if desired, e.g., when samples historically have contained numerous mold spores.
9.4.1.4. USP <61> recommends no more than 50 mold colonies per plate.
9.4.2. Count colonies on all plates inoculated with the positive control sample. The positive control plates should have no more than 100 colonies showing typical *E. coli* or *A. brasiliensis* morphology.
9.4.2.1. *E. coli* ATCC 8739 colonies are non-pigmented, shiny, round and have an entire margin when grown on TSA.
9.4.2.2. *A. brasiliensis* ATCC 16404 colonies are initially white but form black centers upon aging (3-4 days) when grown on SDA.
9.4.2.3. If there are no colonies on the plated positive control showing typical morphology, the sample results are invalidated and the Laboratory Director is notified.
9.4.2.4. If the total number of *E. coli* or *A. brasiliensis* colonies is greater than 100 CFUs/sample aliquot, the Laboratory Director is notified.
9.4.3. Count colonies on all plates inoculated with the matrix spike sample. Colonies of the spiked organism and any organisms indigenous to the sample aliquot will be observed.
9.4.3.1. If there are no colonies showing either typical *E. coli* or *A. brasiliensis* morphology on the matrix spike plates the Laboratory Director is notified.
9.4.4. Record the number of colonies in the matrix spike as CFUs (e.g., LEB-RS-605A).
9.4.4.1. If the number of colonies on the matrix spike plates is less than half of the number of colonies on the positive control plates, the Laboratory Director is notified.
9.4.5. Count and record the number of colonies in the negative control as CFUs (e.g., LEB-RS-605A).

9.4.5.1. If there are colonies on the negative control plate, the Laboratory Director is notified.

9.4.5.1.1. Depending on the extent of contamination, the results may be invalidated at the discretion of the Laboratory Director.

9.4.6. If counting must be delayed, store plates at 4°C for no more than 48 hours.

9.4.7. For plates having no colony forming units, report the count as less than one CFU/dose plated.

9.4.7.1. If one tenth the aliquot volume was plated, report the count as less than ten CFU/dose plated.

9.4.7.2. If one fifth the aliquot volume was plated, report the count as less than five CFU/dose plated.

9.4.8. As a general practice, plates with more than 250 CFU should not be counted.

9.4.8.1. If there is no plate showing 250 or fewer colonies, results must be reported as estimated.

9.4.8.2. If colonies on plates are too numerous to count, dilute an archived sample from the same lot and repeat the analysis.

9.4.9. Colony identification is not required for any of the inoculated APC plates.

9.4.10. For any sample having a result of ≥10 CFUs when 1/10 of the sample aliquot volume is plated for APC analysis, repeat the APC analysis using a full sample volume.

9.4.11. For any sample containing ≥ 5 mold colonies having similar morphology, record colony morphologies and the total number of similar colonies (e.g., LEB-RS-609B). Proceed to NYS DOH LEB-609 and/or NYS DOH LEB-615 for mold identification.

10.0.Data Acquisition, Reduction and Documentation

10.1. Record the accession number, analyst, number of CFUs per plate, total number of CFUs per dose, media lot date, and start/end dates and times of incubation on the Aerobic Bacteria and Mold Plate Count Result Sheet (e.g., LEB-RS-605A).

10.2. Report results of plate counts as CFU/dose.

10.3. Results for samples from which *E. coli* and *A. brasiliensis* were not recovered in the positive control are invalidated.

10.4. Record results of air density plate analyses (e.g., LEB-RS-605A).

11.0.Method Performance

11.1. Demonstration of Capability

11.1.1. Prior to acceptance and use of this method for data reporting, a satisfactory initial demonstration of capability (DOC) is required. Thereafter, an ongoing DOC is to be performed annually.

11.1.2. An initial DOC shall be made prior to using any method, and at any time there is a change in instrument type, personnel or method or any time that

a method has not been performed by the laboratory or analyst in a twelve (12) month period.

11.1.3. All DOCs shall be documented, and all data applicable to the demonstration shall be retained and readily available at the laboratory. Consult state regulations and standards for additional information on performing a DOC for microbiological contaminants.

11.1.4. Consult relevant standards, regulations and Accreditation Body requirements for additional information on performing DOCs for microbial contaminants.

11.2. Laboratory Detection Limits

11.2.1 See section 8.1.

12.0. Waste Management/Pollution Prevention

12.1. It is the responsibility of the laboratory to comply with all federal, state and local regulations governing waste management, particularly the hazardous waste identification rules and land disposal restrictions.

12.2. Bacterial/fungal cultures and contaminated or potentially contaminated disposable materials are disposed of in biohazardous waste cans and autoclaved prior to discarding.

12.3. Dispose of non-hazardous aqueous waste in the laboratory sink followed by flushing with tap water.

12.4. Dispose of glassware in appropriately labeled boxes. Be sure that, whenever possible, the glass has been thoroughly rinsed and is contaminant-free before disposal.

12.5. Consult federal, state and local regulations for additional information or for information on the disposal of products not described in this method.

13.0.References

13.1. APHA. *Standard Methods for the Examination of Water and Wastewater,* 23rd edition.

13.2. United States Pharmacopeia. USP38-NF33, The United States Pharmacopeial Convention, General chapters <61>, <62>, <1111>.

13.3. TNI 2016 Standards – EL-V1M2-2016-Rev2.1: Quality Systems General Requirements

13.4. TNI 2016 Standards – EL-V1M5-2016-Rev2.0: Microbiological Testing

13.5. NYS DOH LEB-603, Preparation of Samples for Medical Marijuana Testing

13.6. NYS DOH LEB-609, Mold Plate Counts and Identification for Medical Marijuana Testing

13.7. Title 10 (Health), Subpart 55-2.15 and Chapter XIII, Section 1004.14 of the official Compilation of Codes, Rules, and Regulations, of the State of New York.

14.0. Appendices

Medical Marijuana Aerobic Bacteria and Mold Plate Count and Mold Presence/Absence Results Sheet
Incubate at 30-35ºC for 72±3 hours and 20-25ºC for 3-7 days (LEB-RS-605A).

Accession #: **Analyst:** **Start Date/Time:**

Aerobic Plate Count	Plate Size	CFUs per plate	Total CFUs per dose
Sample	15 x 100mm		
Matrix Spike (*E. coli*)	15 x 100mm		
Positive Control (*E. coli*) in PBST	15 x 100mm		n/a
Negative Control in PBST	15 x 100mm		n/a
Air Density	15 x 100mm		n/a

End Date/Time: _____

Mold Plate Count**	Plate Size	CFUs per plate	Total CFUs per dose
Sample	15 x 150mm		
Matrix Spike (*A. brasiliensis*)	15 x 150mm		
Positive Control (*A. brasiliensis*) in PBST	15 x 150mm		n/a
Negative Control in PBST	15 x 150mm		n/a
Air Density	15 x 150mm		n/a

End Date/Time: _____

Air Density Open/Close Times/Results: |_____|_____|_____|
Pooling Hood Benchtop Plating Hood

Check if applies: ____ One tenth of the diluted aliquot was plated for APC
__ __ One fifth of the diluted aliquot was plated for MPC

Mucor/Rhizopus Results (circle one): Positive
Negative

Penicillium/Aspergillus/Paecilomyces Results (circle one): Positive
Negative

Comments:

** Mold colonies of similar morphology that number greater than 5 total (all plates) must be tested for the presence of *Aspergillus*, *Penicillium*, and *Mucor* according to NYS DOH LEB-609, NYS DOH LEB-615, and NYS DOH LEB-618. TSA = Trypticase Soy Agar, SDA = Sabouraud Dextrose Agar

Reviewed by: Date:

Analyst APC Count Comparison (LEB-QARS-605A)
(30-35°C Incubator)

MONTH	PLATE #	ANALYST #1	ANALYST #2	ANALYST #3	% DIFFERENCE	INITIALS
JANUARY						
FEBRUARY						
MARCH						
APRIL						
MAY						
JUNE						
JULY						
AUGUST						
SEPTEMBER						
OCTOBER						
NOVEMBER						
DECEMBER						

Plates are incubated at 30-35ºC for 72 ± 3 hours.The percent difference, calculated as the difference between the highest and lowest values, divided by the average of the highest and lowest values, must be 10% or less.

Reviewed by: Date:

Analyst MPC Count Comparison (LEB-QARS-605B)
(20-25°C Incubator)

MONTH	PLATE #	ANALYST #1	ANALYST #2	ANALYST #3	% DIFFERENCE	INITIALS
JANUARY						
FEBRUARY						
MARCH						
APRIL						
MAY						
JUNE						
JULY						
AUGUST						
SEPTEMBER						
OCTOBER						
NOVEMBER						
DECEMBER						

Incubate at 20-25°C for 3-7 days. The percent difference, calculated as the difference between the highest and lowest values, divided by the average of the highest and lowest values, must be 10% or less.

Reviewed by: Date: