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NYS DOH LEB-604

**Microbial Presumptive Presence/Absence Test
for Medical Marijuana Samples
Samples**



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1.0. Scope and Application

- 1.1. This method, NYS DOH LEB-604, Microbial Presumptive Presence/Absence Test of Medical Marijuana Samples (ELAP Method ID 9986) is used after completion of NYS DOH LEB-603 (ELAP Method ID 9926), “Preparation of Samples for Medical Marijuana Testing”, and describes the inoculation of several types of bacteriological media for determining the presence or absence of bile tolerant gram negative bacteria, *Escherichia coli*, *Clostridium botulinum*, *Pseudomonas* species, *Enterococcus* species, *Salmonella* species, and thermophilic actinomycetes in samples of medical marijuana products as required in 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.
- 1.2. Evaluation of medical marijuana products for aerobic bacteria and molds (NYS DOH LEB-605) is also required.
- 1.3. Protocols for the identification of these organisms in samples of medical marijuana products can be found in the NYS DOH LEB-600 series. See Appendix A for Medical Marijuana Microbial Testing Plan flowcharts.

2.0. Summary of the Method

- 2.1. Portions of the diluted sample aliquot of a medical marijuana product and associated matrix spikes are inoculated into four different liquid media and incubated at temperatures and times specific for the detection of bile tolerant gram negative bacteria, *Escherichia coli*, *Clostridium botulinum*, *Pseudomonas* species, *Enterococcus* species, *Salmonella* species, and thermophilic actinomycetes.
- 2.2. Any growth (turbidity) observed is considered a presumptive positive result and is further investigated using Standard Operating Procedure-specific methods (NYS DOH LEB-600 series).

3.0. Definitions

- 3.1. PBST stands for Phosphate Buffered Saline, pH 7.2, containing 0.1% Tween[®] 80.
- 3.2. Polysorbate 80 (Tween[®] 80) is a nonionic surfactant and emulsifier.
- 3.3. A dose is determined by the Registered Organization and is listed in the paperwork that accompanies the delivery of the product. The dose is typically a weight or volume with the exception of capsules or tablets in which the dose is either one or multiple capsules/tablets. The fill weight of a capsule is not the dose.
- 3.4. Pooled product refers to the total combined doses of one lot of product. Typically, 20 doses are pooled.
- 3.5. Batched product refers to the combining of individual doses from the pooled product to be tested. Typically, 15 doses of the pooled product are batched for analysis.
- 3.6. Diluted sample aliquot is one measured dose of sample diluted in PBST in accordance with NYS DOH LEB-603.
- 3.7. TSB stands for Trypticase Soy Broth
- 3.8. EEBM stands for Enterobacteria Enrichment Broth Mossel
- 3.9. RCM stands for Reinforced Clostridial Medium



3.10. RO stands for an organization that is registered to manufacture and dispense medical marijuana in New York State.

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 before and after use with a minimum concentration of 70% ethanol.
 - 4.1.3.** Mouth pipetting is prohibited.
 - 4.1.4.** All accidents, particularly those which may result in infection, shall be reported according to laboratory specific policies and procedures.
- 4.2.** Laboratory safety procedures shall be followed at all times. Regulations required by federal, state and local government agencies shall be implemented and followed.
- 4.3.** The analyst must be familiar with any possible hazards from the reagents and standards used for sample preparation and analysis.
- 4.4.** Always follow guidelines listed in safety data sheets (SDS) for proper storage, handling, and disposal of samples, solvents, reagents, and standards. SDSs are located within the laboratory. These guidelines must be made available to all personnel involved in microbiological analyses.
- 4.5.** Appropriate lab coat, safety glasses and gloves must be worn when performing standard or sample preparations, working on instrumentation, disposing of waste, and cleaning laboratory equipment.

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.** This procedure is initiated upon completion of the Preparation of Medical Marijuana Samples (see NYS DOH LEB-603, sections 9.5. and 9.6.).

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.

- 5.4.2.** Unused, reconstituted BioBalls® can be stored for one week at 1.0-8.0°C.

5.5. Storage



- 5.5.1. If storage is required prior to analysis, isolates or archived plates are stored refrigerated until it has been determined that they are not needed for additional microbiological evaluation.

6.0. Interferences

- 6.1. Some components of medical marijuana products, e.g., ethanol, may inhibit the growth of microorganisms.
- 6.2. Some components of medical marijuana products, e.g., capsule particulates, may create turbidity in the diluted sample aliquots which can be mistaken for microbial growth during analysis.

7.0. Apparatus and Materials

7.1. Equipment and Supplies

- 7.1.1. Incubator, 30.0-35.0°C
- 7.1.2. Incubator, 50.0-55.0°C
- 7.1.3. Incubator, 30.0-35.0°C, 5% CO₂
- 7.1.4. Water Bath, 35.0-40.0°C
- 7.1.5. Water Bath, 78.0-82.0°C
- 7.1.6. Pipette-aid
- 7.1.7. Pipettes, sterile – 25mL, 10mL, and 5mL
- 7.1.8. Automatic pipettors and sterile aerosol-resistant micropipette tips.
- 7.1.9. NIST certified laboratory timer – Krackeler Scientific, Albany, NY, cat no. 291-5004 or equivalent.
- 7.1.10. Disposable powder-free latex gloves
- 7.1.11. Anaerobic incubation chambers
- 7.1.12. GasPak™ EZ Gas Generating sachets – Fisher Scientific, Hanover Park, IL, cat. no. B260678 or equivalent.
- 7.1.13. Anaerobic Indicator Strips – Fisher Scientific, Hanover Park, IL, cat. no. B71051 or equivalent.
- 7.1.14. Glass tubes with kaputs, 16 x 150mm size, sterile, or equivalent
- 7.1.15. Erlenmeyer flasks, foam plugged, various sizes, sterile
- 7.1.16. 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. Trypticase Soy broth, sterile 1X, 2X, and 10X, in 100 mL and 50mL aliquots/Wheaton bottle and 10 mL aliquots/tube. Ensure that the formulation is in agreement with that specified by USP.
- 7.2.3. Enterobacteria Enrichment Mossel broth, sterile 1X and 2X, 100 mL and 50mL aliquots respectively in Wheaton bottles. Ensure that the formulation is in agreement with that specified by USP.



- 7.2.4. Reinforced Clostridial Medium broth, sterile 1X and 2X, 100 mL aliquots and 50mL aliquots respectively in Wheaton bottles. Ensure that the formulation is in agreement with that specified by USP.
- 7.2.5. Laboratory prepared suspensions (prepared according to laboratory specific procedures) or BioBalls® (prepared according to NYS DOH LEB-603) *Clostridium sporogenes*, ATCC 11437; *Enterococcus faecalis*, ATCC 29212; *Escherichia coli*, ATCC 8739; *Klebsiella pneumoniae* ATCC 13883; *Pseudomonas aeruginosa*, ATCC 9027; *Salmonella typhimurium*, ATCC 14028; *Thermoactinomyces vulgaris* ATCC 43649 for use as matrix spikes and/or positive controls.
- 7.2.6. Disinfectants such as Envirocide®, Fisher Scientific cat. no. 19898220. 70% ethanol and/or Clorox.
- 7.2.7. Diluted sample aliquots, matrix spikes, and positive and negative controls of medical marijuana samples, prepared according to NYS DOH LEB-603.

7.3. Forms

- 7.3.1. Medical Marijuana Presumptive Presence/Absence Result Sheet (e.g., LEB-RS-604A)
- 7.3.2. Medical Marijuana Presumptive Presence/Absence with Matrix Spikes Result Sheet (e.g., LEB-RS-604C).
- 7.3.3. Medical Marijuana Sample Set-Up sheet (e.g., LEB-RS-604B or LEB-RS-604D).

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. Temperatures for the 30.0-35.0°C incubator are recorded on the Walk-In Incubator Temperature Record.
 - 8.2.1.1.1. 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.
 - 8.2.1.2. Temperatures for the 30.0-35.0°C incubator with 5% CO₂ are recorded on the Incubator 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.



- 8.2.1.3.** Temperatures for the 50.0-55.0°C incubator are recorded on the Incubator Temperature Record.
 - 8.2.1.3.1.1.** If the incubator temperature does not stay within 50.0-55.0°C, laboratory-specific corrective actions are followed. Analytical results may be invalidated if the incubator temperature exceeds 55.0°C, at the discretion of the laboratory.
- 8.2.2.** Temperatures of the cold room and refrigerators are observed and recorded twice daily separated by at least 4 hours on either the Cold Room or Refrigerator Temperature Record.
 - 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 or refrigerator temperature was out of compliance, at the discretion of the laboratory.
- 8.2.3.** Water bath temperatures shall be observed and recorded twice daily, separated by at least 4 hours.
 - 8.2.3.1.** Temperature of the 35.0-40.0°C water bath is observed and recorded on the Water Bath Temperature Record.
 - 8.2.3.1.1.** If the water bath temperature does not stay within 35.0-40.0°C, laboratory-specific corrective actions are followed. Analytical results may be invalidated if the incubator temperature exceeds 40.0°C, at the discretion of the laboratory.
 - 8.2.3.2.** Temperature of the 80.0°C water bath is observed and recorded on the Water Bath Temperature Record on days that samples are analyzed just prior to sample analyses and after sample analyses are completed.
 - 8.2.3.2.1.** If the water bath temperature does not stay within 78.0-82.0°C, laboratory-specific corrective actions are followed. Analytical results may be invalidated if the incubator temperature exceeds 82.0°C, at the discretion of the laboratory.
- 8.2.4.** Max/Min temperatures are recorded when twice-daily temperature measurement is not possible, such as on holidays and weekends.



- 8.2.5. The accuracy of digital timers is determined as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.
- 8.2.6. Thermometers must be calibrated against a NIST-certified thermometer as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.
- 8.2.7. The volumetric accuracy of automatic pipetters and serological pipettes is determined as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.
- 8.2.8. CO₂ levels are tested monthly as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.
- 8.2.9. Biosafety cabinets are certified 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 PBST, TSB, EEBM, and RCM will be determined as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.
- 8.3.2. Liquid media shall be stored in tightly-capped bottles in the dark at 4°C for up to 3 months from the date of preparation.
- 8.3.3. The use test for deionized water is performed annually, when cartridges are changed, or repairs are made to the deionized water systems or 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.
- 9.1.2. Pre-reduce the RCM under anaerobic conditions by placing them in an anaerobic container with GasPak™ sachets and Dry Anaerobic Indicator Strip and incubate them either refrigerated or at room temperature at least overnight.
 - 9.1.2.1. Typically, the small chambers need one GasPak™ sachet and the larger chambers need three.



- 9.1.2.2. Follow the manufacturer's instructions for GasPaks™ and Dry Anaerobic Indicator Strips.
 - 9.1.2.2.1. Dry Anaerobic Indicator strips are blue under aerobic conditions and white under anaerobic conditions.
 - 9.1.2.2.2. If the color strip indicates the presence of aerobic conditions after incubation, transfer the bottles to a different anaerobic chamber and replace the GasPaks™ and indicator strip.
 - 9.1.2.2.3. If anaerobic conditions are still not achieved, follow laboratory-specific corrective actions.
 - 9.1.3. All work surfaces are disinfected prior to sample set-up for presence/absence analyses.
 - 9.1.4. Every RO has different brands of products which are available in different forms. For example, an RO may have a "Extra Strength" brand that comes in capsules, tinctures, and vaporizers. Each one of the forms under the "Extra Strength" brand is considered a separate product type and is analyzed as such.
 - 9.1.5. Every new product type analyzed for microbial contaminants has corresponding matrix spikes that are analyzed the first three times that new product type is analyzed. After three matrix spikes, the new product type requires a corresponding matrix spikes every 20th time it is received for analysis.
 - 9.1.6. Sample set-up for presence/absence analyses is performed in a different location than preparation of spiking solutions to prevent cross-contamination of incoming products.
- 9.2. Aerobic Bacteria - *E. coli*, *Pseudomonas*, *Salmonella* (1-2 diluted sample aliquots)**
- 9.2.1. Add either one or two diluted sample aliquots (prepared according to NYS DOH LEB-603) to separate labeled tubes or bottles containing TSB (one for a sample, and one for a matrix spike, if needed).
 - 9.2.1.1. The volume of TSB chosen is dependent upon the volume of the diluted sample aliquot being analyzed so that the addition of the diluted sample aliquot will not dilute the TSB more than 10%.
 - 9.2.1.1.1. For example, if the diluted sample aliquot is 10mLs, it should be inoculated into no less than 90mLs of TSB.
 - 9.2.1.2. If the volume of the diluted sample aliquot is greater than 10mLs and less than or equal to 50mLs, then the diluted aliquot can be added to a 50mL bottle of 2X TSB, and enough PBST is added to bring the final volume up to 100mLs (creating a 1X TSB concentration).
 - 9.2.1.2.1. Do not dilute the 2X concentration of TSB more than 1:2 with the diluted sample aliquot.



- 9.2.1.3.** If the volume of the diluted sample aliquot is 50mLs or more, then a volume of 10X TSB can be added directly to the diluted sample aliquot so that the final concentration of TSB is 1X.
- 9.2.1.3.1.** For example, if the volume of the diluted aliquot is 150mLs, then 16.6mLs of 10X TSB can be added directly to the diluted sample aliquot for a final volume of 166mLs.
- 9.2.1.3.2.** Do not dilute the 10X concentration of TSB more than 1:10 with the diluted sample aliquot.
- 9.2.2.** If a matrix spike is needed (see 9.1.), inoculate one of the diluted sample aliquots in TSB with a suspension of no more than 100 CFUs of *E. coli*, *Ps. aeruginosa* and *S. typhimurium*. Use either a lab prepared spike (prepared according to laboratory specific procedures) or BioBall[®] suspensions (prepared according to NYS DOH LEB-603).
- 9.2.3.** Mix diluted sample aliquots in TSB by swirling or vortexing and incubate at 30.0-35.0°C for 48 ± 3 hours without shaking.
- 9.2.4.** After incubation record the turbidity results of both the sample and matrix spike (if applicable) analyses on the Sample Presumptive Presence/Absence Result Sheet (e.g., LEB-RS-604A or LEB-RS-604C) in the “Turbidity” section.
- 9.2.4.1.** The presence of turbidity and is recorded as “Y”, no turbidity is recorded as “N”.
- 9.2.4.2.** Some sample matrices may inhibit microbial growth. If the positive and negative controls for aerobic plate counts and mold plate counts meet QC criteria (see NYS DOH LEB-605), results are considered valid in the absence of turbidity in the matrix spikes.
- 9.2.4.3.** If the positive and negative controls for aerobic plate counts and mold plate counts do not meet QC criteria (see NYS DOH LEB-605), results are considered invalid in the absence of turbidity in the matrix spikes and the analyses must be repeated.
- 9.2.4.3.1.** Additional testing using USP methods designed to decrease inhibitory effects of product that could result in growth in matrix spike samples may be undertaken using archived samples, at the discretion of the laboratory.
- 9.2.5.** If there is no turbidity, the sample is negative for the presence of *E. coli*, *Salmonella spp.*, and *Ps. aeruginosa*.
- 9.2.6.** If the sample enrichment is turbid, proceed to section 9.7.
- 9.2.7.** Record the incubation start and end date and time, volume of diluted aliquot, and results on the Sample Presumptive Presence/Absence Result Sheet (e.g., LEB-RS-604A or LEB-RS-604C).
- 9.2.8.** Record the media lot dates on the Medical Marijuana Sample Set-Up Results Sheet (e.g., LEB-RS-604B or LEB-RS-604D).

9.3. Bile Tolerant Gram Negative Bacteria (1-2 diluted sample aliquots)

- 9.3.1. Add either one or two diluted sample aliquots (prepared according to NYS DOH LEB-603) to separate labeled tubes or flasks containing 10mLs of TSB (one for a sample, and one for a matrix spike, if needed).
 - 9.3.1.1. The volume of TSB chosen is dependent upon the volume of the sample aliquot being analyzed so that the addition of the diluted sample aliquot will not dilute the TSB more than 10%.
 - 9.3.1.1.1. For example, if the diluted sample aliquot is 10mLs, it should be inoculated into at least 90mLs of TSB.
 - 9.3.1.2. If the diluted sample aliquot volume is greater than 10 mL, instead add 1/10 volume of 10X TSB to the sample aliquot.
 - 9.3.1.2.1. For example, if the volume of the diluted sample aliquot is 20mLs, then 2.2mLs of 10X TSB can be added directly to the diluted sample aliquot for a final volume of 22mLs.
 - 9.3.1.2.2. Do not dilute the 10X concentration of TSB more than 1:10 with the diluted sample aliquot.
- 9.3.2. If a matrix spike is needed (see 9.1.), inoculate one of the diluted sample aliquots in TSB with a suspension of no more than 100 CFUs of *K. pneumoniae* (prepared according to laboratory specific procedures).
- 9.3.3. Mix diluted sample aliquots in TSB by swirling or vortexing and incubate benchtop for 2-5 hours without shaking to resuscitate bacteria.
- 9.3.4. After 2-5 hours incubation, inoculate the entire sample and matrix spike (if applicable) in TSB into separate 100mLs EEBM that have been warmed to room temperature.
 - 9.3.4.1. The EEBM should not be diluted by more than 10% after the sample in TSB is added.
 - 9.3.4.2. If the volume of the diluted sample aliquot in TSB is greater than 10mLs, then inoculate it into a 50mL bottle of 2X EEBM, and add enough PBST to bring the final volume up to 100mL (creating a 1X EEBM concentration).
 - 9.3.4.3. If the volume of the diluted sample aliquot in TSB is greater than 50mLs, add 50mL of sample in TSB to multiple 50mL bottles of 2X EEBM.
- 9.3.5. Shake the EEBM to mix and incubate at 30.0-35.0°C for 48 ± 3 hours.
- 9.3.6. After incubation record the turbidity results of both the sample and matrix spike (if applicable) analyses on the Sample Presumptive Presence/Absence Result Sheet (e.g., LEB-RS-604A or LEB-RS-604C) in the “Turbidity” section.
 - 9.3.6.1. The presence of turbidity and is recorded as “Y”, no turbidity is recorded as “N”.
 - 9.3.6.2. Record changes in the medium color.



- 9.3.6.2.1. Typically, medium color changes from green to yellow, and is recorded as “Y” in the “Color Change” section.
- 9.3.6.3. Some sample matrices may inhibit microbial growth. If the positive and negative controls for aerobic plate counts and mold plate counts meet QC criteria (see NYS DOH LEB-605) results are considered valid in the absence of turbidity in the matrix spikes.
- 9.3.6.4. If the positive and negative controls for aerobic plate counts and mold plate counts do not meet QC criteria (see NYS DOH LEB-605), results are considered invalid in the absence of turbidity in the matrix spike and the analyses must be repeated.
 - 9.3.6.4.1. Additional testing using USP methods designed to decrease inhibitory effects of product that could result in growth in matrix spike samples may be undertaken using archived samples, at the discretion of the laboratory.
- 9.3.7. If there is no turbidity, the sample is negative for the presence of bile tolerant gram negative bacteria.
- 9.3.8. If the sample enrichment is turbid (regardless of color change), proceed to section 9.7.
- 9.3.9. Record the incubation start and end date and time, and results on the Sample Presumptive Presence/Absence Result Sheet (e.g., LEB-RS-604A or LEB-RS-604C).
- 9.3.10. Record the media lot dates on the Medical Marijuana Sample Set-Up Results Sheet (e.g., LEB-RS-604B or LEB-RS-604D).
- 9.4. ***Clostridium botulinum* (2-3 diluted sample aliquots)**
 - 9.4.1. Add one diluted sample aliquot (prepared according to NYS DOH LEB-603) to a labeled glass tube.
 - 9.4.1.1. If the diluted sample aliquot is less than 10mLs, add the entire diluted aliquot to a glass tube and add enough PBST to bring the final volume to 10mLs.
 - 9.4.2. Add a second diluted sample aliquot (prepared according to NYS DOH LEB-603) to a labeled glass tube.
 - 9.4.2.1. If the diluted sample aliquot is less than 10mLs, add the entire diluted aliquot to a glass tube and add enough PBST to bring the final volume to 10mLs.
 - 9.4.2.2. Heat this diluted sample aliquot tube at 80.0°C for 10 minutes and cool on ice.
 - 9.4.2.2.1. Only 10mLs of the diluted aliquot can be heated in each glass tube to ensure uniform heating of the sample.
 - 9.4.2.2.2. If the diluted sample aliquot is more than 10mLs, split the diluted aliquot into separate 10mL sub-aliquots.



- 9.4.3.** If a matrix spike is needed (see 9.1.), add a third diluted sample aliquot (prepared according to NYS DOH LEB-603) to a labeled glass tube and inoculate with a suspension of no more than 100 CFUs of *C. sporogenes*. Use a BioBall[®] suspension (prepared according to NYS DOH LEB-603).
- 9.4.4.** Transfer the room temperature diluted sample aliquot, heat-treated diluted sample aliquot, and the matrix spike (if applicable) to separate bottles containing 100mLs of RCM that have been pre-reduced (see section 9.1.2).
- 9.4.4.1.** The RCM should not be diluted by more than 10% when the sample in PBST is added.
- 9.4.4.2.** If the volume of the diluted sample aliquot in PBST is greater than 10mLs, then inoculate it into bottles containing 50mL of 2X RCM, and add enough pre-reduced PBST to bring the final volume up to 100mLs (creating a 1X RCM concentration).
- 9.4.4.3.** If the volume of the diluted sample aliquot in TSB is greater than 50mLs, add 50mLs of sample in PBST to multiple 50mL bottles of 2X RCM
- 9.4.5.** Incubate the RCM samples at 30.0-35.0°C for 48 ± 3 hours under anaerobic conditions (see section 9.1.).
- 9.4.6.** After incubation record the turbidity results of both the sample and matrix spike (if applicable) analyses on the Sample Presumptive Presence/Absence Result Sheet (e.g., LEB-RS-604A or LEB-RS-604C) in the “Turbidity” section.
- 9.4.6.1.** The presence of turbidity and is recorded as “Y”, no turbidity is recorded as “N”.
- 9.4.6.2.** Some sample matrices may inhibit microbial growth. If the positive and negative controls for aerobic plate counts and mold plate counts meet QC criteria (see NYS DOH LEB-605), results are considered valid in the absence of growth (turbidity) in the matrix spikes.
- 9.4.6.3.** If the positive and negative controls for aerobic plate counts and mold plate counts do not meet QC criteria (see NYS DOH LEB-605), results are considered invalid and the analyses must be repeated.
- 9.4.6.3.1.** Additional testing using methods to decrease inhibitory effects of product that could result in growth in matrix spike samples may be undertaken using archived samples, at the discretion of the laboratory.
- 9.4.7.** If there is no turbidity in either the untreated or heat-treated diluted aliquots, the sample is negative for the presence of *C. botulinum*.
- 9.4.8.** If there is turbidity in either the untreated or heat-treated samples, proceed to section 9.7.



- 9.4.9. Record the incubation start and end date and time, volume of diluted aliquot, and results on the Sample Presumptive Presence/Absence Result Sheet (e.g., LEB-RS-604A or LEB-RS-604C).
- 9.4.10. Record the media lot dates on the Medical Marijuana Sample Set-Up Results Sheet (e.g., LEB-RS-604B or LEB-RS-604D)

9.5. *Enterococcus* Species (1-2 diluted sample aliquots)

- 9.5.1. Add either one or two diluted sample aliquots (prepared according to NYS DOH LEB-603) to separate labeled tubes, bottles, or flasks containing TSB (one for a sample, and one for a matrix spike, if needed).
 - 9.5.1.1. The volume of TSB chosen is dependent upon the volume of the sample aliquot being analyzed so that the addition of the diluted sample aliquot will not dilute the TSB more than 10%.
 - 9.5.1.1.1. For example, if the diluted sample aliquot is 10mLs, it should be inoculated into at least 90mLs of TSB.
 - 9.5.1.2. If the volume of the diluted sample aliquot is greater than 10mLs and less than or equal to 50mLs, then the diluted aliquot can be added to a 50mL bottle of 2X TSB, and enough PBST is added to bring the final volume up to 100mLs (creating a 1X TSB concentration).
 - 9.5.1.2.1. Do not dilute the 2X concentration of TSB more than 1:5 with the diluted sample aliquot.
 - 9.5.1.3. If the volume of the diluted sample aliquot is 50mLs or more, then a volume of 10X TSB can be added directly to the diluted sample aliquot so that the final concentration of TSB is 1X.
 - 9.5.1.3.1. For example, if the volume of the diluted aliquot is 150mLs, then 16.6mLs of 10X TSB can be added directly to the diluted sample aliquot for a final volume of 166mLs.
 - 9.5.1.3.2. Do not dilute the 10X concentration of TSB more than 1:10 with the diluted sample aliquot.
- 9.5.2. If a matrix spike is needed (see 9.1.), inoculate one of the diluted sample aliquots in TSB with a suspension of no more than 100 CFUs of *E. faecalis*. Use either a lab prepared spike (prepared according to laboratory specific procedures) or BioBall® suspensions (prepared according to NYS DOH LEB-603).
- 9.5.3. Mix diluted sample aliquots in TSB by vortexing or swirling and incubate at 30.0-35.0°C for 48 ± 3 hours under 5% CO₂.
- 9.5.4. After incubation record the turbidity results of both the sample and matrix spike (if applicable) analyses on the Sample Presumptive



Presence/Absence Result Sheet (e.g., LEB-RS-604A or LEB-RS-604C) in the “Turbidity” section.

9.5.4.1. The presence of turbidity and is recorded as “Y”, no turbidity is recorded as “N”.

9.5.4.2. Some sample matrices inhibit microbial growth. If the positive and negative controls for aerobic plate counts and mold plate counts meet QC criteria (see NYS DOH LEB-605), results are considered valid in the absence of turbidity in the matrix spikes.

9.5.4.3. If the positive and negative controls for aerobic plate counts and mold plate counts do not meet QC criteria (see NYS DOH LEB-605), results are considered invalid and the analyses must be repeated.

9.5.4.3.1. Additional testing using USP methods designed to decrease inhibitory effects of product that could result in growth in matrix spike samples may be undertaken using archived samples, at the discretion of the laboratory.

9.5.5. If there is no turbidity, the sample is negative for the presence of *Enterococcus* spp.

9.5.6. If the sample enrichment is turbid, proceed to section 9.7.

9.5.7. Record the incubation start and end date and time, volume of diluted aliquot, and results on the Sample Presumptive Presence/Absence Result Sheet (e.g., LEB-RS-604A or LEB-RS-604C).

9.5.8. Record the media lot dates on the Medical Marijuana Sample Set-Up Results Sheet (e.g., LEB-RS-604B or LEB-RS-604D).

9.6. Thermophilic Actinomycetes (1-2 diluted sample aliquots)

9.6.1. Add either one or two diluted sample aliquots (prepared according to NYS DOH LEB-603) to separate labeled tubes or bottles containing TSB (one for a sample, and one for a matrix spike, if needed).

9.6.1.1. The volume of TSB chosen is dependent upon the volume of the sample aliquot being analyzed so that the addition of the diluted sample aliquot will not dilute the TSB more than 10%.

9.6.1.1.1. For example, if the diluted sample aliquot is 10mLs, it should be inoculated into at least 90mLs of TSB.

9.6.1.2. If the volume of the diluted sample aliquot is greater than 10mLs and less than or equal to 50mLs, then the diluted aliquot can be added to a 50mL bottle of 2X TSB, and enough PBST is added to bring the final volume up to 100mLs (creating a 1X TSB concentration).

9.6.1.2.1. Do not dilute the 2X concentration of TSB more than 1:2 with the diluted sample aliquot.



- 9.6.1.3.** If the volume of the diluted sample aliquot is 50mLs or more, then a volume of 10X TSB can be added directly to the diluted sample aliquot so that the final concentration of TSB is 1X.
- 9.6.1.3.1.** For example, if the volume of the diluted aliquot is 150mLs, then 16.6mLs of 10X TSB can be added directly to the diluted sample aliquot for a final volume of 166mLs.
- 9.6.1.3.2.** Do not dilute the 10X concentration of TSB more than 1:10 with the diluted sample aliquot.
- 9.6.2.** If a matrix spike is needed (see 9.1.), inoculate one of the diluted sample aliquots in TSB with a lab prepared suspension of no more than 100 CFUs of *T. vulgaris* (prepared according to NYS DOH LEB-603).
- 9.6.2.1.** A matrix spike should be accompanied by a positive control consisting of less than 100 CFUs of *T. vulgaris* (prepared according to NYS DOH LEB-603) spiked into TSB without product.
- 9.6.3.** Mix diluted sample aliquots in TSB by swirling or vortexing and incubate at 50.0-55.0°C for 3-5 days without shaking.
- 9.6.4.** After incubation record the turbidity results of both the sample and matrix spike (if applicable) analyses on the Sample Presumptive Presence/Absence Result Sheet (e.g., LEB-RS-604A or LEB-RS-604C) in the “Turbidity” section.
- 9.6.4.1.** The presence of turbidity and is recorded as “Y”, no turbidity is recorded as “N”.
- 9.6.5.** Some sample matrices may inhibit microbial growth. If the positive and negative controls for aerobic plate counts and mold plate counts meet QC criteria (see NYS DOH LEB-605), results are considered valid in the absence of turbidity in the matrix spikes.
- 9.6.6.** If the positive and negative controls for aerobic plate counts and mold plate counts do not meet QC criteria (see NYS DOH LEB-605), results are considered invalid and the analyses must be repeated.
- 9.6.6.1.** Additional testing using USP methods designed to decrease inhibitory effects of product that could result in growth in matrix spike samples may be undertaken using archived samples, at the discretion of the laboratory.
- 9.6.7.** If there is no turbidity, the sample is negative for the presence of thermophilic actinomycetes.
- 9.6.8.** If the sample enrichment is turbid, proceed to section 9.7.
- 9.6.9.** Record the incubation start and end date and time, volume of diluted aliquot, and results on the Sample Presumptive Presence/Absence Result Sheet (e.g., LEB-RS-604A or LEB-RS-604C).
- 9.6.10.** Record the media lot dates on the Medical Marijuana Sample Set-Up Results Sheet (e.g., LEB-RS-604B or LEB-RS-604D).



9.7. Analyzing Turbidity for Growth

- 9.7.1. Turbidity is not a conclusive indicator of microbial growth as some sample matrices may also result in turbidity upon addition to either the PBST and/or the presence/absence broths.
- 9.7.2. For each turbid presence/absence sample enrichment for aerobes, BTGN, *Enterococcus* spp., and/or thermophilic actinomycetes remove one TSA plate and one TSAB plate from the cold room and warm to room temperature. Dry the plates in the biological safety cabinet.
- 9.7.3. For each turbid presence/absence sample enrichment for *C. botulinum* remove one pre-reduced (see section 9.1.) TSAB plate from the cold room and warm to room temperature. Dry the plates in the biological safety cabinet.
- 9.7.4. Using an automatic micropipettor, sterile tips, and a sterile inoculum spreader, spread 100 μ Ls of the turbid sample enrichment onto each of the TSA and TSAB plates.
- 9.7.5. Allow the plates to dry, invert, and incubate for the following conditions:
 - 9.7.5.1. Aerobes and BTGN - 18-24 hours at 30.0-35.0°C.
 - 9.7.5.2. *Enterococcus* spp. - 18-48 hours at 30.0-35.0°C with 5% CO₂.
 - 9.7.5.3. *C. botulinum* - 18-48 hours at 30.0-35.0°C anaerobically.
 - 9.7.5.4. Thermophilic actinomycetes – 3-5 days at 50.0-55.0°C.
- 9.7.6. Store the turbid presence/absence sample enrichments at 1.0-8.0°C until analysis has been completed.
- 9.7.7. After incubation, record the any growth observed on the TSA and TSAB plates in the “Growth” section of the Sample Presumptive Presence/Absence Result Sheet (e.g., LEB-RS-604A or LEB-RS-604C).
 - 9.7.7.1. The presence of growth is recorded as “Y”, no growth is recorded as “N”.
- 9.7.8. If there is no growth on plates containing sample enrichments, the sample is negative for the presence of their respective target organisms.
- 9.7.9. If there is growth on the TSA and/or TSAB plates for:
 - 9.7.9.1. Aerobic TSB enrichment, proceed to NYS DOH LEB-608, NYS DOH LEB-610, and NYS DOH LEB-611 for identification of *E. coli*, *Ps. aeruginosa*, and *Salmonella* spp. respectively.
 - 9.7.9.2. BTGN EEBM enrichment, proceed to NYS DOH LEB-606 for identification of bile tolerant gram-negative bacteria.
 - 9.7.9.3. *C. botulinum* RCM enrichment, proceed to NYS DOH LEB-607 for identification of *C. botulinum*.
 - 9.7.9.4. *Enterococcus* spp. TSB enrichment, proceed to NYS DOH LEB-612 for identification of *Enterococcus* spp.
 - 9.7.9.5. Thermophilic actinomycetes TSB enrichment, proceed to NYS DOH LEB-613 for identification of thermophilic actinomycetes.
- 9.7.10. The TSA and TSAB plates containing growth are archived at 1.0-8.0°C until analysis has been completed.



9.7.11. In the “Comments” section of the Sample Presumptive Presence/Absence Result Sheet (e.g., LEB-RS-604A or LEB-RS-604C) record the start and end dates and times for the incubation of the TSA and TSAB plates.

10.0. Data Acquisition, Reduction, Analysis, Calculations, Acceptance Criteria and Documentation

- 10.1.** Record the accession number, the incubation start and end dates and times for each analysis, results, and analyst initials on the Sample Presumptive Presence/Absence form (either LEB-RS-604A or C).
- 10.2.** Record the media lot dates, laboratory prepared spike dates, and lots and expiration dates of BioBalls[®] on the Medical Marijuana Sample Set-Up Results Sheet (either LEB-RS-604B or D).

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.

11.1.4 Consult state regulations and standards for additional information on performing a DOC for microbiological contaminants.

11.2. Laboratory Detection Limits

11.2.1. Detection limits are determined in accordance with relevant standards, regulations and accreditation body requirements.

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. United States Pharmacopeia. USP38-NF33, The United States Pharmacopeial Convention, General chapters <61>, <62>, <1111>.
- 13.2. Standard Methods 22nd edition
- 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-606, Identification of Bile Tolerant Gram Negative Bacteria in Medical Marijuana Products
- 13.7. NYS DOH LEB-607, Identification of *Clostridium botulinum* in Medical Marijuana Products
- 13.8. NYS DOH LEB-608, Identification of *Escherichia coli* in Medical Marijuana Products
- 13.9. NYS DOH LEB-610, Identification of *Pseudomonas* in Medical Marijuana Products
- 13.10. NYS DOH LEB-611, Identification of *Salmonella* in Medical Marijuana Products
- 13.11. NYS DOH LEB-612, Identification *Enterococcus* in Medical Marijuana Products
- 13.12. NYS DOH LEB-613, Identification of Thermophilic Actinomycetes in Medical Marijuana Products
- 13.13. 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 Presence/Absence Results Sheet (LEB-RS-604A)

Accession #: _____ Analyst Initials: _____ Start Date/Time: _____

Sample Type/Volume of Diluted Aliquot: _____

Aerobic Bacteria (E. coli, Pseudomonas, Salmonella), 30-35°C, E552, 48 ± 3 hours

	<u>Turbidity</u> [†]		<u>Growth</u> ⁺		End Date/Time: _____
Sample	Y	N	Y	N	
					TSB: 1X or 2X or 10X (circle one)

Bile Tolerant Gram Negative Bacteria, 30-35°C, E552, 48 ± 3 hours

	<u>Turbidity</u> [†]		<u>Color Change</u>		<u>Growth</u> ⁺		End Date/Time: _____
Sample	Y	N	Y	N	Y	N	TSB: 1X or 10X (circle one)
							EEBM: 1X or 2X (circle one)

C. botulinum, 80°C Water Bath, S/N 208556, 30-35°C Incubator, E552, 48 ± 3 hours

	<u>Turbidity</u> [†]		<u>Growth</u> ⁺		End Date/Time: _____
Sample	Y	N	Y	N	
80°C Sample	Y	N	Y	N	
					RCM: 1X or 2X (circle one)

Enterococcus, 30-35°C with 5% CO2 Incubator, S/N 37111-5139, 48 ± 3 hours

	<u>Turbidity</u> [†]		<u>Growth</u> ⁺		End Date/Time: _____
Sample	Y	N	Y	N	
					TSB: 1X or 2X or 10X (circle one)

Thermophilic Actinomycetes, 50-55°C Incubator, S/N 0302193, 3-5 days

	<u>Turbidity</u> [†]		<u>Growth</u> ⁺		End Date/Time: _____
Sample	Y	N	Y	N	
					TSB: 1X or 2X or 10X (circle one)

EEBM = Enterobacteria Enrichment Broth Mossel, RCM = Reinforced Clostridia Medium, TSB = Trypticase Soy Broth. † Samples with no turbidity are reported as "Negative". + Samples with turbidity but no growth are reported as "Negative"

Comments:

Reviewed by _____ Date _____



Sample Set-Up for MMJ Samples (LEB-RS-604B)

Date: _____

Sample 1: BTGN no cells
Sample 2: BTGN, Matrix Spike (if needed) _____ µL of K. pneumoniae LP

Sample 3: C. botulinum no cells
Sample 4: C. botulinum, 80°C no cells
Sample 5: C. botulinum, Matrix Spike (if needed) _____ µL of C. sporogenes BB

Sample 6: Aerobes no cells
Sample 7: Aerobes, Matrix Spike (if needed) _____ µL of E. coli BB or LP
_____ µL of Ps. aeruginosa BB or
_____ µL of S. typhimurium
BB or LP

Sample 8: Enterococcus spp. no cells
Sample 9: Enterococcus spp., Matrix Spike (if needed) _____ µL of Ent. faecalis BB or LP

Sample 10: Thermophilic actinomycetes no cells
Sample 11: Thermophilic actinomycetes, Matrix Spike (if needed) _____ µL of T. vulgaris, LP Dilution

Sample 12: Aerobic Plate Count no cells
Sample 13: Aerobic Plate Count, Matrix Spike _____ µL of E. coli BB or LP
Aerobic Plate Count, Positive Control _____ µL of E. coli BB or LP
Aerobic Plate Count, PBST no cells
Air Density no cells

Sample 14: Mold Plate Count no cells

Sample 15: Mold Plate Count, Matrix Spike _____ µL of A. brasiliensis BB
Mold Plate Count, Positive Control _____ µL of A. brasiliensis BB
Mold Plate Count, PBST no cells
Air Density no cells

Volume of lab prepared spikes are determined according to laboratory-specific procedures.
BB = BioBall® spike, LP = Lab Prepared Spike, BB or LP = BioBall® or Lab Prepared Spike (one is circled)



Department of Health

ANDREW M. CUOMO
Governor

HOWARD A. ZUCKER, M.D., J.D.
Commissioner

SALLY DRESLIN, M.S., R.N.
Executive Deputy Commissioner

Lab Prepared Spike Date or BioBall Lot/Exp. Dates:

A. brasiliensis _____

C. sporogenes _____

E. coli _____

Ent. faecalis _____

K. pneumoniae _____

Ps. aeruginosa _____

S. typhimurium _____

T. vulgaris _____

Media Lot Dates**:

EEBM, 1X _____

EEBM, 2X _____

PBST _____

RCM, 1X _____

RCM, 2X _____

SDA _____

TSA _____

TSAB _____

TSB, 1X _____

TSB, 2X _____

TSB, 10X _____

Supply Lot/Exp. Dates:

Isopropyl Myristate _____

Gas Pak _____

Indicator Strips _____

EEBM stands for Enterobacteria Enrichment Broth Mossel, PBST stands for PBS with 0.1% Tween, RCM stands for Reinforced Clostridia Medium, SDA stands for Sabouraud Dextrose Agar, TSA stands for Trypticase Soy agar, and TSB stands for Trypticase Soy Broth
 ** Expiration dates of liquid media are 3 months after lot dates indicated on the bottles/tubes.



ANDREW M. CUOMO Governor

HOWARD A. ZUCKER, M.D., J.D. Commissioner

SALLY DRESLIN, M.S., R.N. Executive Deputy Commissioner

Medical Marijuana Presence/Absence Results Sheet (LEB-RS-604C)

Accession #: _____ Analyst Initials: _____ Start Date/Time: _____

Sample Type/Volume of Diluted Aliquot: _____

Aerobic Bacteria (E. coli, Pseudomonas, Salmonella), 30-35°C, E552, 48 ± 3 hours

Table with columns: Turbidity†, Growth+, End Date/Time. Rows: Sample, Matrix Spike. Includes TSB: 1X or 2X or 10X (circle one)

Bile Tolerant Gram Negative Bacteria, 30-35°C, E552, 48 ± 3 hours

Table with columns: Turbidity†, Color Change, Growth+, End Date/Time. Rows: Sample, Matrix Spike. Includes TSB: 1X or 10X (circle one) and EEBM: 1X or 2X (circle one)

C. botulinum, 80°C Water Bath, S/N 208556, 30-35°C Incubator, E552, 48 ± 3 hours

Table with columns: Turbidity†, Growth+, End Date/Time. Rows: Sample, 80°C Sample, Matrix Spike. Includes RCM: 1X or 2X (circle one)

Enterococcus, 30-35°C with 5% CO2 Incubator, S/N 37111-5139, 48 ± 3 hours

Table with columns: Turbidity†, Growth+, End Date/Time. Rows: Sample, Matrix Spike. Includes TSB: 1X or 2X or 10X (circle one)

Thermophilic Actinomycetes, 50-55°C Incubator, S/N 0302193, 3-5 days

Table with columns: Turbidity†, Growth+, End Date/Time. Rows: Sample, Matrix Spike, Pos. Control. Includes TSB: 1X or 2X or 10X (circle one)

EEBM = Enterobacteria Enrichment Broth Mossel, RCM = Reinforced Clostridia Medium, TSB = Trypticase Soy Broth

† Samples with no turbidity are reported as "Negative"

+ Samples with turbidity but no growth are reported as "Negative"

Comments:

Reviewed by _____ Date _____



Department of Health

ANDREW M. CUOMO
Governor

HOWARD A. ZUCKER, M.D., J.D.
Commissioner

SALLY DRESLIN, M.S., R.N.
Executive Deputy Commissioner

Sample Set-Up for MMJ Samples (LEB-RS-604D)

Lab Prepared Spike or BioBall Spike:

	Lot/Prep Date, Exp. Date	Sample Spike Date, Volume
<i>A. brasiliensis</i>	_____	_____
<i>C. sporogenes</i>	_____	_____
<i>E. coli</i>	_____	_____
<i>Ent. faecalis</i>	_____	_____
<i>K. pneumoniae</i>	_____	_____
<i>Ps. aeruginosa</i>	_____	_____
<i>S. typhimurium</i>	_____	_____
<i>T. vulgaris</i>	_____	_____

Media Lot Dates**:

EEBM, 1X	_____
EEBM, 2X	_____
PBST	_____
RCM, 1X	_____
RCM, 2X	_____
SDA	_____
TSA	_____
TSAB	_____
TSB, 1X	_____
TSB, 2X	_____
TSB, 10X	_____

Supply Lot/Exp. Dates:

Isopropyl Myristate	_____
Gas Pak	_____
Indicator Strips	_____

EEBM stands for Enterobacteria Enrichment Broth Mossel, PBST stands for PBS with 0.1% Tween, RCM stands for Reinforced Clostridia Medium, SDA stands for Sabouraud Dextrose Agar, TSA stands for Trypticase Soy agar, and TSB stands for Trypticase Soy Broth
 ** Expiration dates of liquid media are 3 months after lot dates indicated on the bottles/tubes.