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NYS DOH LEB-609

Mold Identification Using RT-PCR for Medical Marijuana Testing

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1.0. Scope and Application

1.1. This method, NYS DOH LEB-609, Mold Identification Using RT-PCR for Medical Marijuana Testing (ELAP Method #9989), is used to prepare reagents and extract DNA for the identification of *Aspergillus, Mucor* and *Penicillium* spp. using real-time quantitative PCR (RT-PCR) 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. 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. Unknown fungal colonies growing on Sabouraud Dextrose agar plates (prepared according to NYS DOH LEB-605) are described, enumerated, and a representative colony is chosen for DNA extraction. A small portion of a colony is transferred from the SDA plate to a tube containing extraction buffer. The DNA is extracted and used in NYS DOH LEB-618 for the identification *Aspergillus, Mucor* and *Penicillium* species using RT-PCR).

3.0. Definitions

3.1. SDA stands for Sabouraud Dextrose Agar

3.2. CFU stands for colony forming unit

3.3. RT-PCR stands for real-time, polymerase chain reaction amplification

3.4. AE buffer contains 10mM Tris-Cl and 0.5mM EDTA; pH 9.0.

3.5. Extraction buffer is AE buffer with 200 ng/mL of salmon testes DNA

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

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

4.1.4. Mouth pipetting is prohibited.

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

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 mold plate counts procedure (see NYS DOH LEB-605).

5.4. Preservation

5.4.1. SDA plates used for mold plate counts that are presumptive positive for fungi are stored refrigerated until it has been determined that they are not needed for additional evaluation.

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

7.0. Apparatus and Materials

7.1. Equipment

7.1.1. Automatic pipetters and sterile micropipette tips
7.1.2. Pipette-aid
7.1.3. Pipettes, sterile – 25mL, 10mL, and 5mL
7.1.4. Extraction tubes containing sterile glass beads – Generite Cat. no. S0205
7.1.5. 1.7mL microcentrifuge tubes, sterile
7.1.6. 15mL conical tubes, sterile
7.1.7. Sterile inoculating loops, 10µL
7.1.8. 8-position mini bead beater – Biospec Products Inc., Cat. no. 693 or equivalent.
7.1.9. Rotamix rotating mixer – Appropriate Technical Resources, Laurel MD, cat no. RKVSD, or equivalent
7.1.10. Spectrophotometer and quartz cuvettes
7.1.11. Quebec colony counter
7.1.12. NIST traceable weights
7.1.13. Balance
7.1.14. Biosafety cabinet with HEPA filter

7.2. Reagent and Chemicals

7.2.1. AE buffer, pH 9.0 – Qiagen Cat. no. 19077.

7.2.2. Salmon testes DNA – Sigma-Aldrich, Cat. no. D1626 or equivalent

7.2.3. PCR grade water, OmniPur water from VWR (EM-9610) or equivalent. Water must be DNase/RNase free.

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

7.3. Forms

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

7.3.2. Mold Identification by RT-PCR Result Sheet (e.g., LEB-RS-609B)

7.3.3. Mold RT-PCR Analyses Bench Sheet (e.g., LEB-RS-618A)

7.3.4. Mold Identification RT-PCR Targets Appendix (e.g., LEB-AP-618D)

8.0. Quality Control/Assurance

8.1. Method Detection Limits

8.1.1. Method Detection Limits are product-specific and are determined in accordance with relevant standards, regulations and Accreditation Body requirements.

8.2. Calibration and Standardization

8.2.1. 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 Record.

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

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

8.2.1.3. 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.

8.2.1.4. 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.

8.2.2. Temperature of the freezer is observed and recorded twice daily, separated by at least 4 hours.

8.2.2.1. Temperatures are recorded on the Freezer Temperature Record.

8.2.2.2. If freezer temperature exceeds -15.0, follow laboratory-specific corrective actions.

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 as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

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

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

8.2.7. The spectrophotometer is calibrated at least annually according.

8.2.8. 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. Sterile AE buffer can be stored for several years at 1.0-8.0°C.

8.3.2. The 1mg/mL stock concentration of Salmon Testes DNA can be stored for several years frozen.

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. For any sample containing ≥ 5 mold colonies having similar morphology on SDA plates (prepared according to NYS DOH LEB-605), record colony morphologies and the total number of similar colonies (e.g., LEB-RS-609B) and proceed to 9.2.

9.1.2.1. If one fifth of the diluted sample aliquot was plated (see NYS DOH LEB-605), then every mold colony is equivalent to 5 colonies and must be identified.

9.2. Preparation of Stock Solutions

9.2.1. AE Buffer

9.2.1.1. AE buffer is aseptically dispensed into 15mL conical tubes and stored long term at 1.0-8.0°C.

9.2.2. 1mg/mL Salmon Testes DNA Stock

9.2.2.1. Place an empty, sterile 15mL conical tube on the balance and tare the balance.

9.2.2.2. Using flame-sterilized scissors and forceps cut a small piece of lyophilized salmon testes DNA and place in the 15mL conical tube.

9.2.2.3. Add enough PCR grade water to make a final concentration of 1mg/mL of DNA.

9.2.2.3.1. For example, if the piece of lyophilized DNA weighs 9.5mg, add enough PCR grade water for a final volume of 9.5mL.

9.2.2.4. Place the tube on a rotating mixer, and rotate for 2 hours, at room temperature, to allow the DNA to completely dissolve into solution.

9.2.2.5. Once dissolved, remove three, 10µL aliquots and dilute each to 1000µL with PCR grade water.

9.2.2.6. Using a spectrophotometer and quartz cuvette, check the absorbance (OD_{260}) of each aliquot.

9.2.2.7. Average the three absorbances, and calculate the amount of DNA in each aliquot by using the equation of an OD_{260} of 1 is equivalent to a concentration of 50µg/mL.

9.2.2.7.1. For example:

OD_{260} of the 1:100 dilutions are 0.3050, 0.3056, and 0.3101

(0.3050+0.3056+0.3101)/3 x 100 x 50µg/mL = 1534.5µg/mL

9.2.2.8. Vortex to mix.

9.2.2.9. Adjust the stock solution to bring the final concentration to 1mg/mL DNA using DNase/RNase free water.

9.2.2.10. Aliquot the solution into 1.7mL tubes and store at -20°C.

9.2.2.10.1. Make 10-20 aliquots of 20µL and aliquot the rest as 1mL aliquots.

9.2.2.10.2. Label the tubes with the DNA concentration and date of preparation.

9.2.2.10.3. The 1mg/mL stock concentration can be stored long term at -20°C.

9.2.2.11. Alternatively, pre-made stocks of Salmon testes DNA can be purchased.

9.2.3. Extraction Buffer

9.2.3.1. Extraction buffer is made fresh daily.

9.2.3.1.1. Each sample requires 500µL of extraction buffer.

9.2.3.2. Thaw an aliquot of a 1mg/mL concentration of salmon testes DNA.

9.2.3.2.1. Label the tube with the date it was thawed.

9.2.3.2.2. After the first thaw, this aliquot is good for one month when stored at 1.0-8.0°C.

9.2.3.3. Vortex to mix and briefly centrifuge.

9.2.3.4. Using a pipette-aid and sterile 5mL pipette, aseptically add 5mL of AE buffer to a sterile 15mL conical tube.

9.2.3.5. Using a micropipettor and sterile pipette tips, aseptically add 1µL of the thawed salmon testes DNA to the conical tube containing 5mL AE buffer.

9.2.3.5.1. Increase the volumes of the solutions according to the number of samples being extracted (1 sample = 500 µL extraction buffer).

9.2.3.5.1.1. For example, if there are 20 samples being extracted add 2µL of salmon testes DNA to 10mL of AE buffer.

9.2.3.5.1.2. Final concentration of Salmon Testes DNA (Sketa) in AE buffer =200ng/mL

9.2.3.6. Vortex to mix.

9.2.3.7. Extraction buffer is also used as a positive control for the Sketa assay (see LEB-618). The extraction buffer used as a control should come from the same preparation date as the extracted unknown samples to minimize the differences in CT values.

9.2.4. Preparation of DNA controls

9.2.4.1. The positive and negative control DNA needed for each RT-PCR assay are listed in the Mold Identification RT-PCR Targets Appendix (e.g., LEB-AP-618D).

9.2.4.2. It is recommended that control organisms grown on SDA are incubated for 7 days prior to harvesting the cells to allow for the highest DNA yield.

9.2.4.3. DNA controls are extracted according to section 9.4 **using AE buffer only**. No salmon DNA is added to the AE buffer when extracting the DNA controls.

9.2.4.4. While not required, the extracted DNA for control organisms may also be purified using DNA purification kits.

9.2.4.5. After extraction, the control DNA is analyzed via RT-PCR (see NYS DOH LEB-618) to ensure the CT values are less than 25.

9.2.4.5.1. A good DNA yield will have CT values between 16-22.

9.2.4.5.2. If CT values do not fall between 16-22, reprocess the DNA controls starting from step 9.2.4.1.

9.2.4.6. After extraction the control DNA can be aliquoted and frozen for several years if the routine use of the control DNA doesn't indicate degradation (i.e., increased CT values over time).

9.3. Colony Selection

9.3.1. Record the appearance of each distinct colony morphology observed on the SDA plates (prepared according to NYS DOH LEB-605A) on the Mold Identification Results Sheet (e.g., LEB-RS-609B).

9.3.2. Record if the described morphology was observed on the sample or matrix spike SDA plates on the Mold Identification Results Sheet (e.g., LEB-RS-609B).

9.3.3. Record the number of each distinct morphology type observed on the Mold Identification Results Sheet (e.g., LEB-RS-609B).

9.3.4. A representative from each morphology that has 5 or more observed colonies is used for DNA extraction.

9.3.4.1. It is recommended, but not required, that a representative colony from each morphology is used for DNA extraction regardless if there are 5 or more colonies.

9.3.5. If there are overlapping colonies that will be used for DNA extraction, use a sterile loop to transfer a small portion of each colony to separate SDA plates and streak for isolation.

9.3.6. The SDA plates are incubated at 20.0-25.0°C for 3-7 days.

9.3.7. Record the incubation dates and times on the Mold Identification Results Sheet (e.g., LEB-RS-609B).

9.4. DNA Extraction for RT-PCR Identification

9.4.1. For every day DNA is extracted from unknown samples, an extraction blank is processed along with the unknown samples. The extraction blank contains only 500µL extraction buffer and no DNA.

9.4.2. Aseptically transfer a small portion of the colony from the SDA plate using a sterile inoculation loop and add to an extraction tube containing 300mg glass beads and 500µL of extraction buffer.

9.4.2.1. Do not gouge the agar.

9.4.3. Cap the extraction tube and bead beat at maximum speed for one minute.

9.4.4. Centrifuge the extraction tube for 1 minute at 12,000 x g to pellet the debris.

9.4.5. Aseptically transfer as much supernatant as possible to a sterile 1.7mL microcentrifuge tube.

9.4.6. Centrifuge the sample tube for 5 minutes at 12,000 x g.

9.4.7. Aseptically transfer as much supernatant as possible to a second sterile, labeled, 1.7mL microcentrifuge tube taking care not to transfer any of the pellet or leftover glass beads.

9.4.8. Refrigerate the DNA lysates at 1.0-8.0°C until identification by RT-PCR (NYS DOH LEB-618) is completed.

9.4.8.1. DNA lysates can be stored long term at -20°C.

9.4.9. Proceed to NYS DOH LEB-618 to set up and run the RT-PCR assay using primer and probe sets listed in the Mold Identification RT-PCR Targets Appendix (e.g., LEB-AP-618D).

9.4.10. DNA extracted for control organisms are stored in 5µL aliquots at -20°C.

9.5. Identification of Mold Colonies

9.5.1. Follow NYS DOH LEB-618 to screen individual colony types for the presence of ribosomal DNA sequences indicative of organisms belonging to the *Penicillium, Aspergillus* or the *Mucor* genera.

9.5.2. If the colony DNA is positive for *Aspergillus/Penicillium/Paecilomyces* using the Pan-Asp and/or PenAsp1mgb assays, then the DNA is further analyzed for the presence of *A. flavus*, *A. fumigatus*, *A. niger*, and *A. terreus* according to NYD DOH LEB-618 and using the species-specific primer/probe sets listed in LEB-AP-618D.

10.0. Data Acquisition, Reduction and Documentation

10.1. Record the accession number, analyst initials, colony morphologies, number of colonies of each morphology type, and source (matrix spike or sample) on the Mold Identification Results Sheet (e.g., LEB-RS-609B).

10.2. If individual colonies overlapped and needed to be streaked for isolation, record the start and end dates and times of incubation on the Mold Identification Results Sheet (e.g., LEB-RS-609B).

10.3. From the Mold RT-PCR Analyses Bench Sheet (e.g., LEB-RS-618A), record RT-PCR run end date and time and the name of the run on the Mold Identification Results Sheet (e.g., LEB-RS-609B).

10.4. For each unique colony morphology described on the Mold Identification Results Sheet (e.g., LEB-RS-609B), record the results from the Mold RT-PCR Analyses Bench Sheet (e.g., LEB-RS-618A) as follows:

10.4.1. If the DNA from a colony was positive for the presence of *Aspergillus/Penicillium/Paecilomyces* species (either Pan-Asp and/or PenAspmgb1), but not any of the four individual *Aspergillus* species (*A. flavus*, *A. fumigatus*, *A. niger*, and/or *A. terreus*), record the colony identification as *Aspergillus/Penicillium/Paecilomyces* species.

10.4.2. If the DNA from a colony was positive for the presence of any of the four individual *Aspergillus* species (*A. flavus*, *A. fumigatus*, *A. niger*, and/or *A. terreus*), record the species identified.

10.4.3. If the DNA from a colony was positive for the presence of *Mucor/Rhizopus* species (muc1) record the colony identification as *Mucor/Rhizopus* species.

10.4.4. If the DNA from a colony was negative for the presence of *Aspergillus/Penicillium/Paecilomyces* and/or *Mucor/Rhizopus* DNA record the colony identification as “unidentified” on the Mold Identification Results Sheet (e.g., LEB-RS-609B).

10.5. Record numbers of colonies that were identified as *Aspergillus/Penicillium/Paecilomyces* species, *Mucor/Rhizopus* species, *A. flavus, A. fumigatus, A. niger,* and/or *A. terreus* on the Mold Identification Results Sheet (e.g., LEB-RS-609B).

10.6. In the "Final Results" section at the top of the Mold Identification Results Sheet (e.g., LEB-RS-609B), circle the appropriate positive or negative options based on the identifications listed above.

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

11.2. Laboratory Detection Limits

11.2.1. Approximately 1 spore/ μL.

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. APHA. *Standard Methods for the Examination of Water and Wastewater,* 20th edition.

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

13.4. NYS DOH LEB-618, Set-Up and Analysis of RT-PCR Assays for Mold Identification in Medical Marijuana Products

13.5. 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

Mold Identification Results Sheet

Accession Number: ____________________ Analyst Initials: ____________________

Final Results (circle one): Negative for Molds Positive for *Aspergillus/Penicillium/Paecilomyces*

Positive for *Mucor/Rhizopus*

Colony Isolation (if necessary)

SDA Start Date/Time: ____________________ SDA Lot Date: ____________________
SDA End Date/Time: ____________________

RT-PCR End Date/Time: ____________________ RT-PCR Run Name(s): ____________________

	No. of colonies	Source (M.S. or Sample), Colony Morphology, and qPCR Identification
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

SDA = Sabouraud Dextrose Agar, SDB = Sabouraud Dextrose Broth
All qPCR assay bench sheets (LEB-RS-618A) are attached.

Total no. colonies identified

Aspergillus/Penicillium/Paecilomyces*:** ***Mucor/Rhizopus:

***Asp. niger*:** ***Asp. terreus*:** ***Asp. flavus*:** ***Asp. fumigatus*:**

Reviewed by: Date: