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-609

Mold Identification Using qPCR for Medical Marijuana Testing

Contents

1.0. Scope and Application

1.1. This protocol is used to identify *Aspergillus, Mucor* and *Penicillium* spp. using real-time quantitative PCR (qPCR).

2.0. Summary of the Method

2.1. Fungal colonies growing on Sabouraud Dextrose agar plates (prepared according to NYS DOH LEB-605) are screened for the presence of potentially pathogenic molds. A loopful of a colony is transferred from the plate to a tube containing buffer, DNA is extracted, and the DNA is analyzed using primers and probes specific for *Aspergillus/Penicillium* spp. and *Mucor* spp. using real-time PCR.

3.0. Definitions

3.1. SDA stands for Sabouraud Dextrose Agar
3.2. CFU stands for colony forming unit
3.3. qPCR stands for real-time, quantitative, polymerase chain reaction amplification
3.4. Extraction buffer is AE buffer with 2ng/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 microbiological 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 qPCR Targets Appendix (see NYS DOH LEB-618 Appendices - LEB-AP-618D)
7.3.3. Mold Identification by qPCR Result Sheet (e.g., LEB-RS-609B)

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 (made 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.

9.2.3. Extraction Buffer

9.2.3.1. Extraction buffer is made fresh daily.

9.2.3.2. Thaw a 20µL 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. 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.

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.

9.2.3.6. Vortex to mix.

9.3. DNA Extraction for qPCR Identification

9.3.1. Aseptically remove the colony from the SDA plate using a sterile inoculation loop and add to an extraction tube containing 300mg glass beads and 0.5mL of extraction buffer.

9.3.1.1. For each day DNA extractions are performed, analysts also make an extraction control by adding 500µL of extraction buffer to an extraction tube and following steps 9.3.2-9.3.6.

9.3.2. Cap the extraction tube and bead beat at maximum speed for one minute.
9.3.3. Centrifuge the extraction tube for 1 minute at 12,000 x g to pellet the debris.
9.3.4. Aseptically transfer as much supernatant as possible to a sterile 1.7mL microcentrifuge tube.
9.3.5. Centrifuge the sample tube for 5 minutes at 12,000 x g.
9.3.6. 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.
9.3.7. Refrigerate the DNA lysates at 1.0-8.0°C until identification by qPCR.
9.3.7.1. DNA lysates can be stored long term at -20°C.
9.3.8. Proceed to NYS DOH LEB-618 to set up and run the qPCR assay using primer and probe sets listed in Mold Identification qPCR Targets Appendix (see NYS DOH LEB-618 Appendices - LEB-AP-618D).
9.3.9. The method in this section is also used for extracting DNA from positive and negative control organisms. Positive and negative controls for each qPCR assay are listed in NYS DOH LEB-618 Appendices - LEB-AP-618D.
9.3.10. DNA extracted for control organisms are stored in 5µL aliquots at -20°C.

9.4. Results Recording

9.4.1. Record the number of colonies of each morphology type, source (matrix spike or sample), colony morphology and qPCR identification (e.g., LEB-RS-609B).
9.4.2. If individual colonies overlap, transfer a representative example of each fungal colony showing similar morphology onto SDA for re-isolation.
9.4.3. 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.
9.4.4. If *Penicillium/Aspergillus* are identified using the qPCR screen for the presence of rDNA sequences belonging to the *Penicillium/Aspergillus* group (Pan Asp and PenAsp1mgb), then the DNA is screened for *Asp. terreus, Asp. flavus, Asp. fumigatus*, and *Asp. niger* according to NYS DOH LEB-618 and using the species-specific primer/probe sets listed in LEB-AP-618D (See NYS DOH LEB-618 Appendices).

10.0. Data Acquisition, Reduction and Documentation

10.1. If subculture (colony isolation) was necessary, record SDA start/end dates and times of incubation, and SDA lot date (e.g., LEB-RS-609B).
10.2. Record the analyst initials, qPCR run date, qPCR Run Name, number of colonies of each morphological type picked, source, colony morphology and qPCR identification (e.g., LEB-RS-609B).
10.3. Report numbers of CFUs identified as *Aspergillus/Penicillium* species, *Mucor* species, *Asp. niger, Asp. terreus, Asp. fumigatus,* and/or *Asp. flavus*.

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 qPCR 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 by qPCR Results Sheet (LEB-RS-609B)

Accession Number: _____ Analyst Initials: _____

Final Results (circle one): Negative for Molds Positive for Molds _____

Colony Isolation (if necessary)

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

qPCR End Date/Time: _____ qPCR 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.

Total no. colonies: ***Aspergillus/Penicillium*:** ***Asp. niger*:** ***Asp. terreus*:**

***Mucor*:** ***Asp. flavus*:** ***Asp. fumigatus*:**

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Reviewed by Date