REAL-TIME PCR BASED IDENTIFICATION OF CANDIDA AURIS USING APPLIED BIOSYSTEMS 7500 FAST REAL-TIME PCR PLATFORM

1.0 Purpose
The purpose of this procedure is to describe the process for identification of Candida auris using the Applied Biosystems 7500 Fast Real-time PCR platform. This protocol will be used for reference as well as clinical specimens.

2.0 Scope
This document applies to laboratory personnel responsible for CLIA regulated processing and testing of clinical specimens for C. auris, using the ABI 7500 Fast Real-Time PCR System.

3.0 Responsibility
3.1 It is the responsibility of the Technical Supervisor to approve all deviations, reconcile conflicting results, determine supply substitutions, and review manufacturer’s recommendations and procedures.
3.2 It is the responsibility of the Technical Supervisor and the General Supervisor to ensure the Testing Personnel performing this procedure is sufficiently trained and that training is documented prior to performance.
3.3 It is the responsibility of the Testing Personnel to follow all procedures listed in this document, document all deviations, complete all required documentation, and comply with all laboratory safety procedures.
3.4 It is the responsibility of the Testing Personnel to strictly follow all policies and safety practices governing laboratory operations, including the use of appropriate PPE; reporting to the supervisor any medical restrictions, reportable illnesses, and any event that may suggest a laboratory exposure or result in the creation of a potential hazard.
3.5 It is the responsibility of the Laboratory Safety Officer to review SOPs and Risk Assessments for safe work practices, provide support to lab personnel as needed, and routinely monitor laboratory spaces.

4.0 Definitions
4.1 BLB – Bacterial Lysis Buffer
4.2 BSC – Biological Safety Cabinet
4.3 ECSL – Enhanced Capacity Surge Lab
4.4 NTC – No Template Control (blank)
4.5 PCR – Polymerase Chain Reaction
4.6 **RCF** – Relative Centrifugal Force, the amount of accelerative force applied to a sample in a centrifuge. RCF is measured in multiples of the standard acceleration due to gravity at the Earth’s surface (x g). RCF and “x g” (units of gravity) are used interchangeably in centrifugation protocols.

5.0 **References**
5.1 Biosafety in Microbiological and Biomedical Laboratories (BMBL), current edition
5.2 Biosafety Manual
5.3 Chemical Hygiene Plan
5.4 *MagNa Pure System Application Note No. 5*
5.5 *MagNA Pure 96 Automated Nucleic Acid Extraction SOP*

6.0 **Equipment/Materials**
6.1 Biological Safety Cabinet (BSC), Certified Class II A2
6.2 PCR enclosure
6.3 Applied Biosystems 7500 Fast Real-time PCR instrument with SDS Software version 1.4
6.4 Roche MagNA Pure 96 [or equivalent system to be determined]
6.5 Shaking Incubator (65°C)
6.6 Calibrated Pipets (P2/10, P20, P200, P1000)
6.7 Aerosol barrier (filter) tips
6.8 96 well cooling rack
6.9 Bacterial Lysis Buffer (Roche; Cat# 04659180001)
6.10 2 mL Sarstedt tubes
6.11 RNase/DNase-free 1.5mL microcentrifuge tubes
6.12 ABI MicroAmp Fast Optical 96-Well Reaction Plate, 0.1mL
6.13 ABI MicroAmp Optical 8-cap strip
6.14 ABI MicroAmp Adhesive optical film
6.15 Molecular Grade RNase/DNase free water
6.16 MagNA Pure 96 DNA and Viral RNA Small Volume Kit (Roche; Cat# 06543588001) [Or equivalent kit to be determined]
6.17 *Candida auris* primers and probes
6.18 *Bicoid* specific primers and probes
6.19 5× PerfeCTa Multiplex qPCR ToughMix (Quanta Biosciences; Cat# 95147-250)
6.20 Template DNA (DNA from Specimens)
6.21 *Bicoid* inhibition control plasmid DNA
6.22 BleachRite, 10% bleach solution  
6.23 ELIMINase or similar reagent for removal of DNase and RNase  
6.24 70% Ethanol

Note: If you do not have Bicoid DNA, it is acceptable to substitute another non-human, non-fungal control DNA in this assay along with the appropriate primers and probe.

7.0 Safety Precautions
7.1 Clinical *Candida auris* specimens (Swabs/Sponge) or isolates have the potential to transmit infectious diseases. Wear protective gloves, lab coat, and eyewear when handling samples.
7.2 All BSL-2 practices, safety equipment, and facility design must follow the requirements described in the BMBL and the Biosafety Manual.
7.3 All biohazardous liquid and solid waste and sharps are handled per agency-specific waste handling policies.
7.4 Laboratory personnel should complete appropriate safety training and *Candida*-specific laboratory training.
7.5 Review safety section of equipment manuals prior to performing procedure.
7.6 Review all SDS for chemicals used in this procedure.
7.7 Follow institutional guidelines for Spills and Incident Reporting.
7.8 Work with unidentified and pathogenic yeast must be performed in an operational Class II A2 BSC.

8.0 Procedure

Sample Information
8.1 Specimen tubes received for testing must be intact, labeled with two unique identifiers (e.g. patient ID and specimen ID) and must be accompanied by a submission form.
8.2 All patient skin swabs are collected using BD ESwab collection and transport system (Catalog# 220245; Becton Dickinson and Company, Sparks, MD).
8.3 Following collection, swabs are placed in 1mL Amies buffer for storage and transport.
8.4 Specimens should be stored at 4°-25°C, and shipped with a cold pack to the laboratory for processing within 96 hours of collection.
8.5 Specimen rejection criteria:
  8.5.1 Specimens received >4 days after collection
  8.5.2 Damaged or visible leakage of transport tubes
  8.5.3 Specimen without submission forms
  8.5.4 Specimen without identifiers
8.6 Individual institutions submitting swabs are responsible for the compliance with local human subjects/Institutional Review Board (IRB)
regulations or applying for the IRB exemption for public health outbreak surveillance and emergency response.

Reagent Handling and Storage
8.7 Store all primers and probes at 2-8°C until re-hydrated for use; store all control materials at -20°C.
8.8 Always check the expiration date prior to use. Do not use expired reagents.
8.9 Protect fluorogenic probes from light.
8.10 Primers, probes (including aliquots), and enzyme master mix must be thawed and kept on ice or cold block at all times during preparation and use.
8.11 Controls DNA must be thawed and kept on ice at all times during preparation and use.
8.12 Clean and decontaminate all work surfaces, BSC, pipettes, and other equipment with a proper decontamination solution such as 10% bleach solution before and after use.
8.13 Use separate and dedicated equipment (e.g., BSC, pipettes) and supplies (e.g., microcentrifuge tubes, pipette tips) for master mix preparations, setup of Optical 96-Well reaction plate, and addition of DNA template in the wells.
8.14 Reagents, master mix, and DNA should be maintained on cold block during preparation and/or use to ensure stability.

Quality Control
8.15 A positive and a negative control ESswab must be processed each day a new set of specimen swabs is processed.
8.16 Prepare the positive control:
   8.16.1 Using a C. auris B11220 stock plate that is < 1 month old, touch a single 1 mm colony with the swab and place the soft end of the collection swab into the tube containing transport medium. [Any C. auris isolate from your laboratory can be substituted here.]
   8.16.2 Snap off the end of the swab at the marked line by bending the plastic handle against the edge of the transport tube and secure the tube cap.
8.17 Prepare the negative control:
   8.17.1 Place a clean swab into the ESwab transport medium. Take care not to contaminate the negative control by touching the swab or cap.
   8.17.2 Snap off the end of the swab at the marked line by bending the plastic handle against the edge of the transport tube and secure the tube cap.
8.18 Process the positive and negative control the same way as clinical specimens following Specimen Processing procedure below.
8.19 Positive Control
   8.19.1 There should be a positive PCR result for the positive control.
   8.19.2 If the result is negative, invalidate patient specimen results and retest starting from the primary swab.

8.20 Negative Control
   8.20.1 There should be no PCR result for the negative control.
   8.20.2 If there is a positive result, invalidate patient specimen results and retest starting from the primary swab.

8.21 No Template Control
   8.21.1 NTCs consist of 5µL PCR grade water spiked into the reaction mix in place of 5µL extracted DNA.
   8.21.2 NTCs must be included in each run and return negative results.
   8.21.3 If result is positive, invalidate patient specimen results and rerun the PCR, starting post extraction.

Specimen Processing
8.22 Assign an Experiment Number for testing.
8.23 Specimens may be stored at 2-8°C or ≤ -20°C prior to analysis. Freeze thaw cycles have been shown to not affect the functionality of the assay, as described by Leach L, Zhu Y, Chaturvedi S. Development and Validation of a Real-Time PCR Assay for Rapid Detection of Candida auris from Surveillance Samples. J Clin Microbiol., 2018 Jan 24; 56(2), e01223-17.
8.24 Perform all specimen handling steps below in a BSC.
8.25 Verify all specimen information on the tube matches the specimen form.
8.26 Record the ID for each specimen on a Nucleic Acid Extraction Log

DNA Extraction from Dermal Swabs
8.27 Label a 2mL tube with the specimen ID.
8.28 Working in a BSC, vortex the ESwab to release the specimen from the swab into the Amies buffer.
8.29 Transfer 100µL of the Amies buffer into the labeled 2mL tube.
8.30 Add 100µL Bacterial Lysis Buffer (BLB) to each tube.
8.31 Add 20µL Proteinase K to each tube and cap each tube.
8.32 Vortex for few seconds.
8.33 Place the capped tube in 65°C incubator for 10 min with shaking.
8.34 Return the tube to the BSC and transfer 200µL liquid sample to a MagNA Pure 96 processing cartridge.
8.35 Follow the procedure for MagNA Pure 96 Automated Nucleic Acid Extraction SOP using the following specifications:
   8.35.1 Reagent Kit: DNA and Viral RNA Small Volume Kit
   8.35.2 Protocol: Pathogen Universal 200 3.1
   8.35.3 Elution Volume: 50µL
Note: If you do not have a MagnaPure96, you can validate another extraction platform.

Master Mix Preparation and Setup of 96-Well reaction plate
8.36 Prior to use, decontaminate PCR enclosure and pipets with ELIMINase and 70% ethanol to minimize the risk of nucleic acid contamination.
8.37 Working in a PCR enclosure, place 5× PerfeCTa Multiplex qPCR ToughMix, Primers and Probes on cold block at 4-8°C.
8.38 Briefly centrifuge 5× PerfeCTa Multiplex qPCR ToughMix, Primers and Probes, then place on cold block.
8.39 Complete a Reaction Plate Setup Table
8.40 Assign a detector and a control or sample to a designated well.
8.40.1 All analyses should be run in duplicate
8.41 Determine the number of reactions being performed per assay. Be sure to account for all positive and negative controls, and duplicates. Note: If the number of samples and controls is <14 make enough master mix for one extra reaction, if the total number is >14 make enough for two extra reactions. This will account for pipette error.
8.42 Calculate the amount of each reagent needed to make up each master mix using the Master Mix Preparation Worksheet, Attachment # 1.
8.43 Combine all reagents listed on Attachment # 1 in the corresponding labeled tube using an appropriate size pipette.
8.44 Pulse vortex master mix 3 times for 5 seconds to homogenize.
8.45 After all master mixes have been prepared, prepare the NTC reactions.
8.45.1 Refer to the Reaction Plate Setup Table and dispense appropriate volume of NTC master mix into the wells designated for NTC reactions.
8.45.2 Securely cap all NTC reactions.

DNA Template Addition
8.46 Working in a BSC, gently vortex extracted nucleic acid samples and positive control tubes and place on ice or in a cold rack.
8.47 Set up reaction plate on ice or in a cooler rack.
8.48 Refer to Master Mix Preparation Worksheet, Attachment #1 and add appropriate volume of Bicoid DNA (1µL/Reaction) in appropriate master mix tubes.
8.49 Pulse vortex master mix 3 times for 5 seconds to homogenize.
8.49.1 Dispense 15µL of prepared master mix containing Bicoid DNA into each corresponding well.
8.49.2 Carefully pipette 5µL of samples (Candida auris DNA) and positive controls into the corresponding wells. Change tips between each sample.
8.49.3 After template addition, securely cap the wells to prevent cross contamination.
8.49.4 Spin down the capped/sealed plate in a centrifuge for 30 seconds at 1000 rcf.

**ABI 7500 Fast Run Setup**

8.50 Create a run template.

*Note: Use Reporter dye FAM and Cy3 for Candida auris and Bicoid, respectively.*

8.51 Define the Instrument Settings

8.51.1 Select the **Instrument tab** to define thermal cycling conditions.

8.51.2 Modify the thermal cycling conditions as follows:

8.51.2.1 *In Stage 1, Set to 20 Sec at 95°C; 1 Rep*

8.51.2.2 *In Stage 2, Step 1 set to 03 Sec at 95°C*

8.51.2.3 *In Stage 2, Step 2 set to 30 Sec at 60°C*

8.51.2.4 *In Stage 2, Reps should be changed to 45*

8.51.2.5 *Under Setting, bottom left box, set volume to 20 μL*

8.51.2.6 *Stage 2 Step 2 should be highlighted in yellow to indicate data collection*

8.51.3 Save the file by selecting Save As and give file name as CAP000X_Date (X = Experiment number). Save as type should be SDS Templates (*.sdt).

**Data Analysis**

8.52 After the run has completed perform data analysis.

8.53 The cutoff value established for this assay is XX:

8.53.1 A sample with a Ct value ≤ XX is positive for *C. auris*

8.53.2 A sample with a Ct value > XX is negative for *C. auris*

8.54 Sample duplicates that do not have corresponding outcomes (ie. one positive and one negative) must be rerun for confirmation using the extracted DNA. If still no result, report as Inconclusive.

*Note: The appropriate cutoff value will be determined by each laboratory during the validation phase.*

**Results Review and Approval**

8.55 Enter valid results into ELIMS as results.

8.56 All results will be approved by the designated Technical Supervisor prior to reporting.

9.0 **Attachments**

Attachment 1: Master Mix Preparation Worksheet (1 Page)

10.0 **Revision History**
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**11.0 Approval Signatures**

Approved By: ___________________________ Date: __________

__Author__

__________________________

Print Name and Title

Approved By: ___________________________ Date: __________

__Technical Reviewer__

__________________________

Print Name and Title

Approved By: ___________________________ Date: __________

__Quality Manager / Designee__

__________________________

Print Name and Title

__________________________
# Master Mix Preparation Worksheet

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## Equipment Information

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## Reagent Information

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Team Lead or Designee Review (Sign/Date): _______________________________________

Quality Review (Sign/Date): ______________________________________________________

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Attachment #1

UNCONTROLLED DOCUMENT