Amplification of Selected Gene Fragments from Fungal DNA Using the Polymerase Chain Reaction (PCR)

Reagents/Materials Required:

- Fungal DNA (your isolate)
- Control DNA (genomic DNA from *Penicillium marneffei*)
- Nuclease-free water
- AmpliTaq Gold Master Mix (2X)
- 200 µl PCR tubes, 10 (various colors, if desired)
- 600 µl microfuge tube, 2 (various colors, if desired)
- Primers AAA solution, 20 µM each forward and reverse primer
- Primers LR solution, 20 µM each forward and reverse primer
- Primers CHS solution, 20 µM each forward and reverse primer
- Primers ACT solution, 20 µM each forward and reverse primer
- Model PTC-200 thermocycler
- Pipette tips/mechanical pipette, various sizes/models

Procedure:

Note 1: Be sure not to contaminate any materials with exogenous sources of DNA/primers. Change pipette tips as often as necessary to avoid cross-contamination. Wipe off the barrels of the mechanical pipettes with dilute bleach and/or 70% ethanol.

Note 2: Be sure label and/or color code all your tubes/mixtures. Record these codes in your notebook for future reference.

1. Prepare the experimental reaction mixture by adding the following components, in order, to a sterile 600 µl microfuge tube using an appropriate pipette tip:l

Nuclease-free water	55.0 µl
2X AmpliTaq Gold MasterMix	137.5 µl

2. Pipette 35 µl of the experimental reaction mixture prepared in step 1 to each of five (5) 200 µl PCR microfuge tubes. Discard any remaining experimental reaction mixture.
3. Add primers or nuclease-free water to each 200 µl PCR tube containing the experimental reaction mix (from step 2) as follows:
    a. To one PCR tube, add 10 µl of Primers AAA (20 µM) and 5 µl of the DNA that you previously extracted from your fungal isolate.
    b. To a second PCR tube, add 10 µl of Primers CHS (20 µM) and 5 µl of the DNA that you previously extracted from your fungal isolate.
    c. To a third PCR tube, add 10 µl of Primers LR (20 µM) and 5 µl of the DNA that you previously extracted from your fungal isolate.

d. To the fourth PCR tube, add 10 µl of Primers ACT (20 µM) and 5 µl of the DNA that you previously extracted from your fungal isolate.

e. To the remaining PCR tube, add 10 µl of Primers LR (20 µM) and 5 µl of nuclease-free water. This tube will serve as your negative control reaction that will determine if there has been any contamination in your PCR preparations. That is, you expect that no DNA will be amplified form this reaction tube.

4. Prepare the control (positive) reaction mixture by adding the following components, in order, to a sterile 600 µl microfuge tube using an appropriate pipette tip:

Control fungal DNA	4.5 µl
Nuclease-free water	63 µl
2X AmpliTaq Gold MasterMix	112.5 µl

5. Pipette 40 µl of the control reaction mixture prepared in step 1 to each of four (4) 200 µl PCR microfuge tubes. Discard any remaining control reaction mixture.

6. Add primers to each 200 µl PCR tube containing the control reaction mix (from step 4) as follows:

   a. To one PCR tube, add 10 µl of Primers AAA (20 µM).

   b. To a second PCR tube, add 10 µl of Primers CHS (20 µM).

   c. To a third PCR tube, add 10 µl of Primers LR (20 µM).

   d. To the remaining PCR tube, add 10 µl of Primers ACT (20 µM).

7. One of the Model PTC-200 thermocyclers (labeled ‘Cooper’) is fitted with a dual block head. Turn on this thermocycler using the switch on the back of the machine. Permit it to run through its diagnostic cycle.

8. Evenly space all of your PCR tubes in the holes under one of the lids of the dual block head, either the left one (Block A) or the right one (Block B). Appropriately adjust, then close the lid of the head carefully so as not to be too snug against the tubes.

9. Begin the PCR by running program 6948-59 on the thermocycler as follows:

   a. Use the “Select” buttons (above the key pad) to place the cursor next to the text marked as “RUN”. Press the “Proceed” button (to the right of the key pad) to continue.

   b. Move the cursor using the “Select” buttons next to the text marked as “<MAIN>”. Press the “Proceed” button to continue.

   c. Move the cursor using the “Select” buttons next to the text marked “6948-59”. Press the “Proceed” button to continue.

   d. The screen and a blinking light will indicate which block, Block A or Block B, is currently set. If you wish to switch to a different block, tap on the “Block” button to the left of the key pad. The screen and the blinking light

should indicate the newly set block to be used. Press the “Proceed” button to continue.

e. The screen will indicate the “Vessel Type” currently set. If the text “TUBES” is not shown in capital letters, move the cursor using the “Select” buttons next to it, then press the “Proceed” button to continue.

f. The screen will indicate the “Volume” currently set. If the number is not “50”, then use the key pad to enter this value. Once set, press the “Proceed” button to continue.

g. The screen will ask for “Heated lid?” Move the cursor using the “Select” buttons next to “YES”, then press the “Proceed” button to continue. The amplification program will begin immediately. It will take approximately 2 hours and 7 minutes to complete. The tubes will then be held at 4°C until retrieved.

10. As soon as possible, these tubes should be removed from the thermocycler as follows:

    a. Press the “Proceed” button and the program will move to the last step, “END”, and the thermocycler will cease holding at 4°C.

       Note: Be sure that you have selected the block that you are using prior to pressing the “Proceed” button. If not, you may inadvertently cause the program on the other block to cease running.

    b. Remove the PCR tubes and either process them for analysis by horizontal gel electrophoresis or store them at -20°C until the analysis can be performed.

11. Turn off the thermocycler.

    Note: Do not turn off the thermocycler if the other block is being used.