Fungal DNA sequencing from laser capture microdissection samples

Introduction

The direct identification of human fungal pathogens by DNA sequencing is currently limited to specimens from sputum, cerebral spinal fluid, or other types of body fluids [1]. Currently, there are difficulties in using solid tissues for fungal identification by DNA sequencing or other available methods. Histopathological fungal identification based on their morphological appearance in tissues is frequently inconclusive due to low discrimination power and high error rate. Likewise, the use of cultures from fresh biopsy tissues can take weeks for results and the success rate of recovering fungal pathogens from these tissues is only estimated to be about 50% [personal communication, Sean Zhang MD, PhD].

This application note provides a workflow for a rapid and accurate method for pathogenic fungi identification by DNA sequencing from standard formalin-fixed, paraffin-embedded (FFPE) histology tissue samples using the Applied Biosystems™ ArcturusXT™ Laser Capture Microdissection (LCM) System, Applied Biosystems™ PicoPure™ DNA Extraction Kit, Applied Biosystems™ BigDye™ Direct Cycle Sequencing Kit, and the Applied Biosystems™ 3500 Genetic Analyzer.

Figure 1. The ArcturusXT Laser Capture Microdissection System.

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Workflow for LCM sample collection and capillary electrophoresis (Sanger) sequencing

LCM provides a rapid and reliable method to procure purified cell populations from a heterogenous tissue sample, allowing a targeted approach to genomic and proteomic profiling. Thermo Fisher Scientific offers the ArcturusXT LCM instrument (Figure 1), providing the power of two microdissection lasers in a single, microscope-based platform. The exclusive infrared (IR) capture laser delivers a gentle cutting technique, preserving biomolecule integrity, and is ideal for single cells or a small number of cells. The optional ultraviolet (UV) laser permits unprecedented speed and precision, well suited for microdissection of larger numbers of cells and dense tissue structures.

The LCM workflow is simple and intuitive, and allows sample custody throughout the LCM process. After loading the slides onto the microscope stage, cells or areas of interest are located, marked, and then microdissected onto an Applied Biosystems™ CapSure™ Macro LCM Cap using the tools in the user interface. The microdissected areas are then confirmed by inspecting the LCM Cap in QC prior to downstream processing for genomic or proteomic analysis (Figure 2).

Sample preparation for LCM

FFPE blocks containing tissues with unidentified fungal infections were used for this application. Using RNase-free conditions, sections were cut at 8 µm using a standard rotary microtome. The sections were briefly floated on a heated water bath containing nuclease-free water and then mounted onto polyethylene naphthalate (PEN) membrane frame slides. They were then air dried for a minimum of 2 hours at room temperature to allow the sections to adhere to the slide. Just prior to performing LCM, the sections were stained using the Applied Biosystems™ Arcturus™ Paradise™ PLUS stain.

Laser capture microdissection

The stained tissues sections were loaded onto the ArcturusXT LCM instrument. A hematoxylin and eosin (H&E) reference slide with infected areas identified was used as a guide in locating the fungal-infected areas on the Arcturus Paradise-stained tissue sections. Using the features in the Arcturus LCM software, the fungal areas were located, marked, and microdissected using both the IR and UV lasers onto a CapSure Macro LCM Cap. To document the microdissection process, images were taken of the tissue before microdissection and of the LCM Cap with the microdissected area (Figure 3). From each tissue specimen, triplicate LCM samples were collected with the microdissected areas ranging from 3 to 5 mm^2.

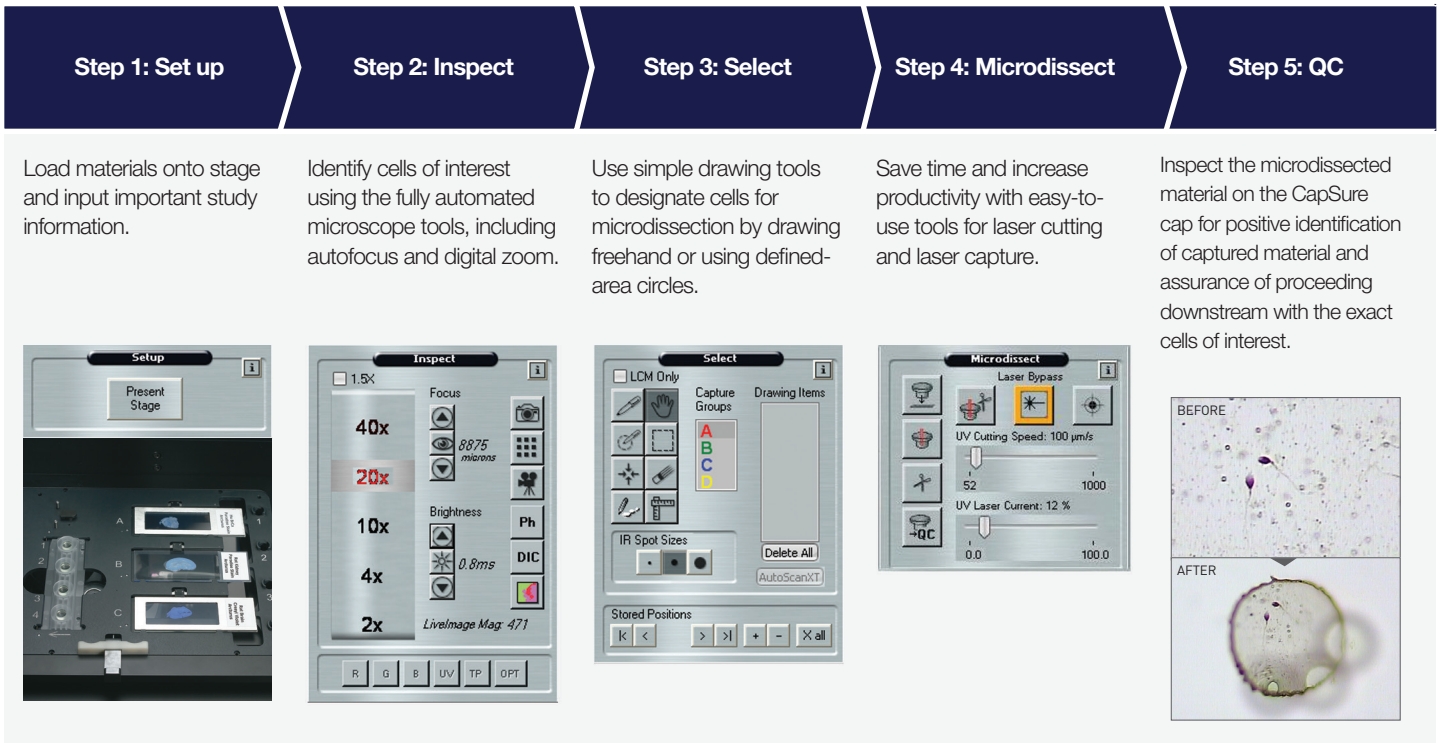


Figure 2. The LCM workflow—sample loading to extraction in five simple steps.

DNA extraction from LCM samples

The DNA was extracted directly from LCM Caps using the PicoPure DNA Extraction Kit. Each cap was coupled with a 500 µL microcentrifuge tube containing 50 µL of extraction solution, incubated overnight at 65°C, and then heated to 95°C to inactivate the Proteinase K in the extraction solution. The lysates were taken directly into the BigDye Direct Sequencing workflow without any DNA purification.

Sequencing D1-D2 and ITS2 regions with the BigDye Direct kit

The ribosomal large-subunit (D1-D2 region) and internal transcribed spacer 2 (ITS2) region are currently thought to be the most informative molecular targets for species or genus identification of fungi [2]. Here we demonstrate the use of the streamlined BigDye Direct Sequencing workflow for these targets. Figure 4 shows the four steps of the process. The entire workflow can be completed typically in less than 5 hours with as little as 60 minutes of hands-on time. Details of each step can be found in the appendix.

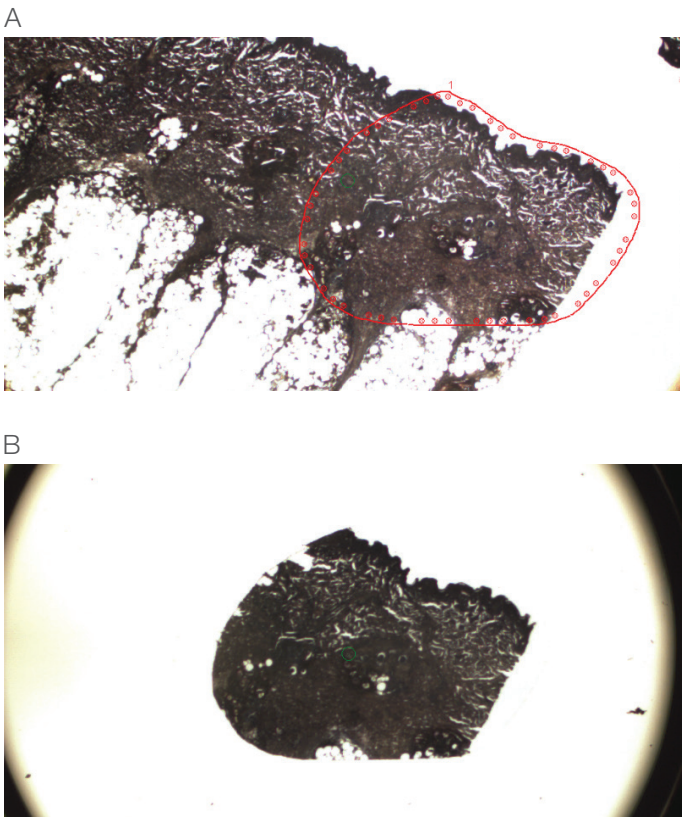


Figure 3. Laser capture microdissection of an infected tissue area. (A) Human dermis infected with fungi. Area for microdissection is indicated in red; perimeter is the UV cut line and smaller internal circles are the IR capture spots. (B) CapSure Macro LCM Cap with microdissected area. Images are shown at 2x magnification.

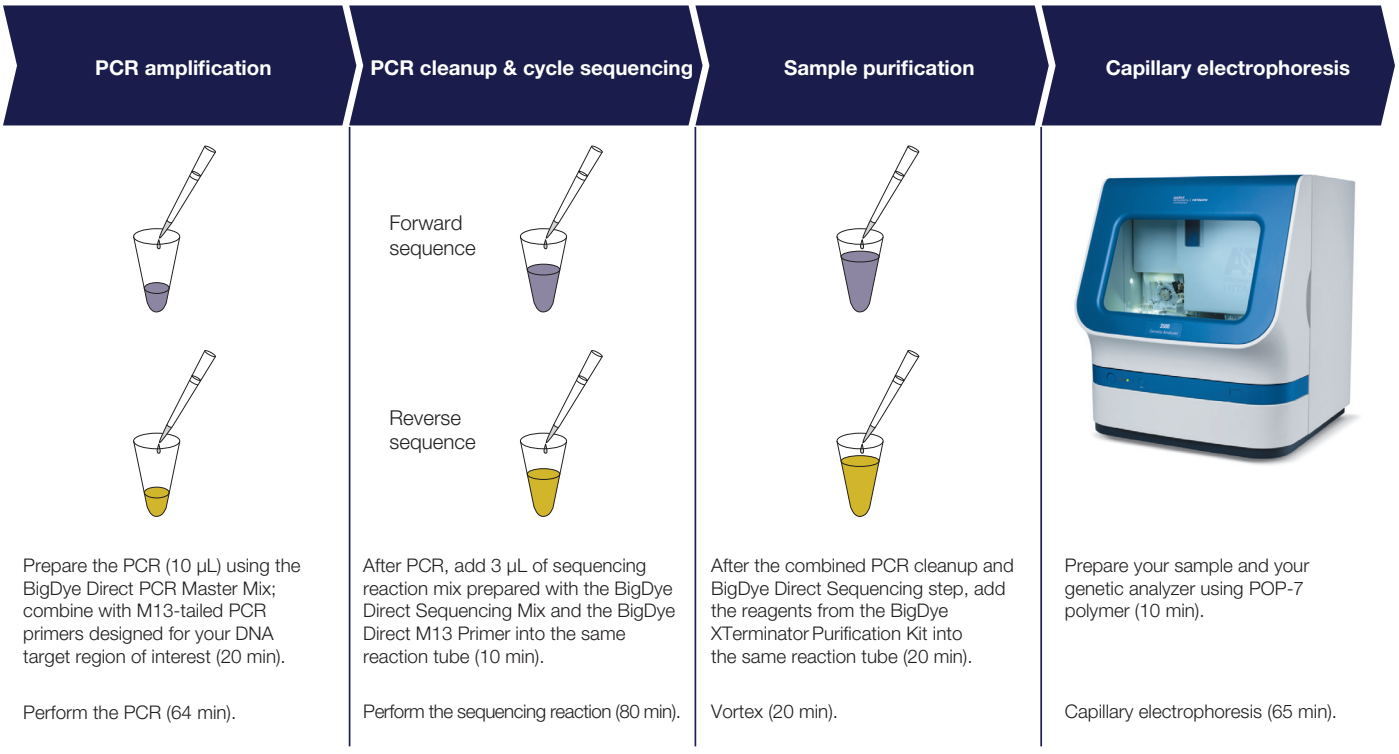


Figure 4. The BigDye Direct Sequencing workflow in four easy-to-operate steps.

Data analysis

The sequencing trace files (.ab1) were imported into a project folder within the Geneious™ software environment [3]. The sequencing traces were trimmed at the 5' and 3' ends to remove "noisy" mixed sequences. The overall quality of human GAPDH control sequencing reactions was acceptable or good for most specimens (data not shown), indicating that the entire process from sample preparation to sequence result was successful. However, the quality of the fungal DNA sequences was often mixed, ranging from excellent to poor, and not consistent from replicate to replicate. As shown in Figure 5, a given primer (e.g., D1D2_R) may yield good data with one sample (1-A) from a triplicate but fail with another (1-B). This was not unexpected since the samples were FFPE-treated hyphal filaments containing limited amounts of target nucleic acid. There may be variability in the amount of amplifiable fungal DNA in that particular sample or damage and degradation of the target sequence. Therefore, it is important to analyze multiple samples from a specimen during an investigation.

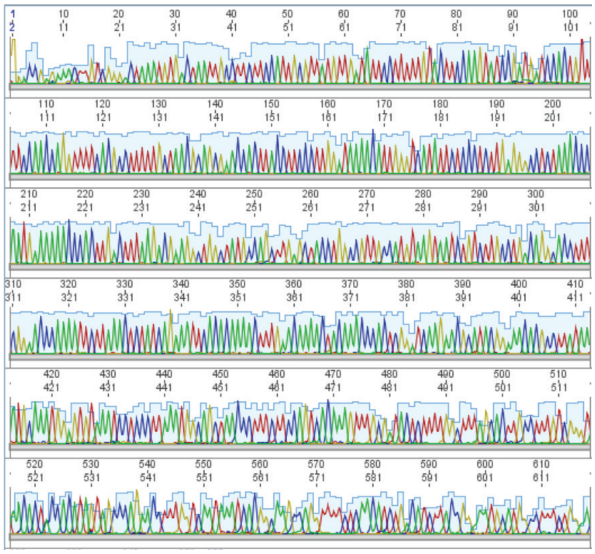
The sequence files were analyzed individually using the "Sequence Search" function in Geneious analysis software. Default parameters were selected for the search program and database (MegaBLAST search, "nr" database for all entries in GenBank and Ensembl), and ambiguous query input was accepted.

In order to sort out (i.e., reject) misleading or nonsense results from the MegaBLAST search, it was important to give weight and preference (i.e., "trust") to only sequence traces that meet these criteria:

- Long trace length (>600 nt for D1-D2 region; >350 nt for ITS2 region)
- High quality value (HQ%), if possible
- Minimal number of ambiguities after trimming 5' and 3' ends

Using a MegaBLAST search with the GenBank sequence repository may not ultimately be the preferred and recommended procedure for the purpose of routine identification of fungal species. However, we used this approach here to demonstrate the principal feasibility of obtaining species identification from sequence data. For higher confidence, we recommend using properly filtered and curated sequence databases such as the proprietary SmartGene™ IDNS software [4].

A



B

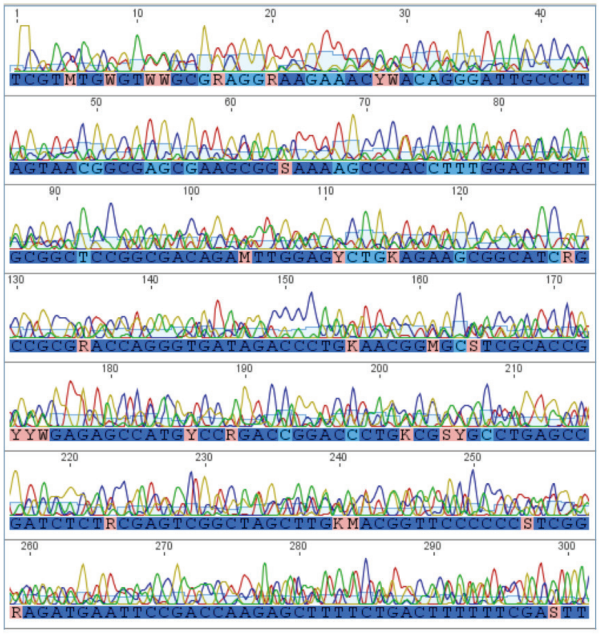


Figure 5. Sequencing data obtained from LCM samples. (A) An example of excellent sequencing data from sample 1-A. A MegaBLAST sequence search identified the organism as *Rhizopus oryzae*. (B) Poor data from a different sample (1-B) using the same primer (D1D2_R).

Results

The results of the sequence analysis are shown in Table 1, and sequencing data are shown in Figure 6. Sample 1 was identified as *Rhizopus oryzae* in the MegaBLAST search of the GenBank database with both D1-D2 and ITS2 target sequences. Sample 2 could not be identified unambiguously; there was only one usable sequence trace, which was identified as "uncultured marine fungus or *Malessezia*". Sample 3 was identified as *Aspergillus flavus* with two target sequences, indicating the likely presence of this fungus in the sample.

In summary, this data shows that the BigDye Direct Cycle Sequencing Kit can be successfully used to obtain DNA sequence information from the D1-D2 and ITS2 regions of fungal samples prepared from FFPE specimens using the ArcturusXT LCM System. These sequencing results were presented to the provider of the samples used in this study, and 2 out of the 3 samples were correctly identified. The sample in which the fungus could not be identified (sample 2) only produced a single usable sequence trace, likely due to overfixation of the tissue.

Conclusion

One of the challenges in fungal identification by sequencing is obtaining enough DNA, especially from solid tissues, for analysis. LCM is an ideal solution to this problem since purified or concentrated fungal samples from standard histopathology tissue sections can be used. The workflow presented in this application note provides a rapid and accurate method for DNA sequencing of fungi, which can aid both researchers in development of antifungal agents as well as public health officials in tracking fungal outbreaks.

Figure 6. Sequencing results from different LCM samples. (A) Sequence traces of the ITS2 region from two samples (1-A and 1-B). The specimen was identified as *Rhizopus oryzae* from database searches. (B) Sequence traces from sample 2, which was not definitively identified. (C) Sample 3 was identified as *Aspergillus flavus*.

Table 1. Summary of sequence analysis results.

Tissue sample	Target	No. of bases*	% High quality**	Identification	% Pairwise identity
Sample 1	D1-D2	655	79.8	*Rhizopus oryzae*	99.9
	ITS2	393	25.2	*Rhizopus oryzae*	99.7
Sample 2	D1-D2	–	–	No ID possible	–
	ITS2	480	69.2	Uncultured marine fungus or *Malassezia*	100
Sample 3	D1-D2	663	82	Uncultured compost fungus	100
	D1-D2	631	50	*Aspergillus flavus*	99.7
	ITS2	371	70.9	*Aspergillus flavus*	100

*Number of bases used for MegaBLAST search after removing mixed sequences at the 5' and 3' ends.
**Determined by the Geneious software, based on QV20 score.

Author's note

The data presented in this application note were developed in collaboration with Sean Zhang, MD, PhD of the Department of Pathology, the Johns Hopkins School of Medicine, Baltimore, MD.

References

1. Brown GD, Denning DW, Levitz SM (2012) Tackling human fungal infections. *Science* 336:647.
2. Hinrikson HP, Hurst SF, Lott TJ et al. (2005) Assessment of ribosomal large-subunit D1-D2, internal transcribed spacer 1, and internal transcribed spacer 2 regions as targets for molecular identification of medically important Aspergillus species. *J Clin Microbiol* 43:2092–2103.
3. Geneious version 5.6 created by Biomatters. Available from http://www.geneious.com/
4. Kwiatkowski NP, Babiker WM, Merz WG et al. (2012) Evaluation of nucleic acid sequencing of the D1/D2 region of the large subunit of the 28S rDNA and the internal transcribed spacer region using SmartGene IDNS [corrected] software for identification of filamentous fungi in a clinical laboratory. *J Mol Diagn* 14:393–401.

Appendix: Detailed sequencing protocol

Step 1: PCR

Primers used [see Ref. 3]

ITS2_3C_F	TGTAAAACGACGGCCAGTGCATCGATGAAGAACGCAGC
ITS2_4C_R	CAGGAAACAGCTATGACCTCCTCCGCTTATTGATATGC
D1D2_NL1_F	TGTAAAACGACGGCCAGTGCATATCAATAAGCGGAGGAAAAG
D1D2_NL4_R	CAGGAAACAGCTATGACCGGTCCGTGTTTCAAGACGG
HuGAPDH_F	TGTAAAACGACGGCCAGTGAAGGTGAAGGTCGGAGTC
HuGAPDH_R	CAGGAAACAGCTATGACCGAAGATGGTGATGGGATTTC

ITS2 primers bind to the fungal internal transcribed spacer (ITS) region and D1D2 primers bind to the D1-D2 region of the large subunit of the 28S rRNA gene. HuGAPDH primers bind to the human GAPDH gene and serve as amplification and sequencing controls. Since the specimens in the investigation were excised from human tissue, we expect positive detection in all samples. The sequences are shown in 5' to 3' direction; the M13 tag (F = forward; R = reverse) is indicated in red and the target-specific region is in black. Primer pairs were formulated to 1 µM each and 1 µL was used for PCR.

The BigDye Direct workflow requires a dedicated PCR reaction for the subsequent forward and the reverse sequencing reactions. The PCR reagent mix for this is provided in the BigDye Direct Cycle Sequencing Kit.

For PCR, 2 µL of DNA lysate was used as the template. PCR cycling conditions were:

1. 95°C 10 min
2. 96°C 3 sec, 54°C 20 sec, 68°C 45 sec: 14 cycles
3. 96°C 3 sec, 62°C 15 sec, 68°C 45 sec: 26 cycles
4. 72°C 2 min
5. 4°C hold

Step 2: Post-PCR cleanup and cycle sequencing in one step

Following PCR, a sequencing master mix was prepared by combining the appropriate multiples of 1 µL M13 forward sequencing primer (provided with kit) and 2 µL BigDye Direct ready reagent (provided). This was repeated for the reverse sequencing reaction. The mix (3 µL each of forward or reverse master mix) was added directly to the PCR sample (no need to transfer sample), put back into the PCR thermal cycler, and incubated as follows:

1. 37°C 15 min (enzymatic degradation of PCR primers; the sequencing primer is protected)
2. 80°C 2 min (inactivation of the nuclease)
3. 96°C 1 min
4. 96°C 10 sec, 50°C 5 sec, 68°C 75 sec: 25 cycles (cycle sequencing)
5. 72°C 2 min

By combining the steps of PCR reaction cleanup with the sequencing reaction, the BigDye Direct Cycle Sequencing Kit reduces the amount of hands-on time. A significant benefit of the kit is that researchers can use a single sample plate from start to finish, which reduces the potential for error when transferring samples between plates, and creates a streamlined workflow that fits within a daily work schedule.

Step 3: Post-sequencing cleanup

At completion of the sequencing reaction, the sequencing products were purified using the Applied Biosystems™ BigDye XTerminator™ Purification Kit. Specifically, the BigDye Direct–sequenced products were purified by addition of 45 µL SAM™ Solution and 10 µL BigDye XTerminator solution. Both components can be premixed and dispensed at 55 µL volume, but care must be taken to keep the beads in suspension. The 96-well plate was placed on a vortexer at high setting for 20 min, followed by centrifugation at 1,000 x *g* for 2 min.

Step 4: Capillary electrophoresis and analysis

Electrophoresis was performed on the 3500 Genetic Analyzer with Applied Biosystems™ POP-7™ Polymer and Applied Biosystems™ 3500 Genetic Analyzer 8-Capillary Array, 50 cm using BDxShortReadSeq50_POP7 run module and KB_3500_POP7_BDTv3direct.mob. New mobility files and base callers were installed to enhance the sequencing trace resolution and base calling accuracy for the BigDye Direct Cycle Sequencing Kit (free download at www.thermofisher.com/bigdyedirectinstaller).

The sequencing results for each sample were analyzed using Geneious software to verify them and to identify the fungus for each sample.

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Ordering information

Product	Cat. No.
PEN Membrane Frame Slides	LCM0521
Arcturus Paradise PLUS Stain	KIT0312-J
CapSure Macro LCM Caps	LCM0211
Arcturus PicoPure DNA Extraction Kit	KIT0103
BigDye Direct Cycle Sequencing Kit	4458687
BigDye XTerminatorPurification Kit	4376486
POP-7 Polymer	4393708
ArcturusXT Laser Capture Microdissection Instrument	thermofisher.com/arcturus
Applied Biosystems 3500 Genetic Analyzer	thermofisher.com/3500series

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