Video Article

Collection and Extraction of Occupational Air Samples for Analysis of Fungal DNA

Angela R. Lemons¹, William G. Lindsley¹, Brett J. Green¹

¹Allergy and Clinical Immunology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention

Correspondence to: Angela R. Lemons at wrw0@cdc.gov

URL: <https://www.jove.com/video/56730>

DOI: [doi:10.3791/56730](https://doi.org/10.3791/56730)

Keywords: Environmental Sciences, Issue 135, Occupational air sampling, air microbiology, genomic DNA extraction, fungal hazard identification, fungal internal transcribed spacer regions, fungal DNA sequencing, occupational air contaminants

Date Published: 5/2/2018

Citation: Lemons, A.R., Lindsley, W.G., Green, B.J. Collection and Extraction of Occupational Air Samples for Analysis of Fungal DNA. *J. Vis. Exp.* (135), e56730, doi:10.3791/56730 (2018).

Abstract

Traditional methods of identifying fungal exposures in occupational environments, such as culture and microscopy-based approaches, have several limitations that have resulted in the exclusion of many species. Advances in the field over the last two decades have led occupational health researchers to turn to molecular-based approaches for identifying fungal hazards. These methods have resulted in the detection of many species within indoor and occupational environments that have not been detected using traditional methods. This protocol details an approach for determining fungal diversity within air samples through genomic DNA extraction, amplification, sequencing, and taxonomic identification of fungal internal transcribed spacer (ITS) regions. ITS sequencing results in the detection of many fungal species that are either not detected or difficult to identify to species level using culture or microscopy. While these methods do not provide quantitative measures of fungal burden, they offer a new approach to hazard identification and can be used to determine overall species richness and diversity within an occupational environment.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56730/>

Introduction

Fungal exposures in indoor and occupational environments can result in respiratory morbidities, including allergic sensitization and asthma¹. Identification of fungal hazards is important for assessing risk and preventing worker exposure. These fungal hazards may be a result of indoor contamination, outdoor air intrusion, or environmental disturbances that result in the transport of fungal materials into areas where workers are present². Methods to assess fungal exposure have included viable culture sampling as well as microscopic identification of fungal spores. These approaches have several limitations and often overlook many fungal species that could be contributing to the overall fungal burden³. Culture-based approaches can only differentiate those viable fungal organisms that can be cultivated on nutrient media. Identifying fungal spores to species level via microscopy can be confounded by spores sharing similar morphologies. Both methods are highly dependent on mycologists to analyze and identify the fungal species, with many remaining unidentified.

To improve upon existing methodologies used in occupational hazard identification and exposure assessments, many researchers have turned to molecular-based technologies. Sequencing-based approaches for assessing microbial diversity within indoor and occupational environments have revealed a broader spectrum of fungal species encountered compared to methods such as microscopy and viable culture^{3,4,5}. The method presented here describes the air sampling of occupational environments and extraction of genomic DNA for the identification of potential fungal hazards. Hazard identification is accomplished by sequencing the nuclear ribosomal internal transcribed spacer, or ITS, regions that are highly variable among fungi and have been commonly used to differentiate fungal species^{6,7,8,9}. Many species found in occupational settings, such as some species belonging to the phylum Basidiomycota, are not identifiable in viable culture and are difficult to differentiate microscopically. These fungi have been observed in high relative abundance within indoor and occupational environments assessed by sequencing fungal ITS regions^{3,4,10}. ITS sequencing has provided greater knowledge into the diversity of fungi encountered within indoor and occupational environments.

The protocol described here details the methods used to collect, extract, and amplify fungal ITS regions from bioaerosols for sequence analysis. This approach utilizes the National Institute for Occupational Safety and Health (NIOSH) two-stage cyclone aerosol sampler to collect particulates in the air. This sampler was developed to collect bioaerosols and separate respirable ($\leq 4 \mu\text{m}$ aerodynamic diameter) and non-respirable ($>4 \mu\text{m}$ aerodynamic diameter) particles, which allows for identification of fungal organisms within indoor environments that are most likely to be inhaled by a worker¹¹. Other air samplers, including cyclone samplers, are available on the market that have the ability to collect particles within the respirable range ($<4 \mu\text{m}$) using filters^{12,13}. In contrast, the NIOSH two-stage cyclone aerosol sampler separates

fungal species based on their aerodynamic diameter into disposable, polypropylene tubes that can be immediately processed for downstream applications¹⁴.

The processes of extracting genomic DNA and amplifying the fungal ITS regions are detailed in this protocol. The extraction methodologies presented have been developed specifically for the extraction of genomic DNA from fungi and bacteria, as many commercial kits target mammalian cells, bacteria, or specifically yeasts¹⁵. The primers used in this study are selected based on their overall coverage of both the fungal ITS 1 and ITS 2 regions^{4,5}. Sequencing of these regions allows for the comparison of many banked ITS sequences, including those that sequence the ITS 1 region, the ITS 2 region, or both the ITS 1 and ITS 2 regions. The fungal diversity of air samples collected in an indoor setting using these methods are shown, revealing a substantial number of sequences placed in the phyla Ascomycota and Basidiomycota as well as other sequences belonging to less dominant fungal phyla, such as Zygomycota. The broad diversity of fungal sequences identified using this approach would not be captured using traditional hazard identification methodologies like cultivation or microscopy. Sequencing of fungal ITS regions provides an enhanced method to identify fungal hazards and allow for a better understanding of indoor and occupational fungal exposures.

Protocol

1. Preparing the NIOSH aerosol sampler

NOTE: The NIOSH aerosol sampler is a two-stage cyclone aerosol sampler that collects bioaerosols using two sampling tubes and a polytetrafluoroethylene (PTFE) filter.

1. Before assembly, carefully inspect the sampler. Check the sampler to ensure it is not damaged, all screws are in place and snug, and the sealing tape around the seam formed by the two halves is intact. Inspect the sampler O-ring to ensure it does not have any nicks, cracks or tears and that it has a very light coating of silicone grease.

NOTE: The sampler includes a 37 mm 3 μ m PTFE filter in a polystyrene or polypropylene three-piece filter cassette.

1. Assemble the filter cassette by placing a cellulose or plastic filter support pad (included with the filters) on the gridded surface of the base piece (see **Figure 1**). Use filter forceps to put the filter on top of the filter support pad with the collection side facing up.
2. Seal the filter cassettes using a manual or pneumatic press. Hand closure will allow air to leak around the filter and reduce the collection efficiency. Insert the ring-shaped piece (the extension cowl), and use the press to push it down tightly and evenly. Press down the extension cowl onto the filter tightly enough to ensure air does not leak around the filter.

NOTE: The top piece of the cassette is not used while sampling but should be saved to cover the filter once sampling is complete.

3. Fit the assembled cassette onto the top of the sampler and push it down as far as possible. Wrap a piece of 19 mm (3/4 inch) tape around the outside of the filter cassette and sampler to hold the filter cassette in place and to act as a backup seal to prevent leaks.
4. Screw each tube tightly and fully into the sampler until it bottoms out and wrap sealing tape around each tube to act as a secondary seal against leaks.

NOTE: The first tube, a 15 mL polypropylene tube, collects non-respirable particles with an aerodynamic diameter of $>4 \mu$ m. The second tube, a 1.5 mL polypropylene microcentrifuge tube, collects respirable particles between 1 and 4 μ m. The remaining smaller particles, $<1 \mu$ m, are collected on the PTFE filter (see **Figure 2**).

2. Calibrate the airflow through the NIOSH sampler with a calibration jar before each use as differences in the filters and samplers will cause the airflow to vary.
 1. To use a calibration jar, insert the jar's Luer fitting into the top of the filter cassette, place the sampler in the jar, and seal the jar.
 2. Connect the calibration jar to a calibration flow meter and the sampling pump. Turn the flow meter on and allow it to warm up for a few minutes. Note that the flow meter must always have a filter attached to its inlet to avoid contaminating the flow meter and the sampler.
 3. Turn the sampling pump on and allow it to run for a few minutes to warm up and stabilize.
 4. Adjust the pump to set the correct flow rate at 3.5 L/min.

NOTE: The flow rate for the NIOSH aerosol sampler is usually set to 3.5 L/min. At this flow rate, the sampler conforms to the ACGIH/ISO criteria for respirable particle sampling¹⁶. Other flow rates can be used if different cut-off sizes are desired or to reduce the noise level, but be aware that if a different flow rate is used, then the sampler will no longer conform to the respirable sampling criteria.

 5. When calibration is finished, turn off the pump and flow meter.

2. Static and personal aerosol sampling

1. Set up the NIOSH aerosol sampler for either personal or static area sampling.

NOTE: Static sampling refers to using the aerosol sampler while it is attached to a tripod or other holding device (see **Figure 3**), as versus personal sampling when the sampler and pump are mounted on the person being studied (see **Figure 4**).

 1. For static area sampling, keep the samplers as close as possible to the specific area being studied. Keep the samplers away from air inlets, room entrances and places that might be in the path of the airflow.

NOTE: Aerosol concentrations can vary considerably even over short distances, especially if the aerosol source is in the room¹⁷.
 2. For personal sampling, set up the sampler within the person's breathing zone. Attach the sampler to the lapel, shoulder or chest, and place the sampling pump at the waist or in a backpack.

NOTE: The breathing zone is commonly assumed to be a 30 cm hemisphere surrounding a person's nose from which the majority of air is drawn during inhalation¹⁷.
2. Make sure that the sampler tubing is well clear of the sampler inlets and that nothing is obstructing the inlets or interfering with the airflow into the sampler. The sampler tubing must not be pinched or kinked. Any blockage will cause the pump to stop.
3. Turn on the pumps. After a minute or two, check if the pumps are still running.

NOTE: Conduct air sampling for time periods ranging from as little as 10 min to a full 8 h work shift, depending on the environment^{10,18,19}. The results presented in **Figure 6** are representative of a 60 min sampling period in an indoor environment.

- After air sampling is completed, collect the sample tubes and filter. Remove the sealing tape, unscrew the tubes, and cap them. Place the third piece of the filter cassette over the filter. Store samples at 4 °C until ready for processing.

3. Extraction of genomic DNA from air samples

- Extract genomic DNA (gDNA) from each stage of the NIOSH BC251 sampler. Process the air sampling collection tubes and filter separately to allow for determination of respirable and non-respirable microbial aerosols in the sample.

NOTE: Alternatively, the three stages can be combined and extracted if sample inoculum is too small.

- In a class II biological safety cabinet or laminar flow clean bench, aseptically remove the filter. Wipe the sampling cassette down using 70% ethanol and pry the sampling cassette open using a cassette-opening tool. Use a filter lifter to push the filter and support pad upwards. Grasp the filter using filter forceps and place it in a sterile petri dish. Cut the filter into 6 equal pieces and place them in a 2 mL reinforced tube containing 300 mg glass beads (0.2 - 0.5 mm).
 - Place the tube containing the filter in liquid nitrogen for 30 s and immediately place it in a bead mill homogenizer set at 4.5 m/s for 30 s.
 - Repeat step 3.1.2 once or twice until the filter is shredded. Small pieces of intact filter may remain. Add 0.5 mL of lysis buffer (4 M urea, 200 mM Tris, 20 mM NaCl, 200 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4).
 - Add 0.3 mL of lysis buffer to the 15 mL and 1.5 mL air sampler tubes. Vortex the tube for 10- 15 s while upright and then another 10 - 15 s inverted. Transfer the contents of each tube to 2 mL reinforced tubes containing 300 mg glass beads.
- NOTE: Most of the material collected in each sampler tube will accumulate near the top of the tube.
- Process all tubes in the bead mill homogenizer at 4.5 m/s for 30 s and centrifuge them at 20,000 x g for 1 min at 22 °C. Transfer supernatants to sterile 1.5 mL microcentrifuge tubes and centrifuge the tubes at 20,000 x g again for 1 min at 22 °C. Repeat this step.
 - Add 30 µL of lysis reagent (see **Table of Materials**) to each tube and incubate the tubes at 37 °C for 15 min.
 - Add 0.2 mL of binding buffer (10 M urea, 6 M guanidine-HCl, 10 mM Tris-HCl, 20% Triton X-100, pH 4.4) and proteinase K (100 µg/mL) to each tube and incubate the tubes at 70 °C for 10 min. Add 100 µL of isopropanol to each tube.
 - Transfer the extract solutions into glass fiber filter tubes (700 µL capacity) placed in 2 mL collection tubes and centrifuge the collection tubes at 20,000 x g for 30 s at 22 °C. Discard the collection tubes and place the filter tubes into new 2 mL collection tubes.
 - Add 0.5 mL of inhibitor removal buffer (5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6, 38% ethanol) to each tube and centrifuge the collection tubes at 20,000 x g for 30 s at 22 °C. Discard the collection tubes and place the filter tubes into new 2 mL collection tubes.
 - Add 0.5 mL of wash buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5, 80% ethanol) to each tube and centrifuge the collection tubes at 20,000 x g for 30 s at 22 °C. Discard the collection tubes and place the filter tubes into new 2 mL collection tubes. Repeat this process.
 - Centrifuge the collection tubes at 20,000 x g for an additional 1 min at 22 °C to remove any residual wash buffer. Discard the collection tubes and place the filter tubes into new collection tubes.
 - Add 100 µL warm (≥70 °C) elution buffer (10 mM Tris-HCl, pH 8.5) to each tube and incubate them at room temperature for 1 - 2 min. Centrifuge the collection tubes at 20,000 x g for 30 s at 22 °C. Transfer the empty filter tubes to a sterile 1.5 mL microcentrifuge tube and re-apply eluates from step 3.8. Incubate at room temperature for 1 - 2 min. Centrifuge at 20,000 x g for 30 s at 22 °C.
- NOTE: Eluates can be used immediately for step 4 or stored at -20 °C until ready to use. Genomic DNA can be stored for up to a year at -20 °C. It is recommended that DNA be stored at -80 °C if long-term storage is required.

4. Amplification of fungal ribosomal DNA

- Use universal fungal primers to amplify ITS regions 1 and 2 from the extracted gDNA.
- NOTE: For this protocol, the primer pair Fun18Sf (5'-TTGCTCTTCAACGAGGAAT-3')/ITS4R (5'-TCCTCCGCTTATTGATATGC-3') are used to provide the greatest coverage of the ITS regions^{4,5}. Other primer sets, such as those that amplify ITS1 or ITS2 regions alone, can be used.
- Set up polymerase chain reactions (PCR) for each sample in triplicate 50 µL reactions in sterile 0.5 mL PCR tubes or 96-well PCR plates as follows: 5 µL of extracted template DNA (from step 3), 33.3 µL of PCR grade water, 5 µL of 10x PCR buffer, 1.5 µL of 50 mM MgCl₂, 1 µL of 10 mM 2'-deoxynucleoside 5'-triphosphates, 0.5 µL of 20 µM Fun18Sf forward primer, 0.5 µL of 20 µM ITS4R reverse primer, and 0.2 µL of *Taq* DNA polymerase.
 - Perform reactions in a thermal cycler: denaturation at 95 °C for 3 min; 6 cycles of denaturation (96 °C, 30 s) annealing (50 °C, 45 s) and primer extension (72 °C, 3 min); 20 cycles of denaturation (96 °C, 30 s), annealing (50 °C, 45 s), and primer extension (72 °C, 1 min); and primer extension at 72 °C for 10 min. Keep the reaction mixtures at 4 °C until next step.
- Combine the triplicate PCR reactions purify using a silica membrane-based purification kit.
- NOTE: This procedure allows for the removal of PCR components (primers, dNTPs, enzymes, salts, etc.) and other contaminants from the amplified DNA preparations.
- Combine the three reactions for each sample (150 µL total) in sterile 1.5 mL microcentrifuge tubes. Add binding buffer (5 M guanidine-HCl, 30% isopropanol) at a 5x volume (750 µL) and mix the solution by pipetting up and down ten times.
 - Add 450 µL of the mixture to spin columns placed in collection tubes and centrifuge the tubes at 17,900 x g for 30 s. Discard the filtrates. Add the remaining 450 µL to the columns and centrifuge at 17,900 x g for 30 s at 22 °C.
- NOTE: The spin columns contain a silica membrane that allows for adsorption of the DNA to the columns.
- Discard the filtrates and add 750 µL washing buffer (10 mM Tris-HCl, pH 7.5, 80% ethanol) to the spin columns. Centrifuge the columns at 17,900 x g for 30 s at 22 °C.
- NOTE: This process removes the contaminants from the PCR reaction mentioned above.
- Discard the filtrate and centrifuge the empty columns at 17,900 x g for 1 min at 22 °C.
- NOTE: This step is to remove any residual washing buffer that may interfere with downstream applications.
- Transfer the spin columns to sterile 1.5 mL microcentrifuge tubes. Add 45 µL of elution buffer (10 mM Tris-Cl, pH 8.5) and incubate the tubes at room temperature for 5 min. Centrifuge the spin columns at 17,900 x g for 1 min at 22 °C.
 - Use the eluted DNA immediately for steps 4 and 5 or store at -20 °C until ready for use.

NOTE: The amplicons can be stored for up to a year at -20 °C. It is recommended that DNA be stored at -80 °C if long-term storage is required.

5. Verification of fungal ITS amplification using agarose gel electrophoresis

1. Cast a 1% agarose gel containing 1 µg/mL ethidium bromide. Dissolve agarose in boiling 1x Tris-acetate-EDTA (TAE) buffer. Once the solution has cooled to approximately 50 °C, add the ethidium bromide and pour into gel cast.
2. After the agarose gel has solidified, immerse the gel in 1x TAE and prepare the amplicons to be loaded on the gel. To 8 µL of amplified DNA, add 2 µL of 5x loading buffer.
NOTE: These volumes can be adjusted depending on the concentration of the desired loading buffer.
3. Load the samples, all 10 µL, onto the gel along with a DNA ladder for size reference. Run the gel at 75 V (6 V/cm) for approximately 90 min and visualize bands, typically seen between 750 and 1,000 base pairs, using ultraviolet light (see **Figure 5**).

6. Sequencing and analysis of fungal ITS regions

1. Sequence the extracted gDNA or amplified fungal ITS regions and analyze the sequences using current and appropriate methods.

Representative Results

The species distribution within an environment can be assessed using relative abundance by determining the number of clones of each OTU identified in the air samples. **Figure 6** is a Krona chart representing the taxonomically placed species within an indoor environment following 60 min of air sampling. It can be observed that the environment contains a variety of species within two major fungal phyla, Ascomycota and Basidiomycota, as well as species belonging to the phylum Zygomycota (*Rhizopus microsporus*). Traditional methods of assessment are biased toward Ascomycota species, as many Basidiomycota are not culturable or cannot be differentiated using microscopy-based methods. Sequencing of fungal ITS regions allows for the detection of numerous fungal species, specifically some Basidiomycota, that often go undetected. Analysis of this environment shows 68% of the fungal sequences identified belonged to the Basidiomycota with most of them placed in the order Polyporales.

The taxonomic data obtained using sequencing-based approaches can be used to determine the taxonomic diversity within an environment. Diversity indices, such as those presented in Table 1, determine how rich the environment is, which describes the number of species identified in each sample, as well as how diverse the environment is, which takes into account the number of species and the abundance of each. **Table 1** shows the Chao 1 richness indices and Shannon diversity indices for two indoor environments. The indices indicate higher richness and diversity in air-conditioned environments compared to evaporative cooler environments. Also included are Bray-Curtis dissimilarity coefficients. These describe how dissimilar samples within the environment are regarding the species identified within each sample. The samples within both of the environments described in Table 1 are highly dissimilar at 98% and 97% for air conditioner and evaporative cooler environments, respectively.

Figure 1: Schematic representation of a three-piece filter cassette assembly. The filter cassette is made up of three pieces: a top or cap piece (inlet), an extension cowl and a base piece (outlet). A support pad and filter are placed on the gridded surface of the base piece. The extension cowl is then sealed onto the base using a manual or pneumatic press. For use with the NIOSH bioaerosol cyclone sampler, the top piece is left off during sampling and is then replaced for storage and transport. [Please click here to view a larger version of this figure.](#)

Figure 2: Schematic representation of the NIOSH bioaerosol cyclone sampler. The NIOSH cyclone aerosol sampler collects airborne particulate by drawing air into the sampler through the inlet, and producing a cyclone that deposits particles on the wall of the sample tubes. Air is first drawn into a 15 mL polypropylene tube, where large particles ($>4\ \mu\text{m}$) collect on the walls of the tube. Air then flows out of the first tube and is drawn into a 1.5 mL microcentrifuge tube, where smaller particles (1 to $4\ \mu\text{m}$) collect. The remaining particles ($<1\ \mu\text{m}$) collect on a PTFE filter as the air is drawn out of the sampler. [Please click here to view a larger version of this figure.](#)

Figure 3: Example of a static sampler set-up. The image demonstrates samplers set up for static area sampling. The NIOSH samplers are set up to collect 40 inches (102 cm) and 60 inches (152 cm) from the floor, representing heights of a sitting and standing adult, respectively. [Please click here to view a larger version of this figure.](#)

Figure 4: Example of a personal sampler assembly. The image illustrates a NIOSH sampler worn on a backpack by an individual. The sampler and power switch are located on the straps of the pack while the sampling pump is located within the backpack itself. [Please click here to view a larger version of this figure.](#)

Figure 5: Example of an agarose gel visualizing amplified ITS DNA from air samples. The image shows DNA bands between 750 and 1,000 base pairs. These bands represent amplified fungal ITS regions from extracted air samples. ITS regions vary in size depending on the species origin. [Please click here to view a larger version of this figure.](#)

Figure 6: Example Krona chart presenting taxonomically placed fungal species identified within an indoor environment. The Krona chart depicts the relative abundance of fungal species found in 4 samples collected from homes following a 60 min air sampling period with the NIOSH bioaerosol cyclone sampler. 68% of the species identified belong to the phylum Basidiomycota with the remaining belonging to the Ascomycota (33%) and Zygomycota (1%). The most abundant ITS sequences in this environment belonged to the order Polyporales and were identified as *Phanerodonta chrysosporium* and *Gelatoporia dichroa*. [Please click here to view a larger version of this figure.](#)

	Chao-1 Richness	Shannon Diversity	Bray-Curtis Distance
	mean (min, max)	mean (min, max)	mean (min, max)
Air Conditioner (n = 9)	33.86 (15.0, 102.0)	1.39 (0.68, 2.51)	0.9778 (0.8313, 1.00)
Evaporative Cooler (n = 10)	25.46 (12.5, 49.5)	1.10 (0.50, 1.72)	0.9624 (0.3804, 1.00)

Table 1: Example data presenting diversity and species richness indices comparing indoor environments. The table shows Chao 1 richness indices (number of species per sample), Shannon diversity indices (number of species and abundance of each), and Bray-Curtis dissimilarity coefficients (how dissimilar the species distribution is between samples on a scale of 0 - 1). These data demonstrate higher richness and diversity in air-conditioned environments and high dissimilarity among samples in both environments. This table has been modified from Lemons *et al.*¹⁰ with permission from the Royal Society of Chemistry.

Discussion

Determining the fungal diversity within an occupational environment using sequencing-based approaches has improved fungal hazard identification and exposure assessment. Using this approach has allowed for the detection of many additional fungal species that are often not detected using culture or microscopy-based methods of assessment. A method for sampling bioaerosols from occupational and indoor environments and the extraction of genomic DNA from air samples for ITS amplification and sequencing is presented here. Determinations of fungal diversity using these methods is highly dependent on: (1) successful and complete extraction of genomic DNA from the air samples, (2) amplification of the gDNA with primers that allow for comparison of the amplified ITS sequences to banked sequences in the database and limit amplification biases, and (3) correct taxonomic identification of the sequences within the databases.

Successful genomic DNA extraction is reliant on the extraction methodologies and reagents employed in a study. As is mentioned in the protocol, a lot of the bioaerosols and other biological and non-biological particulate collected in the tube phases of the NIOSH two-stage cyclone sampler collect at the top of the tube. It is very important that the tubes be carefully opened when adding the lysis buffer as not to lose any particulate and that they be vortexed in a manner that allows for all the particulate to fall into the lysis buffer (right-side up and upside down). It is also important that the filter be carefully handled using aseptic methods as not to disrupt any bioaerosols that have collected on the surface prior to extraction or introduce contaminating DNA. It has been demonstrated that there is a large amount of variation between many of the commercially

available genomic DNA extraction kits¹⁵. Some extraction procedures bias toward specific fungal species and result in varying DNA yields²⁰. If the DNA yield following extraction is low, higher PCR reaction replicates can be performed. Not only do the extraction yields vary, but many of the kits contribute a substantial amount of microbial DNA contamination. For this reason, it is important to include proper controls throughout the procedure to identify and remove potential reagent contaminants from the analysis.

ITS amplification is another critical step of the protocol. The extraction methodologies used can vary in their ability to remove PCR inhibitors from the environmental samples. This is particularly concerning with environmental dust samples²¹. PCR amplification of samples extracted using the method presented here exhibited some PCR inhibition after the addition of 10 mg of dust¹⁵. Although PCR inhibition is of greatest concern in environmental dust samples, it should still be a consideration when amplifying DNA extracted from air samples for determining fungal diversity. The PCR primer set used in this method provides coverage of both the ITS 1 and ITS 2 regions. This allows for comparison to many of the sequences placed in the sequence databases. As with the extraction procedure, many amplification and primer biases have been previously described²². The fungal genome size and gene copy number could also influence ITS amplification²³. These biases should be taken into consideration when interpreting the resultant sequence data. Taxonomic identification of ITS sequences is perhaps the most critical component of this method. It is important to keep identification criteria consistent. The ability to identify ITS sequences is dependent on the sequences banked in the databases. Many sequences cannot be identified to the species level. Having specific criteria when making taxonomic identifications based on banked sequences, like percentage identity cutoffs, is critical for keeping datasets consistent from study to study.

Compared to traditional assessment approaches, extracting and sequencing fungal DNA from occupational air samples provides a more complete representation of fungal diversity within an environment. In addition to the limitations discussed above, it must be noted that the results of these types of analyses are semi-quantitative unlike culture, spore counts, or quantitative PCR²⁴ that can yield quantitative data. Sequencing data can be used to determine relative abundance per sample as well as calculate fungal diversity metrics. These methods can be used in conjunction with other methods, like qPCR, to get more quantifiable data. The datasets created using these approaches can be used in occupational health studies to gain a better understanding of fungal hazards in specific occupational environments. Developing more standardized approaches of extraction and primer selection are necessary to better characterize and compare sequencing-based studies of fungal diversity.

Disclosures

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

Acknowledgements

This work was supported in part by an interagency agreement between NIOSH and NIEHS (AES12007001-1-0-6).

References

- Mendell, M.J., Mirer, A.G., Cheung, K., Tong, M., Douwes, J. Respiratory and allergic health effects of dampness, mold, and dampness-related agents: a review of the epidemiologic evidence. *Environ Health Perspect.* **119** (6), 748-756 (2011).
- Green, B.J., Lemons, A.R., Park, Y., Cox-Ganser, J.M., Park, J.H. Assessment of fungal diversity in a water-damaged office building. *J Occup Environ Hyg.* **14** (4), 285-293 (2017).
- Green, B.J., Lemons, A.R., Park, Y., Cox-Ganser, J.M., Park, J.H. Assessment of fungal diversity in a water-damaged office building. *J Occup Environ Hyg.* **0** (2016).
- Pitkaranta, M. *et al.* Analysis of fungal flora in indoor dust by ribosomal DNA sequence analysis, quantitative PCR, and culture. *Appl Environ Microbiol.* **74** (1), 233-244 (2008).
- Rittenour, W.R. *et al.* Internal transcribed spacer rRNA gene sequencing analysis of fungal diversity in Kansas City indoor environments. *Environ Sci Process Impacts.* **16** (1), 33-43 (2014).
- Martin, K.J., Rygiel, P.T. Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiol.* **5** 28 (2005).
- Monard, C., Gantner, S., Stenlid, J. Utilizing ITS1 and ITS2 to study environmental fungal diversity using pyrosequencing. *FEMS Microbiol Ecol.* **84** (1), 165-175 (2013).
- Op De Beeck, M., Lievens, B., Busschaert, P., Declerck, S., Vangronsveld, J., Colpaert, J.V. Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies. *PLoS One.* **9** (6), e97629 (2014).
- Toju, H., Tanabe, A.S., Yamamoto, S., Sato, H. High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PLoS One.* **7** (7), e40863 (2012).
- Lemons, A.R. *et al.* Microbial rRNA sequencing analysis of evaporative cooler indoor environments located in the Great Basin Desert region of the United States. *Environ Sci Process Impacts.* (2017).
- Cao, G., Noti, J.D., Blachere, F.M., Lindsley, W.G., Beezhold, D.H. Development of an improved methodology to detect infectious airborne influenza virus using the NIOSH bioaerosol sampler. *J Environ Monit.* **13** (12), 3321-3328 (2011).
- Soo, J.C., Lee, T., Kashon, M., Kusti, M., Harper, M. Quartz in coal dust deposited on internal surface of respirable size selective samplers. *J Occup Environ Hyg.* **11** (12), D215-219 (2014).
- Wang, C.H. *et al.* Field evaluation of personal sampling methods for multiple bioaerosols. *PLoS One.* **10** (3), e0120308 (2015).
- Lindsley, W.G., Schmechel, D., Chen, B.T. A two-stage cyclone using microcentrifuge tubes for personal bioaerosol sampling. *J Environ Monit.* **8** (11), 1136-1142 (2006).
- Rittenour, W.R., Park, J.H., Cox-Ganser, J.M., Beezhold, D.H., Green, B.J. Comparison of DNA extraction methodologies used for assessing fungal diversity via ITS sequencing. *J Environ Monit.* **14** (3), 766-774 (2012).

16. ACGIH. *Documentation of the threshold limit values and biological exposure indices. Appendix C: Particle size-selective sampling criteria for airborne particulate matter*. 7th edn, American Conference of Governmental Industrial Hygienists (2001).
17. Rodes, C.E., Thornburg, J.W. in *Aerosols handbook : measurement, dosimetry, and health effects*. (eds Lev S. Ruzer, & Naomi H. Harley) CRC Press (2005).
18. Broadwater, K. *et al.* Investigating a persistent odor at an aircraft seat manufacturer. *J Occup Environ Hyg.* **13** (10), D159-165 (2016).
19. Couch, J. *et al.* *Health Hazard Evaluation Program: Evaluation of Potential Hazards during Harvesting and Processing Cannabis at an Outdoor Organic Farm. Report No. 2015-0111-3271*. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Cincinnati, OH (2017).
20. Feinstein, L.M., Sul, W.J., Blackwood, C.B. Assessment of bias associated with incomplete extraction of microbial DNA from soil. *Appl Environ Microbiol.* **75** (16), 5428-5433 (2009).
21. Keswani, J., Kashon, M.L., Chen, B.T. Evaluation of interference to conventional and real-time PCR for detection and quantification of fungi in dust. *J Environ Monit.* **7** (4), 311-318 (2005).
22. Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P., Kauserud, H. ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. *BMC Microbiol.* **10** 189 (2010).
23. Farrelly, V., Rainey, F.A., Stackebrandt, E. Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Appl Environ Microbiol.* **61** (7), 2798-2801 (1995).
24. Vesper, S. *et al.* Development of an Environmental Relative Moldiness index for US homes. *J Occup Environ Med.* **49** (8), 829-833 (2007).