2021

Optimizing DNA extraction from microorganisms living in Wind Cave

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OPTIMIZING DNA EXTRACTION FROM MICROORGANISMS LIVING IN WIND CAVE

A Thesis Submitted
in Partial Fulfillment
of the Requirements for the Designation
University Honors

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May 2021

This Study by: Allison Warming

Entitled: Optimizing DNA Extraction from Microorganisms Living in Wind Cave

Has been approved as meeting the thesis or project requirement for the Designation University Honors

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Date. Dr. Justin Peters, Honors Thesis Advisor, Chemistry and Biochemistry

_____ _____

Date. Dr. Jessica Moon, Director, University Honors Program

Acknowledgements

I would like to thank Dr. Justin Peters for all of his help and guidance, as well as the many hours dedicated to this project. Additionally, I would like to thank Dr. Joshua Sebree for organizing the trip to the Wind Cave and providing an amazing opportunity for students to perform research in the field. I would also like to thank the Wind Cave National Park Staff for allowing us to take more than just pictures of the cave, thank you for the samples! I couldn't have done this project without the help of the team members who went to the Wind Cave last spring: Katie Dyer, Hanna Lovstad, Pratima Raut, and Bridget Shoemaker… thank you all! Thank you to the College of Humanities, Arts and Sciences for the funding received through the SOAR grant. I would also like to thank the Department of Chemistry & Biochemistry for providing me with many opportunities to enhance my time here at the University of Northern Iowa. This project is based upon work currently supported by the Iowa Space Grant Consortium under NASA Award No. 80NSSC20M0107.

Abstract

Wind Cave National Park in South Dakota is home to many creatures, from the giant buffalo to microscopic single-celled organism. This study focused on the single-celled organisms living in Wind Cave. A total of 81 samples were collected and grown on culture media. The goal of this project was to determine which commercially available kit would yield the highest amount of purified DNA from the samples cultured from the cave. Following the protocols provided by the manufacturer, a control sample as well as samples from three plates had their DNA isolated and purified using seven commercially available DNA purification kits. Two kits yielded an optimal amount of DNA for PCR and sequence analysis, the Omega Bio-tek E.Z.N.A. Fungal DNA Mini kit and the Qiagen DNeasy PowerLyzer PowerSoil kit. Further isolation of other samples was done using both kits, and a total 21 samples were sent off for sequencing analysis.

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Introduction

On March 14th, 2020, the biochemistry laboratory research team left from the University of Northern Iowa to travel to Wind Cave National Park in South Dakota. We spent our spring break traveling down into the cave and collecting samples. At the end of two days in the cave, we returned home. On the journey back to Iowa, classes were moved online for the remainder of the semester, thus halting progress on the Wind Cave project. Upon return to campus in the Fall of 2020, samples were cultured and DNA extraction became the main focus. Our goal was to determine which commercial kits are most effective at isolating DNA from the samples collected in Wind Cave. Results were evaluated using polymerase chain reaction (PCR) amplification and gel electrophoresis as well as spectrophotometric quantification. The kits with the best yield will be used on further study of Wind Cave microorganisms at UNI, saving time and money.

Literature Review

While there have been many studies done on cave systems throughout the world, there have been few that focus exclusively on Wind Cave near Hot Springs, South Dakota. Although far from sunlight, the relative heat of the cave system as well as nitrates, phosphorous, and sodium create conditions where niches of microorganisms can survive. There are many visible indicators that there is microbial activity within the cave system. Dots on surfaces, unusual coloration, precipitates, corrosion residues, structural changes, and biofilms all point to potential colonies of microorganisms (Barton, 2006).

Wind Cave in South Dakota has over 150 miles of surveyed area as of February of 2019 (Farrell, 2019). Tours cover under 2 miles of the 150-mile-long cave. Prior research has been done on the cave system, both along the guided trails and deep into the cave off the public path (Palmer and Palmer, 1989; Beck, 2011; Ohms, 2016). One study, done between 2008 and 2011, evaluated the Madison Aquifer over 500 feet underground for microorganisms (Barton, 2011; Hershey et al., 2018). This work, done by Dr. Hazel Barton, focused on the comparison of microorganisms from the Madison Aquifer and Wind Cave lakes to other wells and bodies of water in the area (Barton and Northup, 2007; Hersey et al., 2017; Hershey et al. 2018). Researchers identified about 350 bacteria species from the bodies of water that were surveyed, many of which were new discoveries (U.S. National Park Service, 2020). Another study focused on identifying bacteria and archaea found in dripping water near Rainbow Falls, a multi-colored

flowstone (Chelius & Moore, 2004). This area is adjacent to the tour trails within the cave, as opposed to off-the-trail wild cave routes where there is very little human interference. Researchers used a soil DNA extraction kit in order to analyze collected samples and evaluated the different bacterial groups present in the cave samples collected from Rainbow Falls (Chelius & Moore, 2004).

Of the seven DNA isolation kits purchased to be used on this project, one brand is used frequently among other researchers of cave microbes. “Cave Drip Water-Related Samples as a Natural Environment for Aromatic Hydrocarbon-Degrading Bacteria” (Marques et al., 2019), “Molecular Phylogenetic Analysis of Archaea and Bacteria in Wind Cave, South Dakota” (Chelius & Moore, 2004), and “Metagenomic Analysis from the Interior of a Speleothem in Tjuv-Ante's Cave, Northern Sweden” (Mendoza et al., 2016) are all studies done on cave microorganisms using DNA extraction kits produced by Qiagen.

Methodology

Prior to leaving for Wind Cave National Park, our biochemistry lab class prepared slants and plates to collect our samples. Yeast extract-peptone-dextrose (YPD), potato dextrose agar (PDA), and luria broth (LB) agar were all prepared. Other supplies included sterile disposable inoculating loops of various sizes, conical vials to collect water samples, and many pairs of disposable gloves. All media and supplies were then packaged up into coolers and brought to South Dakota.

Figure 1: Collecting a sample from Wind Cave

Plates and slants were inoculated using the sterile disposable loops, as shown in Figure 1. The plates and slants were then labeled, recorded, and parafilmed shut. The sealed plates and slants were then placed in a plastic bag inside someone on the team's backpack to be taken back to the cooler at the end of the day.

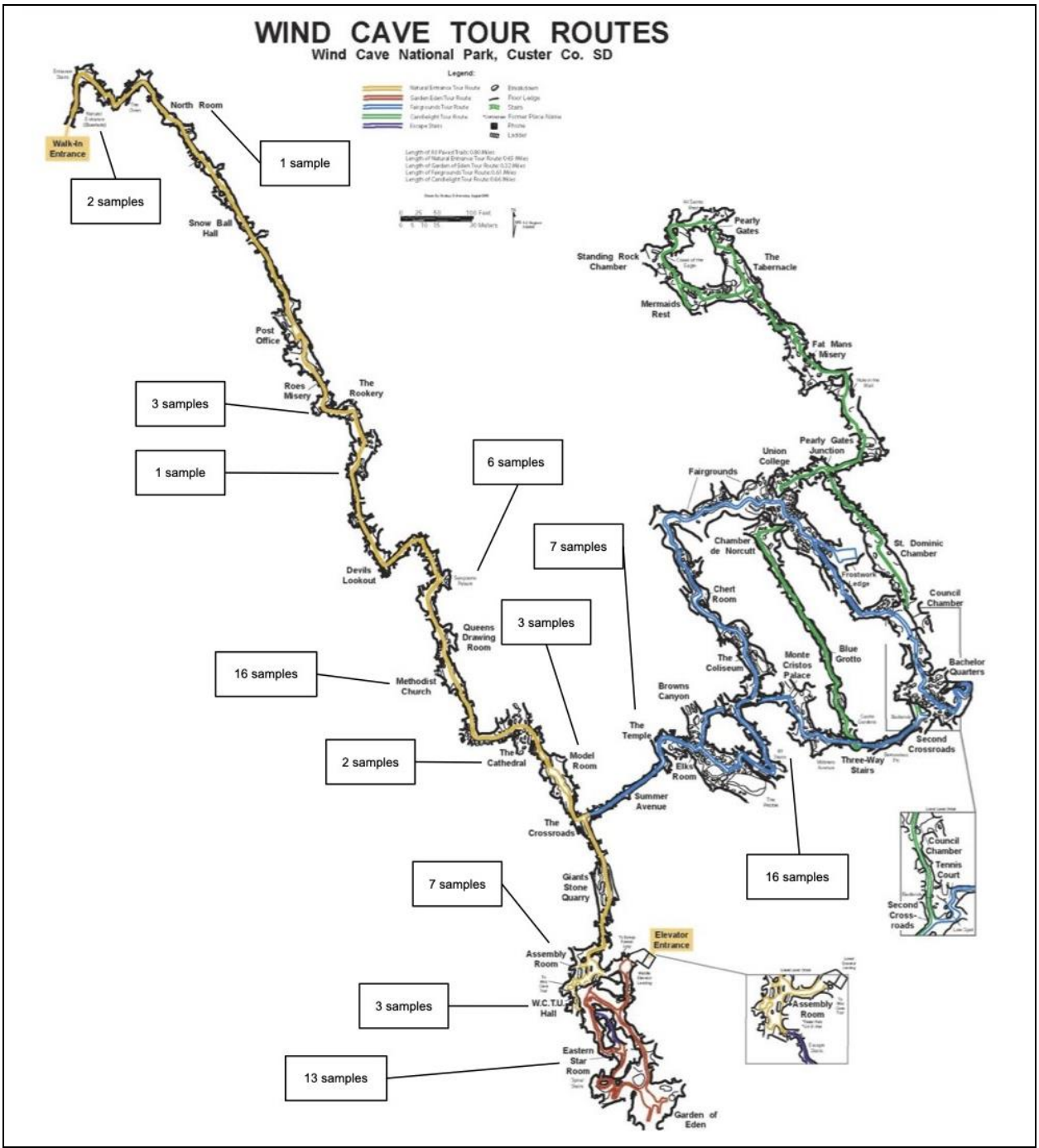

Figure 2: Map of Wind Cave trails labeled with where samples were collected

Figure 2 shows a map of the Wind Cave tour routes. Each area where samples were collected are labeled on the map along with the quantity of samples. While exploring the cave, the biochemistry research team inoculated the plates with material we thought might have a high level of microorganisms.

In December of 2020, three cultures were selected from the samples taken from Wind Cave. The selections included plates #6, #14, and #60, and were used with every DNA extraction kit purchased. Samples were chosen by determining whether there was enough organic material for use in the DNA purification and isolation process, as well as homogeneity in appearance across the plate.

The kits were then utilized in accordance with their individual sets of instructions. Each of the kits was run using material from all 3 cultures plus a control simultaneously. Each starting point was approximately 100 mg of a combination of organic material and media.

The first kit to be evaluated was the SurePrep Soil DNA Isolation Kit by Fisher BioReagents, followed by E.Z.N.A Fungal DNA Mini Kit by Omega Bio-tek, E.Z.N.A. Universal Pathogen Kit by Omega Bio-tek, GeneJET Genomic Purification Kit by Thermo Scientific, E.Z.N.A. Soil DNA Kit Omega Bio-tek, DNeasy PowerLyzer PowerSoil Kit by Qiagen, concluding with the final kit to be evaluated the DNeasy UltraClean Microbial Kit by Qiagen. The protocol for these kits can be found in the Appendix.

Spectroscopy was done on the samples as the kits were completed using a NanoDrop spectrophotometer. One microliter of elution buffer was used as the blank, and then one microliter of each sample was used to measure nucleic acid content. After all of the kits had been run and tabulated (Table 1), polymerase chain reaction (PCR) analysis of the DNA began.

PCR was performed on the samples in order to send to sequencing. Primers were used to target a specific, conserved region of the 16S small ribosomal subunit of prokaryotes, or an internal transcribed spacer (ITS) for identifying fungal eukaryotes. Initially, many nonspecific products were getting amplified, while some samples showed no amplification at all. Modifications were made to the PCR protocol including a reduction of magnesium ions and selection of suitable annealing temperature. The final protocol can be found in the Appendix. Following this optimized PCR protocol, the PCR reactions were then evaluated by gel electrophoresis. These samples were further purified using the GeneJET PCR Purification Kit by Thermo Scientific and sent for sequencing analysis.

Results

Table 1: NanoDrop Data

Sample ID	Nucleic Acid (ng/μL)	Resuspension Volume (μL)	Mass DNA (ng)	Starting Amount (mg)	Mass DNA/Starting Amount (ng/mg)
Fisher SurePrep 6	8	50	400	122.3	3.27
Fisher SurePrep 14	3.2	50	160	106.0	1.51
Fisher SurePrep 60	1.8	50	90	148.5	0.61
Fisher SurePrep control	9.5	50	475	102.9	4.62
EZNA Fungal 6	293.5	100	29350	100.5	292.03
EZNA Fungal 14	3.1	100	310	101.1	3.07
EZNA Fungal 60	60.6	100	6060	129.1	46.94
EZNA Fungal control	52	100	5200	107.9	48.19
EZNA Pathogen 6	4	100	400	98.3	4.07
EZNA Pathogen 14	6.2	100	620	98.2	6.31
EZNA Pathogen 60	29.1	100	2910	103.7	28.06
EZNA Pathogen control	11.8	100	1180	97.4	12.11
GeneJet 6	1.4	200	280	97.4	2.87
GeneJet 14	1.9	200	380	102.0	3.72
GeneJet 60	8.7	200	1740	96.7	17.99
GeneJet control	1	200	200	95.0	2.10
EZNA Soil 6	1.5	100	150	98.1	1.53
EZNA Soil 14	2.2	100	220	98.6	2.23
EZNA Soil 60	0.6	100	60	101.0	0.59
EZNA Soil control	6.6	100	660	98.0	6.73
DNeasy Soil 6	101.8	100	10180	101.9	99.90
DNeasy Soil 14	58.6	100	5860	112.0	48.03
DNeasy Soil 60	99.4	100	9940	113.6	87.5
DNeasy Soil control	17.5	100	1750	99.9	17.51
DNeasy Microbe 6	7.7	50	385	100.2	3.84
DNeasy Microbe 14	39.4	50	1970	108.1	18.22
DNeasy Microbe 60	10.2	50	510	104.5	4.88
DNeasy Microbe control	4.9	50	245	106.2	2.31

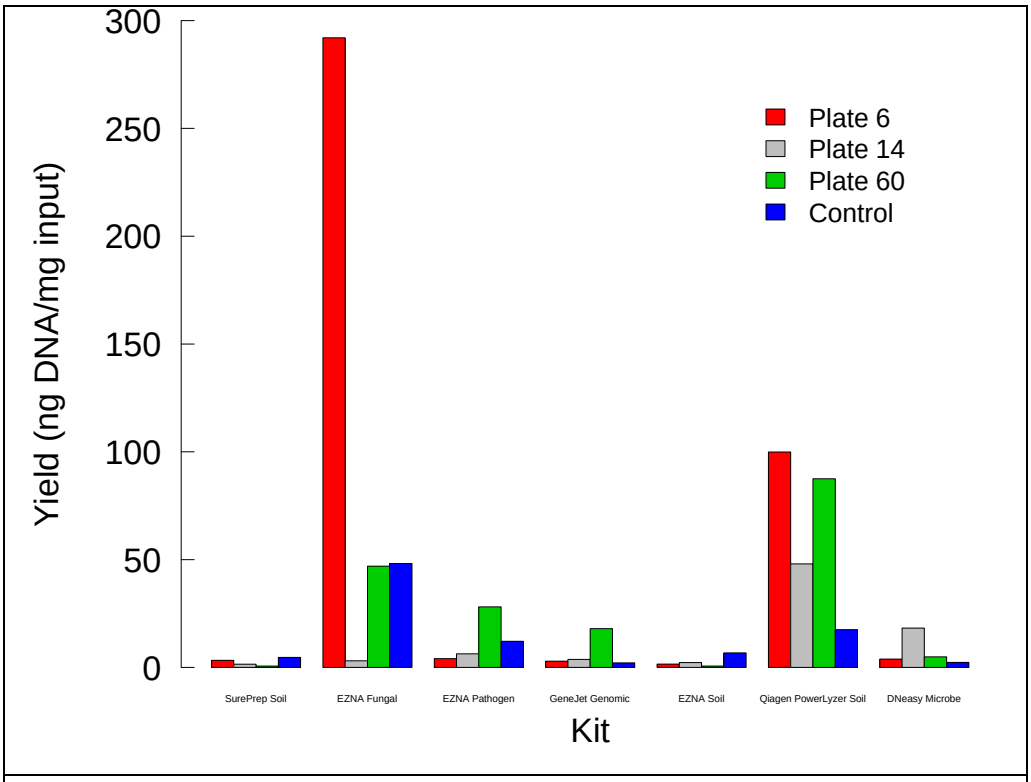

Figure 3: Kit Used vs. Yield

As shown in Table 1 and Figure 3 above, while each kit started with roughly the same amount of raw genetic material, only two kits stand out in terms of quantity of purified nucleic acid content. The Omega Bio-tek E.Z.N.A. Fungal DNA Mini kit demonstrated large yields from plate #6. For plate #14 yield was low, but the yield was lower from plate #14 across all kits. Plate #60 and the control both had high enough yields that PCR could be performed. The Qiagen DNeasy PowerLyzer PowerSoil Kit also produced high yields, across all samples tested, as shown in Table 1 and Figure 3. All samples from this kit had sufficient DNA purified that PCR could be performed.

Both high performing kits were used to isolate the DNA of more samples that were collected from the cave. An additional 11 samples were purified.

Next, polymerase chain reaction (PCR) was performed. Several protocols were tested in order to obtain a pure product. The final PCR protocol was shown to be selective for the desired product, and this protocol can be found in the Appendix.

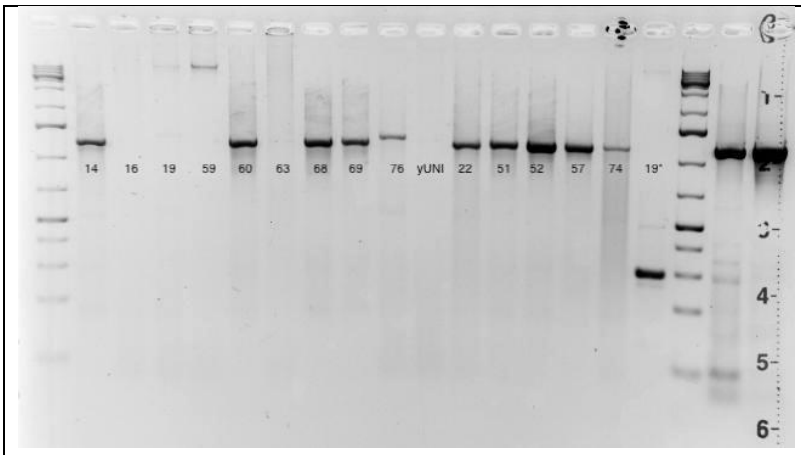

Figure 4: Results of preliminary PCR procedure 3/18

*with universal primer

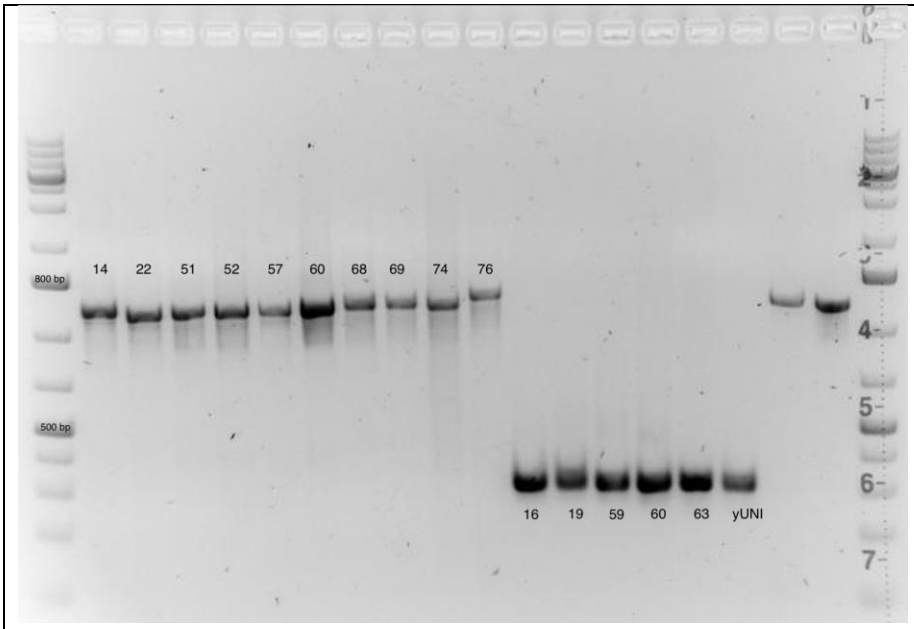

Figure 5: Results of final PCR procedure 3/26

Figure 4 shows the results of a preliminary PCR procedure. Samples 6, 19, 59, 60, and 63 did not produce a product when ITS primers were used, suggesting that these samples were not eukaryotic. In order to obtain a suitable product to sequence, prokaryotic universal primers were used on those samples (Figure 5). Sample #19 was run twice on the same gel, in one column with ITS primers and in another column with the prokaryotic universal primers. Figure 5 shows the results of the final PCR amplification done on all samples that were purified. Samples 14, 22, 51,

52, 57, 60, 68, 69, 74, and 76 were all processed using the ITS primers. Samples 16, 19, 59, 60, and 63 were processed using the prokaryotic universal primers. The products shown on the gel were purified with a GeneJET PCR Purification kit before being sent for sequencing analysis.

Preliminary results from sequencing show that there were both bacterial (firmicutes and proteobacteria) and fungal (ascomycetes and basidiomycetes) samples processed by both kits successfully. More work remains to be done to determine the specific identity of the organisms and create a genetic map of the different microorganisms found in the cave.

Discussion

Following day one of being down in the cave collecting samples, the results looked bleak. None of the plates collected showed early signs of growth 24 hours after inoculation. The growth came slower than expected when compared to microorganisms commonly used in lab. However, many plates eventually started to show some growth, after we had left South Dakota.

Each of the 3 samples from the Wind Cave used to determine which kit recovered the highest amount of DNA were grown on yeast extract-peptone-dextrose (YPD) media but were taken from two different areas of the cave. Plates #6 and #14 were inoculated with material from the Assembly Room, and plate #60 with material from the top of the 89 stairs. Since the location within the cave could influence the type of organisms cultured, we selected many more plates to take organic material from to purify and perform sequencing analysis.

When testing the kits, we did not initially know what kind of microorganism we were culturing. Some of the kits were marketed towards specific organisms, such as fungi or bloodborne pathogens. Different samples therefore may have shown a higher yield when using these targeted kits than the Wind Cave samples selected to be run on all seven of the kits. With both the original 3 samples and the additional 11 samples, both the DNeasy PowerLyzer PowerSoil Kit (Qiagen) and the E.Z.N.A Fungal DNA Mini Kit (Omega Bio-tek) showed good product yield across all organisms assayed. This indicates that the DNeasy PowerLyzer PowerSoil Kit and the E.Z.N.A Fungal DNA Mini Kit each provide a good quantity of DNA regardless of the particular cave microorganism being studied, as preliminary results from sequencing show that there were both bacterial and fungal samples processed by both kits successfully.

In addition to the quantity of purified DNA after the kit was run, the protocols were also evaluated for their ease of use. While performing the protocols provided by the manufacturer, it was noted that some were easier to follow than others. Time taken to complete the kits was also a factor. After completing all the protocols, I reflected on which I found the most efficient as well as the easiest to use. It is my opinion that the Qiagen DNeasy PowerLyzer PowerSoil Kit had the most user-friendly protocol. There was very little wait time, as compared to some other kits like the GeneJET kit that had incubation steps of up to an hour. There are also fewer steps in comparison to the E.Z.N.A Fungal DNA Mini Kit. Although ease of use is not as important as yield to consider when selecting a DNA purification kit, it is important to note when there are two kits that both performed well.

A sample of particular interest was #60, as it was found to have both fungus and bacteria growing on it. This is demonstrated in the success of both the ITS primers and prokaryotic universal primers in amplifying a clean product. Other samples that successfully amplified a product from the prokaryotic universal primers showed no product after amplification with the ITS primers, as shown in Figure 4. Preliminary results of the sequencing concur with this assessment, as the organisms identified from the ITS product was fungal and the organism identified from the prokaryotic universal product was bacterial.

Conclusion

Our goal was to determine which of the seven commercially-available kits is the most effective at isolating DNA from the samples collected in Wind Cave. Kits were used in accordance with the manufacturer provided instructions. Of the seven kits tested, two reliably provided enough DNA for PCR and sequencing. The E.Z.N.A Fungal DNA Mini Kit and the DNeasy PowerLyzer PowerSoil Kit were the two kits that showed the best results when working with samples from the Wind Cave. DNA from more samples taken throughout the Wind Cave was then isolated using these two kits. All showed good yield, so it was concluded that the kits worked well across many samples taken from all areas of the cave. Preliminary results from sequencing analysis show that there were both fungal and bacterial microorganisms with genetic material isolated by the kits.

Upon return from any future travels to the Wind Cave, the two optimal kits and optimized PCR protocol can be used to quickly purify and amplify the DNA from the cultured organisms.

More time can then be spent analyzing the sequence of many different microorganisms that were found in the cave. Understanding what organisms grow in the extreme environment of this cave will hopefully provide insight into what organisms may be growing in other extreme environments.

Future Work

When the biochemistry team went to collect samples from Wind Cave National Park, we had little knowledge of what to expect in the cave. We inoculated plates with material from all throughout the cave as we were exploring what areas might have a high level of microorganisms. Future groups could spend time looking at previous work done in the cave environment to better identify where to find microorganisms in the cave setting.

Furthermore, upon returning to the cave, future groups may venture further into the cave and take samples from areas outside the public paths. It is probable that some microorganisms grown are common organisms that are already known to us and have been brought into the cave by public tours. To take a look at what organisms may be more native and adapted to cave life, future groups may want to venture off the well-traveled, beaten paths.

As previously discussed, plate #60 likely had two organisms growing on it, one fungal and one bacterial. Other purified samples that showed results with the ITS primers could be re-run using the universal primers. This would show if bacterial organisms were present on the media in addition to the already identified fungal organisms.

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Appendix

SurePrep Soil DNA Isolation Kit (Fisher BioReagents)

Notes Prior to Use

- All centrifugation steps are carried out in a bench top micro centrifuge at 14,000 x g (~12,000 RPM) except where noted. All centrifugation steps are performed at room temperature.
- A variable speed centrifuge should be used for maximum kit performance. If a variable speed centrifuge is not available a fixed speed centrifuge can be used, however reduced yields may be observed.
- Ensure that all solutions are at room temperature prior to use.
- Prepare a working concentration of Wash Solution II by adding 42 mL of 95 - 100 % ethanol (provided by the user) to the supplied bottle containing the concentrated Wash Solution
- Recommended soil input varies depending on the soil type. For clay, loam and sand the recommended soil input is 250 mg. For fecal samples and compost, it is recommended that 100-150 mg of sample be used.

Protocol:

1. Weigh up to 250 mg of soil sample (please see Notes Prior to Use) into a 2 mL Screw Cap Tube provided with the kit. Note: In case of a wet soil sample, transfer the sample to a clean 1.7 mL micro-centrifuge tube and centrifuge for 30 seconds at 14,000 × g (~12,000 RPM). Remove the water carefully using a pipette, and weigh the remaining soil.
2. Add a 2:1 ratio of the provided Beads into the screw cap tube with the soil (500 mg of glass beads is added to 250 mg of soil).
3. Add 700 µL of Lysis Solution to the tube. Vortex briefly to mix soil and Lysis Solution.
4. Add 100 µL of Lysis Additive and vortex briefly.

5. Secure tube horizontally on a flat-bed vortex pad with tape, or secure the tube in any commercially available bead beater equipment (e.g. Scientific Industries' Disruptor GenieTM). Vortex for 5 minutes at maximum speed.
6. Centrifuge the tube for 1 minute at 14,000 × g (~12,000 RPM).
7. Transfer up to 450 µL of supernatant to a DNase-free micro-centrifuge tube
8. Add 100 µL of Binding Solution, mix by inverting the tube a few times, and incubate for 5 minutes on ice.
9. Spin the lysate for 1 minute to pellet any protein and soil particles.
10. Using a pipette, transfer up to 450 µL of supernatant into a DNase-free micro-centrifuge tube. Note: Avoid any contact with the pellet when collecting the supernatant. Also, depending on the soil type, some residue may be present on top of the supernatant. It is important to avoid collection of this residue while collecting the supernatant.
11. Add an equal volume of 70% ethanol (provided by the user) to the lysate collected above (100 µL of ethanol is added to every 100 µL of lysate). Vortex to mix.
12. Assemble a spin column with one of the provided collection tubes. Apply up to 600 µL of the clarified lysate with ethanol onto the column and centrifuge for 1 minute at 14,000 × g (~12,000 RPM). Discard the flow-through and reassemble the spin column with the collection tube. Note: Ensure the entire lysate volume has passed through into the collection tube by inspecting the column. If the entire lysate volume has not passed, spin for an additional minute.
13. Depending on your lysate volume, repeat if necessary.
14. Apply 500 µL of Wash Solution I to the column and centrifuge for 1 minute. Note: Ensure the entire wash solution has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute.
15. Discard the flow-through and reassemble the spin column with its collection tube.
16. Apply 500 µL of Wash Solution II to the column and centrifuge for 1 minute.
17. Discard the flow-through and reassemble the spin column with its collection tube.
18. Repeat 16 and 17.
19. Spin the column for 2 minutes in order to thoroughly dry the resin. Discard the collection tube.
20. Place the column into a fresh 1.7 mL Elution Tube provided with the kit.

21. Add 50 µL of Elution Buffer to the column.
22. Centrifuge for 2 minutes at 200 x g (~1,500 RPM), followed by a one minute spin at 14,000 x g (~12,000 RPM). Note the volume eluted from the column. If the entire volume has not been eluted, spin the column at 14,000 x g (~12,000 RPM) for one additional minute.

Per manufacturers website: product discontinued.
https://www.fishersci.com/shop/products/fisher-bioreagents-sureprep-soil-dna-isolation-kit-50-preps/bp281550?ef_id=CjwKCAjw9r-DBhBxEiwA9qYUpS06OA7ORqC1mb73M1V3o4fwlcp8h8j5SuCrm4TbV7NkCWYj5clAkxoCYUIQAvD_BwE:G:s&s_kwcid=AL!3652!3!326690343760!b!!g!!%2Bdna%

E.Z.N.A Fungal DNA Mini Kit (Omega)

Before Starting:

- Prepare the DNA Wash Buffer according to the instructions on Page 4
- Heat the sterile deionized water and Elution Buffer to 65°C
- Prepare an ice bucket

Protocol:

1. Prepare 10-50 mg powdered dry tissue in a 1.5 or 2 mL microcentrifuge tube.
2. Add 800 µL FG1 Buffer. Vortex vigorously to mix. Make sure to disperse all clumps. Note: Process in sets of four to six tubes: grind, add FG1 Buffer, then proceed to Step 3 before starting another set. Do not exceed 50 mg dried tissue.
3. Incubate at 65°C for 10 min. Mix sample twice during incubation by inverting tube.
4. Add 180 µL FG2 Buffer. Vortex to mix thoroughly.
5. Let sit on ice for 5 minutes.
6. Centrifuge at 10,000 x g for 10 minutes.
7. Transfer supernatant to a new microcentrifuge tube, making sure not to disturb the pellet or transfer any debris.
8. Add 0.7 volumes isopropanol. Vortex to precipitate DNA. Note: In most cases 700 µL supernatant can easily be removed. This will require 490 µL isopropanol. Note that depending on the sample, the volume of supernatant may vary. After transferring to a fresh tube, measure the volume and add the correct amount of isopropanol. This step will

remove much of the polysaccharide content and improves spin-column performance by increasing DNA binding capacity (and hence yield) in the steps that follow. No incubation is required after addition of isopropanol.

9. Immediately centrifuge at 10,000 x g for 2 minutes. Longer centrifugation does not improve yields.
10. Aspirate and discard the supernatant, making sure not to dislodge the DNA pellet.
11. Invert the microcentrifuge tube on a paper towel for 1 minute to allow residual liquid to drain. It is not necessary to dry the DNA pellet.
12. Add 300 µL sterile deionized water heated to 65°C. Vortex to resuspend the pellet. Note: A brief incubation at 65°C may be necessary to effectively dissolve the DNA.
13. Add 4 µL RNase A. Vortex to mix thoroughly.
14. Add 150 µL FG3 Buffer and 300 µL 100% ethanol. Vortex to mix thoroughly. Note: A precipitate may form upon addition of ethanol; it will not interfere with DNA isolation.
15. Insert a HiBind® DNA Mini Column into a 2 mL Collection Tube.
16. Transfer the entire sample (including any precipitate that may have formed) to the HiBind® DNA Mini Column.
17. Centrifuge at 10,000 x g for 1 minute.
18. Discard the filtrate and the Collection Tube.
19. Transfer the HiBind® DNA Mini Column to a new 2 mL Collection Tube.
20. Add 750 µL DNA Wash Buffer. Note: DNA Wash Buffer must be diluted with 100% ethanol prior to use. Please refer to Page 4 or the bottle label for instructions.
21. Centrifuge at 10,000 x g for 1 minute.
22. Discard the filtrate and reuse the Collection Tube.
23. Repeat Steps 20-22 for a second DNA Wash Buffer wash step.
24. Centrifuge the empty HiBind® DNA Mini Column at maximum speed for 2 minutes to dry the membrane. Note: It is critical to remove any trace of ethanol that may otherwise interfere with downstream applications.
25. Transfer the HiBind® DNA Mini Column to a nuclease-free 1.5 or 2 mL microcentrifuge tube.

26. Add 100 µL Elution Buffer (or sterile deionized water) heated to 65°C. Note: Smaller elution volumes will increase DNA concentration but decrease yield. Elution volumes greater than 200 µL are not recommended.
27. Let sit for 3 to 5 minutes.
28. Centrifuge at 10,000 x g for 1 minute.
29. Repeat Steps 26-28 for a second elution step. Note: Any combination of the following steps can be used to help increase DNA yield.
    - After adding the Elution Buffer, incubate the column for 5 minutes.
    - Increase the elution volume.
    - Repeat the elution step with fresh Elution Buffer (this may increase the yield, but decrease the concentration).
    - Repeat the elution step using the eluate from the first elution (this may increase yield while maintaining elution volume).
30. Store DNA at -20°C.

Protocol found on manufacturers website: https://www.omegabiotek.com/product/e-z-n-a-fungal-dna-mini-kit/?cn-reloaded=1#protocols

GeneJET Genomic Purification Kit (Thermo Scientific)

1. Harvest up to 1 x 10^8 yeast cells in a 1.5 or 2 mL microcentrifuge tube by centrifugation for 5-10 s at maximum speed ≥12000 g. Discard the supernatant.
2. Resuspend the pellet in 500 µL of Yeast lysis buffer. Incubate for 1 hour at 37 °C.
3. Centrifuge cells for 10 min at 3000 x g. Discard the supernatant.
4. Resuspend the pellet in 180 µL of Digestion Solution. Add 20 µL of Proteinase K Solution and mix thoroughly by vortexing or pipetting to obtain a uniform suspension.
5. Incubate the sample at 56 °C while vortexing occasionally or use a shaking water bath, rocking platform or thermomixer until the cells are completely lysed (x45 min).
6. Add 20 µL of RNase A Solution, mix by vortexing and incubate the mixture for 10 min at room temperature.
7. Add 200 µL of Lysis Solution. Mix thoroughly by vortexing for 15 s until a homogeneous mixture is obtained.
8. Add 400 µL of 50% ethanol and mix by pipetting or vortexing.

9. Transfer the prepared lysate to a GeneJET Genomic DNA Purification Column inserted in a collection tube.
10. Centrifuge the column for 1 min at 6000 x g. Discard the collection tube containing the flow-through solution.
11. Place the GeneJET Genomic DNA Purification Column into a new 2 mL collection tube (included). Note. Close the bag with GeneJET Genomic DNA Purification Columns tightly after each use!
12. Add 500 µL of Wash Buffer I (with ethanol added). Centrifuge for 1 min at 8000 x g. Discard the flow-through and place the purification column back into the collection tube.
13. Add 500 µL of Wash Buffer II (with ethanol added) to the GeneJET Genomic DNA Purification Column.
14. Centrifuge for 3 min at maximum speed (≥12000 x g). Optional. If residual solution is seen in the purification column, empty the collection tube and re-spin the column for 1 min. at maximum speed. Discard the collection tube containing the flow-through solution and transfer the GeneJETGenomic DNA Purification Column to a sterile 1.5 mL microcentrifuge tube (not included).
15. Add 200 µL of Elution Buffer to the center of the GeneJET Genomic DNA Purification Column membrane to elute genomic DNA.
16. Incubate for 2 min at room temperature and centrifuge for 1 min at 8000 x g. Note: For maximum DNA yield, repeat the elution step with additional 200 µL of Elution Buffer. If more concentrated DNA is required or DNA is isolated from a small amount of starting material the volume of the Elution Buffer added to the column can be reduced to 50-100 µL. Please be aware that smaller volumes of Elution Buffer will result in smaller final quantity of eluted DNA.
17. Discard the purification column. Use the purified DNA immediately in downstream or store at -20 °C

Protocol found on manufactures website:
https://www.thermofisher.com/order/catalog/product/K0721#/K0721

E.Z.N.A. Universal Pathogen Kit (Omega)

Before Starting:

- Prepare HBC Buffer and DNA Wash Buffer
- Set an incubator to 70°C.
- Heat Elution Buffer to 70°C.

Protocol:

1. Briefly spin the Disruptor Tube to remove any glass beads from the wall of the tube. Uncap the Disruptor Tube and save the cap for use in Step 3.
2. Add 25-30 mg tissue.
3. Add 725 µL SLX-Mlus Buffer. Seal the Disruptor Tube with the cap removed in Step 1.
4. Vortex at maximum speed for 3-5 minutes to lyse and homogenize the samples. For best results, a Mixer Mill, such as Spex CertiPrep Geno/Grinder® 2010 or Qiagen Tissuelyser, should be used. Note: Depending on the sample type and amount, the volume of SLX-Mlus Buffer may need to be adjusted so that 300 µL can be recovered during Step 12.
5. Centrifuge at 1,000-2,000 x g for 15 seconds at room temperature.
6. Uncap the Disruptor Tube and save the cap for use in Step 8.
7. Add 72 µL DS Buffer and 20 µL Proteinase K Solution.
8. Seal the Disruptor Tube with the cap removed in Step 6.
9. Vortex for 60 seconds to mix thoroughly.
10. Incubate at 70°C for 15 minutes. Mix once during incubation.
11. Centrifuge at 12,000 x g for 5 minutes.
12. Transfer 300 µL cleared supernatant to a 1.5 mL centrifuge tube (not provided). Note: Do not transfer any debris as it can reduce yield and purity.
13. Add 600 µL RBB Buffer. Vortex to mix thoroughly.
14. Let sit at room temperature for 5 minutes.
15. Insert a MicroElute® LE DNA Column into a 2 mL Collection Tube.
16. Transfer 700 µL sample from Step 14 to the MicroElute® LE DNA Column.
17. Centrifuge at maximum speed for 1 minute.
18. Discard the filtrate and reuse the collection tube.
19. Transfer the remaining lysate from Step 14 to the MicroElute® LE DNA Column.
20. Centrifuge at maximum speed for 1 minute.
21. Discard the filtrate and reuse the collection tube.

22. Add 500 µL HBC Buffer. Note: HBC Buffer must be diluted with 100% isopropanol before use. Please see Page 4 for instructions.
23. Centrifuge at maximum speed for 30 seconds.
24. Discard the filtrate and collection tube.
25. Insert the MicroElute® LE DNA Column into a new 2 mL Collection Tube.
26. Add 700 µL DNA Wash Buffer. Note: DNA Wash Buffer must be diluted with 100% ethanol before use. Please see Page 4 for instructions.
27. Centrifuge at maximum speed for 30 seconds.
28. Discard the filtrate and reuse the collection tube.
29. Repeat Steps 26-28 for a second DNA Wash Buffer wash step.
30. Centrifuge the empty MicroElute® LE DNA Column at maximum speed for 2 minutes to dry the column. Note: This step is critical for removal of trace ethanol that may interfere with downstream applications.
31. Transfer the MicroElute® LE DNA Column into a nuclease-free 1.5 mL microcentrifuge tube (not provided).
32. Add 15-100 µL Elution Buffer heated to 70°C.
33. Let sit at room temperature for 2 minutes.
34. Centrifuge at maximum speed for 1 minute.
35. Repeat Steps 32-34 for a second elution step. Note: Each 200 µL elution will typically yield of 60-70% of the DNA bound to the column. Two elutions will generally yield ~90%. However, increasing the elution volume will reduce the concentration of the final product. To obtain DNA at higher concentrations, elution can be carried out using 50-100 µL Elution Buffer which slightly reduces overall DNA yield. Volumes lower than 50 µL greatly reduce yields. In some instances yields may be increased by incubating the column at 70°C (rather than room temperature) upon the addition of Elution Buffer.
36. Store eluted DNA at -20°C.

Protocol found on manufactures website: https://www.omegabiotek.com/product/e-z-n-a-universal-pathogen-kit/#protocols

E.Z.N.A. Soil DNA Kit (Omega)

Before Starting:

- Prepare HBC Buffer and DNA Wash Buffer as instructed in the “Preparing Reagents” section on Page 4
- Set a incubator to 70°C
- Heat Elution Buffer to 70°C
- Prepare an ice bucket
- Chill P2 Buffer in an ice bucket

Protocol:

1. Add 100-250 mg soil sample to a Disruptor Tube.
2. Add 725 µL SLX-Mlus Buffer. Vortex at maximum speed for 3-5 minutes to lyse samples. Note: For best results, a mixer mill, such as GenoGrinder 2010, Fastprep-24®, or Omni Bead Ruptor should be used.
3. Spin at 500 x g for 5 seconds to remove drops of liquid from the lid.
4. Add 72 µL DS Buffer. Vortex to mix thoroughly.
5. Incubate at 70°C for 10 minutes. Briefly vortex the tube once during incubation.
6. Centrifuge at 10,000 x g for 5 minutes at room temperature.
7. Transfer 400 µL supernatant into a new 1.5 mL microcentrifuge tube (not provided).
8. Add 135 µL chilled P2 Buffer. Vortex to mix thoroughly.
9. Let sit on ice for 3 minutes.
10. Centrifuge at maximum speed (≥13,000 x g) for 1minute.
11. Carefully transfer the supernatant to a new 1.5 mL microcentrifuge tube.
12. Add 200 µL HTR Reagent. Vortex to mix thoroughly. Note: Completely resuspend HTR Reagent by shaking the bottle before use. It may be necessary to cut the end of the pipette tip to aspirate and dispense HTR Reagent.
13. Let sit at room temperature for 2 minutes.
14. Centrifuge at maximum speed for 1 minute.
15. Transfer cleared supernatant (~500 µL) to a new 1.5 mL microcentrifuge tube. Note: If supernatant still has a dark color from the soil, repeat Steps 12-14 for a second HTR Reagent step. This will require additional HTR Reagent that can be purchased separately.
16. Add an equal volume XP1 Buffer. Vortex to mix thoroughly.
17. Insert a HiBind® DNA Mini Column into a 2 mL Collection Tube (provided).
18. Transfer up to 700 µL sample from Step 16 to the HiBind® DNA Mini Column.

19. Centrifuge at 10,000 x g for 1 minute at room temperature.
20. Discard the filtrate and reuse the Collection Tube.
21. Repeat Steps 18-20 until all the lysate from Step 16 has passed through the HiBind® DNA Mini Column.
22. Add 500 µL HBC Buffer. Note: HBC Buffer must be diluted with 100% isopropanol before use. Please see the "Preparing Reagents" section on Page 4 for instructions.
23. Centrifuge at 10,000 x g for 1 minute.
24. Discard the filtrate and the Collection Tube.
25. Transfer the HiBind® DNA Mini Column into a new 2 mL Collection Tube.
26. Add 700 µL DNA Wash Buffer. Note: DNA Wash Buffer must be diluted with 100% ethanol before use. Please see the "Preparing Reagents" section on Page 4 for instructions.
27. Centrifuge at 10,000 x g for 1 minute.
28. Discard the filtrate and reuse the Collection Tube.
29. Repeat Steps 26-28 for a second DNA Wash Buffer wash step.
30. Centrifuge the empty HiBind® DNA Mini Column at maximum speed for 2 minutes at room temperature. Note: This step is critical in removing residual ethanol that may interfere with downstream applications.
31. Transfer the HiBind® DNA Mini Column into a clean 1.5 mL microcentrifuge tube.
32. Add 50-100 µL Elution Buffer heated to 70°C directly onto the center of HiBind® matrix.
33. Let sit at room temperature for 1-2 minutes.
34. Centrifuge at maximum speed for 1 minute.
35. Take the filtrate from Step 34 and place onto the center of the same HiBind® DNA Mini Column used in the procedure.
36. Let sit at room temperature for 1 minute.
37. Centrifuge at maximum speed for 1 minute.
38. Store eluted DNA at -20°C.

Protocol found on manufacturers website: https://www.omegabiotek.com/product/e-z-n-a-soil-dna-kit/#protocols

DNeasy PowerLyzer PowerSoil Kit (Qiagen)

Important points before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If Solution C1 has precipitated, heat at 60°C until precipitate dissolves.
- Shake to mix Solution C4 before use.

Procedure

1. Add up to 0.25 g of soil sample to the PowerBead Tube provided.
2. Add 750 µl of PowerBead Solution to the PowerBead Tube.
3. Add 60 µl of Solution C1 and invert several times or vortex briefly.
4. Bead beating options:
    a. PowerLyzer 24 Homogenizer: Place the PowerBead Tubes into the tube holder for the PowerLyzer 24 Homogenizer. The PowerBead Tubes must be balanced in the tube holder. Run the samples for a time and RPM suitable for your soil type. Note: For clay soils, 4,000 RPM for 45 s is the best starting point. For loose, granular and high organic soils, 2,500 RPM for 45 s will provide an optimal result.
    b. Vortex: Secure the PowerBead Tubes horizontally using a Vortex Adapter (cat. no. 13000-V1-24). Vortex at maximum speed for 10 min. Note: If you are using a 24-place Vortex Adapter for more than 12 preps, increase the vortex time by 5–10 min.
5. Make sure the PowerBead Tubes rotate freely in the centrifuge without rubbing. Centrifuge at 10,000 x g for 30 s. Do not exceed 10,000 x g. Note: Centrifuge for 3 min at 10,000 x g for clay soils or if your soil is not completely pelleted after 30 s.
6. Transfer the supernatant to a clean 2 ml Collection Tube (provided). Note: Expect 400–500 µl. Supernatant may still contain some soil particles.
7. Add 250 µl of Solution C2 and vortex for 5 s. Incubate at 2–8°C for 5 min. Note: You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with the incubation we recommend you retain the step.
8. Centrifuge the tubes for 1 min at 10,000 x g. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml Collection Tube (provided).

9. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min. Note: You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with the incubation we recommend you retain the step.
10. Centrifuge the tubes for 1 min at 10,000 x g. Avoiding the pellet, transfer up to 750 µl of supernatant into a clean 2 ml Collection Tube (provided).
11. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 s.
12. Load 675 µl of the supernatant onto an MB Spin Column and centrifuge at 10,000 x g for 1 min. Discard the flow-through and add an additional 675 µl of supernatant.
13. Centrifuge at 10,000 x g for 1 minute. Load the remaining supernatant onto the MB Spin Column and centrifuge at 10,000 x g for 1 min. Note: A total of three loads for each sample processed is required.
14. Add 500 µl of Solution C5 and centrifuge for 30 s at 10,000 x g.
15. Discard the flow-through. Centrifuge again for 1 min at 10,000 x g.
16. Carefully place the MB Spin Column in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the MB Spin Column.
17. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you may use sterile DNA-free PCR-grade water (cat. no. 17000-10) or TE buffer.
18. Centrifuge for 30 s at 10,000 x g. Discard the MB Spin Column.
19. The DNA is now ready for downstream applications.

Protocol found on manufacturers website:
https://www.qiagen.com/us/resources/resourcedetail?id=329362e4-03e6-4ae1-9e4e-bbce41abe4b7&lang=en

DNeasy UltraClean Microbial Kit (Qiagen):

Important points before starting

- If Solution SL has precipitated, heat at 55°C for 5–10 min.
- Shake to mix Solution SB before use.
- Perform all centrifugation steps at room temperature (15–25°C).

Procedure

1. Add 1.8 ml of microbial (bacteria, yeast) culture to a 2 ml Collection Tube (provided) and centrifuge at 10,000 x g for 30 s. Decant the supernatant and spin the tubes again at 10,000 x g for 30 s. Completely remove the supernatant with a pipette tip. Note: Depending on the type of microbial culture, it may be necessary to centrifuge longer than 30 s.
2. Resuspend the cell pellet in 300 µl of PowerBead Solution and gently vortex to mix. Transfer resuspended cells to a PowerBead Tube.
3. Add 50 µl of Solution SL to the PowerBead Tube. Note: To increase yields, to minimize DNA shearing or for difficult cells, refer to the Troubleshooting Guide, page 18.
4. Secure PowerBead Tubes horizontally using the Vortex Adapter (cat. no. 13000-V1-24). Vortex at maximum speed for 10 min.
5. Make sure the 2 ml PowerBead Tubes rotate freely in the centrifuge without rubbing. Centrifuge the tubes at a maximum of 10,000 x g for 30 s.
6. Transfer the supernatant to a clean 2 ml Collection Tube (provided). Note: Expect 300–350 µl of supernatant.
7. Add 100 µl of Solution IRS to the supernatant and vortex for 5 s. Incubate at 4°C for 5 min.
8. Centrifuge the tubes at 10,000 x g for 1 min.
9. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2 ml Collection Tube (provided). Note: Expect 450 µl of supernatant.
10. Add 900 µl of Solution SB to the supernatant and vortex for 5 s.
11. Load about 700 µl into an MB Spin Column and centrifuge at 10,000 x g for 30 s. Discard the flow-through, add the remaining supernatant to the MB Spin Column, and centrifuge again at 10,000 x g for 30 s. Note: Each sample processed will require 2–3 loads. Discard all flow-through.
12. Add 300 µl of Solution CB and centrifuge at 10,000 x g for 30 s.
13. Discard the flow-through. Centrifuge at 10,000 x g for 1 min.
14. Place the MB Spin Column in a new 2 ml Collection Tube (provided). Note: Be careful not to splash any of the liquid on the MB Spin Column.
15. Add 50 µl of Solution EB to the center of the white filter membrane.
16. Centrifuge at 10,000 x g for 30 s.

17. Discard the MB Spin Column. The DNA is now ready for downstream applications.
    Note: We recommend storing DNA frozen (–30°C to –15°C or –90°C to –65°C) as Solution EB does not contain EDTA.

Protocol found on manufacturers website:
https://www.qiagen.com/us/resources/resourcedetail?id=a733f3a9-5a8b-4003-b28e-55bb050bad1e&lang=en

PCR Amplification for ITS region

Protocol:

1. Set up PCR reaction amplify ~1200 bp

Reagents	**Volume**
DNA (~20 ng/µl)	1 µl
UNI135 (ITS5) (10µM)	1 µl
UNI136 (LR3) (10µM)	1 µl
10X *Taq* buffer	2.5 µl
10X BSA	2.5 µl
50 mM $MgCl_2$	0.5 µl
10 mM dNTP mix	0.5 µl
Taq DNA polymerase	0.5 µl
MQ H_2O	15.5 µl
Total Volume	**25 µl**

2. Amplify the reaction using the following cycling parameters for *Taq* DNA polymerase

Temperature	Time	Cycles
94 °C	2 minutes	1
94 °C	30 seconds	
50 °C	30 seconds	35
72 °C	30 seconds	
72 °C	2 minutes	1
4 °C	hold	

3. Purify samples using Thermo GeneJET PCR Purification Kit
    a. Add 1 volume of Binding Buffer to 1 volume of the PCR sample.Check that the color of the mixture is yellow (similar to Binding Buffer without PCR sample), otherwise add 10 µl of 3 M NaOAc, pH 5.2
    b. Transfer up to 800 µl of the solution from step 1 to the GeneJET purification column. Centrifuge for 60 s. Discard flow-through.
    c. Add 700 µl of Wash Buffer (diluted with ethanol as described) to the GeneJET purification column. Centrifuge for 60 s. Discard flow-through and place the purification column back into the collection tube.
    d. Centrifuge empty GeneJET purification column for an additional 1 min to completely remove any residual wash buffer.
    e. Transfer the GeneJET purification column to a clean 1.5 mL microcentrifuge tube. Add 30 µl of Elution Buffer to the center of the GeneJET purification column membrane and centrifuge for 1 min.
    f. Discard GeneJET purification column and store purified DNA at -20 °C.
4. Check mixtures on a 2% agarose gel in 1X TAE + EtBr
    a. Add 1 µl of agarose loading dye to each 5 µl sample
    b. Use DNA ladder to detect sequences of interest