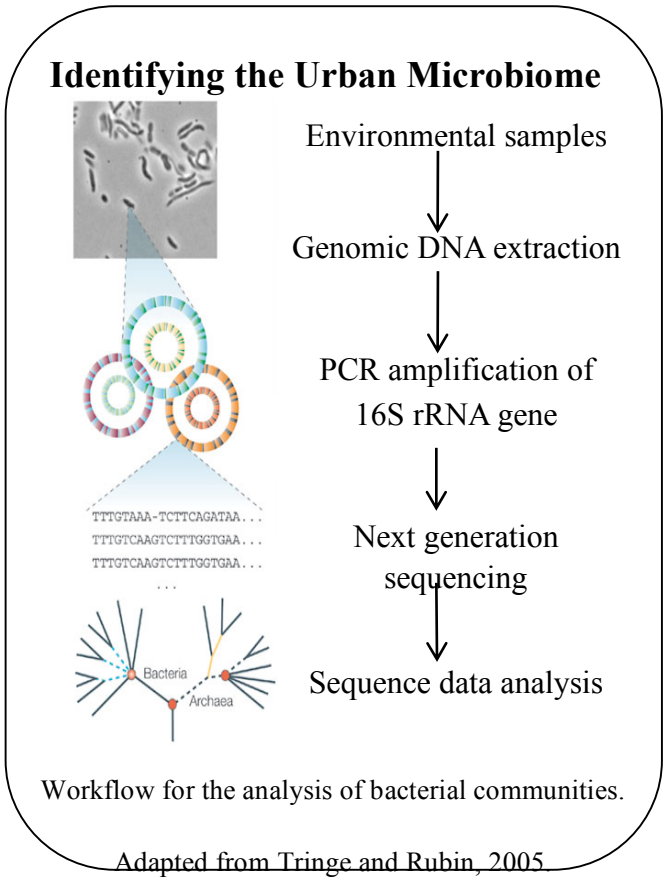
Urban Microbiome DNA Isolation Protocol 9.10.20
Muth & Smyth Lab 2020

Introduction:

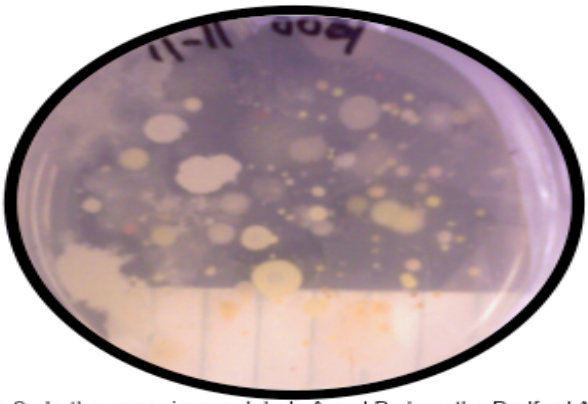
Microorganisms are present in almost every natural environment and they are often the most numerous type of organism. Until recently, studies of microbial diversity were limited to methods where only microorganisms whose growth could be detected in the lab in nutrient media could be identified, quantified and studied. It is estimated that only <10% of the microbial diversity in any given sample can be cultivated in the lab and detected in this way, therefore much of the microbial diversity has gone undetected. **Metagenomic** approaches have been introduced that detect a much greater portion of the actual diversity in a sample. Metagenomic approaches are culture-independent, which means that it is not necessary to cultivate the microorganisms to detect their presence. Instead, genomic DNA (or RNA in the case of some viruses) is isolated from the environmental sample being studied and using "signature" DNA sequences as a proxy to quantify and characterize the microorganisms present in the sample. The isolated DNA contains genomes representing, in principle, all of the microorganisms that were present in the sample (hence the prefix "meta", in metagenomics).



Workflow for the analysis of bacterial communities.

Adapted from Tringe and Rubin, 2005.

Metagenomic approaches have now been used to analyze numerous natural environments across the globe as well as the microbiomes of humans and a variety of other organisms. More research is focused on microbial communities in the ocean and distant regions of the earth than the urban microbial community – despite the fact that a majority of the world's population now resides in cities (Yergeau *et al.*, 2010; Tringe *et al.*, 2005).



re 2: In the upper image, labels A and B show the Bedford Avenu
Nostrand Avenue sites, respectively, where samples were collecte
the street and sidewalk. C shows the approximate location of the
to the Flatbush subway station where the platform was sampled.
se sites were sampled every month for a year. The lower image
ws a nutrient agar plate growing bacteria isolated from an urban
obial community sample. This likely represents less than 10% of
diversity present in the sample.

The metagenomic approach can be used to analyze any set of organisms from the environment, however, for the work in this lab we will begin by focusing our investigation on the bacterial members of the urban microbial community and leave viruses, fungi and other groups microorganisms to future studies. A specific region of each microbial genome is examined to focus on a subset of organisms– in the case of bacteria this is most often the gene coding for the 16S rRNA. The 16S rRNA gene is used because it is present in all bacteria and it has regions of sequence that are shared among all bacteria as well as variable regions in the gene that differ from species to species.

To investigate the urban bacterial communities of your surrounding area you will collect samples from the environment and prepare and analyze these samples. Each of your samples contains the genomic DNA from all of the organisms present. After collecting the environmental sample the DNA from the bacteria needs to be purified away from the bacterial cells and all the other unwanted dirt and debris that is collected. We will use universal PCR primers to amplify a variable region within the bacterial 16S rRNA gene. The universal primers are designed to be variable in several positions (that is, a given position in the sequence of the primer could be A, C, G or T for example, and the resulting primers will not all be identical in sequence) for the amplification of 16S rRNA gene from as broad a range as possible of the bacterial species represented in the sample. Each PCR sample will be visually examined by gel electrophoresis to confirm the correct band size and each sample will be quantified using a NanoDrop 2000 spectrophotometer. The company that sequences our DNA samples uses the Illumina MiSeq technology. The sequence data from the 16S rRNA genes can them be compared to a database to determine the bacterial diversity of the sample.

<u>Required</u> background reading:

1. N. Pace review article: Mapping the Tree of Life: Progress and Prospects; Microbiology and Molecular Biology Reviews, Dec. 2009, p. 565–576
2. J. Handelsman review article: Metagenomics: Application of Genomics to Uncultured Microorganisms; Microbiology and Molecular Biology Reviews, Dec. 2004, p. 669–685

3. Microbiology; A Human Perspective, 6th edition, Nester et al., chapter 1 and chapter 11 (or equivalent chapters from other introductory microbiology texts).

Recommended background reading:
* These reading cover next generation sequencing technologies and metagenomics data analysis. They can be technical, but reading them to get the general sense of things can be very useful.

1. Logares, R., Haverkamp, T. H. A., Kumar, S. et. al.. (2012). Environmental microbiology through the lens of high-throughput DNA sequencing: Synopsis of current platforms and bioinformatics approaches. Journal of Microbiological Methods, 91(1), 106-113.
2. Metzker, M. L. (2010). Sequencing technologies the next generation. Nature Reviews Genetics, 11(1), 31-46.
3. Scholz, M. B., Lo, C., & Chain, P. S. G. (2012). Next generation sequencing and bioinformatic bottlenecks: The current state of metagenomic data analysis. Current Opinion in Biotechnology, 23(1), 9-15.
4. Shokralla, S., Spall, J. L., Gibson, J. F., & Hajibabaei, M. (2012). Next-generation sequencing technologies for environmental DNA research. Molecular Ecology, 21(8), 1794-1805.

Experiment

A study of the bacterial diversity from urban soil samples collected from around your urban environment.

Procedure - Discuss as a class an overall research question to guide sampling locations. Collect soil samples. Extract microbiome community DNA and sequence with Illumina MiSeq.

*Students work in a group of 3 students (each bench island forms a group; 6 groups per Micro 3004 lab section)

Questions

1) What is your group's hypothesis for this experiment?

2) What are the controls for this experiment? What are key environmental data to collect that cannot be controlled between samples?

Part 1: Experiment Setup

1. Collect exposed soil or sand avoiding roots, twigs, stones, and other debris. Using sterile tube or plastic bag. Label the containers with your sample location, group number/identifier, date, and lab instructor's name.

2. Store at 4°C (refrigerator) until Part 2 & 3.

Part 2: Bacteria Initial Viability

1. Using the **lid** of a sterile 1.7 ml microcentrifuge tube, transfer 2 lid full of soil from the container into a test tube with 9mL of sterile water & appropriately labeled. Flick tube vigorously to mix. The soil will settle to the bottom. Perform a 1:10 dilution: Transfer 1 ml of "dirty" water from the first test tube to a second test tube of 9 ml of sterile water (labeled 1:10). Flick tube vigorously to mix.

2. Spread plate 100 µl (0.1 ml) from your 2 tubes (undiluted and 1:10) on to a labeled **Nutrient agar plate**.

Part 3: DNA Extraction

Follow the Qiagen DNeasy PowerSoil protocol for isolation of genomic DNA form your sample. *Note: This part of the protocol may also be used on urban environmental DNA samples collected via cotton swabs. Attatched after this section will be visuals of DNA extraction for both types of samples.*

1. Transfer the entire resuspended ~100 µl from your microcentrifuge tubes to a PowerBead tube.

   After your sample has been loaded into the PowerBead tube the next step is homogenization and lysis. The PowerBead tube contains a buffer that will (a) help disperse the debris particles, (b) begin to dissolve any humic acids that may be present and (c) protect the nucleic acids (genomic DNA) from degradation.

   Note from Davida – there are different ways to isolate DNA – most commonly used are enzymatic lysis and mechanical lysis. In this case, you're using a physical means to lyse the cell therefore it's mechanical.

2. Gently vortex or invert PowerBead tubes to mix.

   What's happening: gentle vortexing mixes the components in the PowerBead tube and begins to disperse the sample in the PowerBead solution.

3. Obtain **solution C1** from your instructor. If solution C1 has a precipitate in it then notify your instructor – it may be necessary to heat the C1 solution to 60°C.

   Solution C1 contains SDS and other disruption agents required to complete cell lysis of the bacteria in your sample. In addition, SDS is an anionic detergent (lipophilic part of the molecule is an anion) that breaks down fatty acids and lipids associated with the cell membrane of many bacteria. If it gets cold it will form a white precipitate in the bottom of the tube. If this is the case then heating to 60°C will dissolve the precipitate of SDS and the C1 solution can then be used. Be careful as SDS is pretty nasty. The quaternary ammonium compounds we're studying in the lab are cationic detergents.

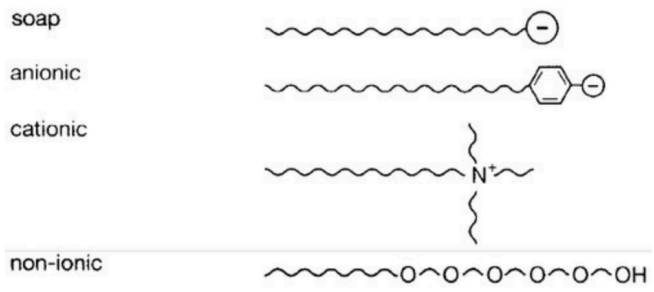


Figure from Ref 1.

*Watch this helpful video to help you understand the interactions of membranes with detergents (*https://www.youtube.com/watch?v=yAXnYcUjn5k*)* and this one on membranes in general (https://www.youtube.com/watch?v=nsklF1w4eok).

4. Add 60 µl of **solution C1** to your PowerBead tube and invert several times to mix or vortex briefly.

5. Secure PowerBead tube horizontally using the vortex adapter. Vortex for 10 minutes at maximum speed.

   What's happening: the vortexing step is critical for complete homogenization and cell lysis. Cells are lysed using a combination of chemical agents from steps 1-4 and mechanical shaking. By randomly shaking the beads in the presence of disruption agents, collisions of the beads with the microbial cells will cause the cells to break open. This is called "mechanical lysis".

6. Place your PowerBead tube in the centrifuge as shown by your instructor. Make sure your tube is balanced against another PowerBead tube (from another group) containing approximately the same volume (if there is not another group's tube to balance with, then use a microcentrifuge tube containing water that weighs the same as your PowerBead tube). Centrifuge your tube at 10,000 x g for 30 seconds at room temperature. CAUTION: be sure not to exceed 10,000 x g or the tubes may crack.

   Note from Davida: How to use a centrifuge https://www.youtube.com/watch?v=IhJNFGfsUus. What kind of mistakes can be made? What are the different types for?

7. Transfer the supernatant to a clean, labeled, 2 ml collection tube.

   You can expect between 400-500 µl of supernatant at this step. The exact recovered volume depends on the absorbency of your starting material and is not critical for the procedure to be effective. The supernatant may be dark in appearance and still contain some particulate debris. Subsequent steps in the protocol will remove the particulate matter.

   Note from Davida: What impact might there be if you were unable to recover a lot of your volume?

8. Obtain solution C2 from your instructor. Add 250 µl of **solution C2** and vortex for 5 seconds. Incubate at 4°C (in the refrigerator) for 5 minutes.

   Solution C2 is a patented inhibitor Removal Technology (IRT) (Which means I don't know what's in there). It contains a reagent to precipitate non-DNA organic and inorganic material, cell debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce the DNA purity and inhibit downstream DNA applications, such as PCR and DNA sequencing.

   Note from Davida: Many sample types are known to contain inhibitors such as blood, fabrics, tissues and soil. Humic acid we mentioned is the organic components of soil. Blood can have heme. Feces can have bile salts. As you're working with the kit, kit components can also inhibit PCR such as excess salts such as KCl, NaCl, ionic detergents such as sodium deoxycholate, sarkosyl and SDS, along with ethanol and isopropanol, phenol and others.

9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g. Make sure to face the hinge of the tube to the outside (away from the center of the rotor – this is so any pellet will collect at the bottom or side of the tube underneath the hinge).

10. Avoiding the pellet (there may not be a visible pellet), transfer up to 600 µl of the supernatant (liquid) to a clean 2 ml collection tube.

    The pellet at this point contains non-DNA organic and inorganic material including cell debris and protein. For the best DNA yields and quality, avoid transferring any of the pellet to the collection tube.

    Note from Davida: Why do you want to avoid transferring the pellet over?

11. Obtain solution C3 from your instructor. Add 200 µl of **solution C3** and vortex briefly. Incubate at 4°C (in the refrigerator) for 5 minutes.

    Solution C3 is patented inhibitor removal technology (IRT) and is a second reagent to precipitate additional non-DNA organic and inorganic material, including cell debris and protein. It is important to remove contaminating organic and inorganic matter that may reduce the DNA purity and inhibit downstream DNA applications, such as PCR and DNA sequencing.

12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.

13. Transfer up to 750 ul of supernatant to a clean 2 ml collection tube.

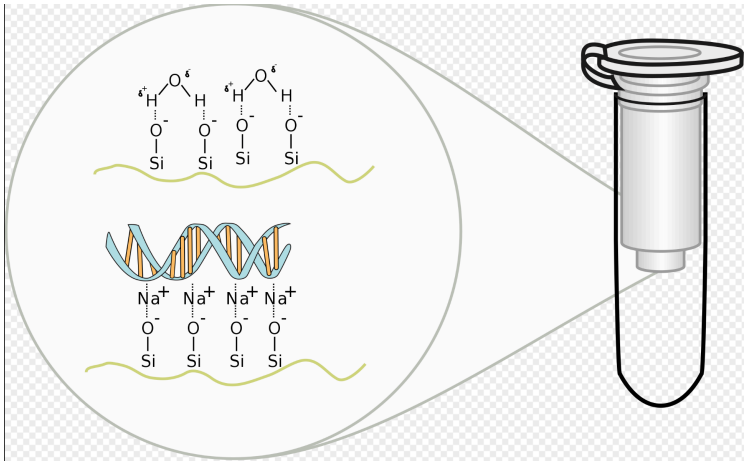
    T*he pellet at this point contains non-DNA organic and inorganic material including cell debris and protein. For the best DNA yields and quality, avoid transferring any of the pellet to the collection tube.*

14. Obtain solution C4 from your instructor. Shake to mix **solution C4** before use. Add 1.2 ml (2x 600 ul) of solution C4 to the supernatant, being careful not to overflow the tube). Close tube and vortex for 5 seconds.

    Solution C4 is a high concentration salt solution. Since DNA binds tightly to silica (is an oxide of silicon) at high salt concentrations, this will adjust the DNA solution salt concentration to allow binding of DNA, but not contaminants that may still be present, to the spin filter.

15. Load approximately 675 ul of your DNA containing solution into a spin filter tube and centrifuge at 10,000 x g for 1 minute at room temperature. Remove the filter insert and discard flow through from the collection tube – dump into a waste container or in the sink. Replace the filter insert to its collection tube base. Add another 675 ul of your DNA containing solution into the filter and spin at 10,000 x g for 1 minute. Dump the flow through as before and replace the spin filter into the collection tube base. Add the remaining DNA containing solution to the filter and spin at 10,000 x g for 1 minute. Dump the flow through as above.

    A total of three loads for each sample processed are required. The DNA is selectively bound to the silica membrane in the spin filter device in the high salt solution. Contaminants pass through the filter membrane leaving only DNA bound to the spin filter.



This figure shows what happens to the spin column when wet with water and when in the presence of a salt solution. In our case we're using guanidinium HCl (GuHCl), which acts as a chaotrope. A chaotrope is a molecule in water solution that can disrupt hydrogen bonding. As a consequence they *denatures biomolecules by interfering*

with non-covalent forces involved in protein folding (hydrogen bonds, van der Waals forces). This allows positively charged ions to form a salt bridge between the negatively charged silica and the negatively charged DNA backbone in high salt concentration. The DNA can then be washed with high salt and ethanol, and ultimately eluted with low salt.

Note from Davida: You've met silica before in those little packets that come with your shoes. What is their purpose? Look up Diatomaceous earth

16. Obtain solution C5 from your instructor. Add 500 µl of solution C5 to the spin filter and centrifuge at 10,000 x g for 30 seconds at room temperature.

    Solution C5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter. This wash removes residual salt and other contaminants while allowing the DNA to stay bound to the silica filter.

    Note from Davida: Why is it critical to remove salt from the filter? What is the salt doing?

17. Discard the flow through from the collection tube as described above.

18. Centrifuge the spin filter at 10,000 x g for 1 minute at room temperature to remove any residual liquid from the filter or the sides of the filter.

    This spin step removes the residual solution C5 (ethanol wash). It is critical to remove all traces of wash solution C5 because ethanol interferes with many downstream DNA applications such as PCR, restriction digests and gel electrophoresis.

Pro Tip: After this step, I leave the spin columns on a paper towel to dry for about 20 min. This is to ensure that there is absolutely no ethanol carry over.

19. Carefully place the dry spin filter in a clean, labeled, 2 ml collection tube. Avoid getting any residual C5 solution on the filter insert.

    If you do, re-spin the column and use a fresh collection tube.

20. Obtain solution C6 from your instructor. Add 100 µl of **solution C6** to the center of the white filter membrane.

Note: placing the solution C6 in the center of the small white membrane will make sure the entire membrane is wetted. This will result in more efficient and complete release of the DNA from the silica filter membrane. As solution C6 (elution buffer) passes through the silica membrane, DNA that was bound to the filter in the presence of high salt is selectively released by solution C6 (10 mM Tris Buffer) that lacks salt.

Note from Davida: This step is the one where your concentration/yield can be impacted by not ensuring the elution buffer actually reaches the membrane. It can cling to the side of the tube. So make sure you look at it as you're doing it, don't just drop it in. Sometimes you can even do this step twice to increase your yield.

21. Centrifuge at room temperature for 30 seconds at 10,000 x g. Discard the spin filter. The DNA is in the collection tube is now ready for downstream applications.

22. Store your DNA in the collection tube at -20°C.

23. Once you have eluted your final purified metagenomic DNA sample, quantify the sample using the Qubit according to the DNA quantification protocol and/or by running your sample on a 1% agarose gel.

Pro Tip: Label both the side and top of your tube. Sometimes the lids break or ethanol can remove your labels and you may not be able to identify your sample.

Further information and reference materials

1. On detergents - https://nsb.wikidot.com/c-9-5-5-5

On inhibitors of PCR and sequencing - https://www.promega.es/-/media/files/resources/profiles-in-dna/1001/an-introduction-to-pcr-inhibitors.pdf?la=es-es

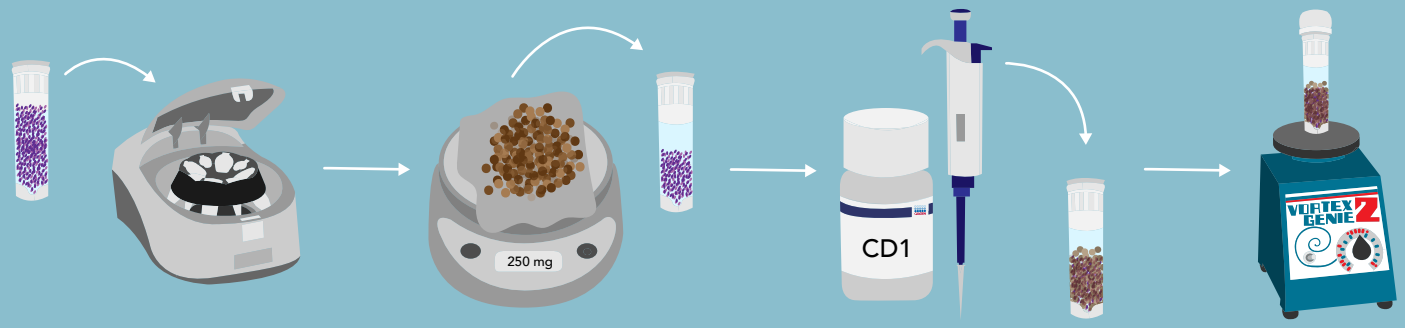
Questions

1) What type of conclusions can be made from initially culturing on nutrient agar (e.g., qualitative assessment, quantitative assessment, preliminary, estimate, descriptive, or bacterial diversity)?

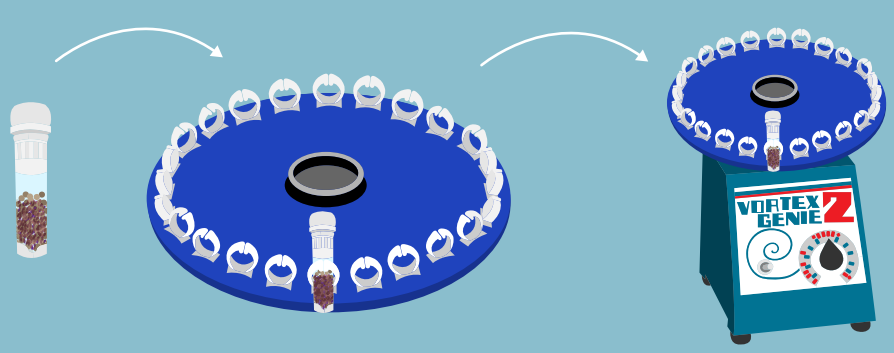
2) What is the purpose of the ethanol during the DNA extraction?

3) Our primers target the 16S rRNA gene, are there other genes used for metagenomic sequencing?

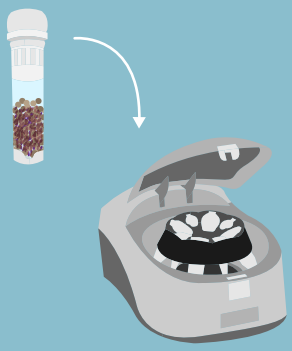
DNA Isolation with the Qiagen DNEASY Powersoil Pro Kit



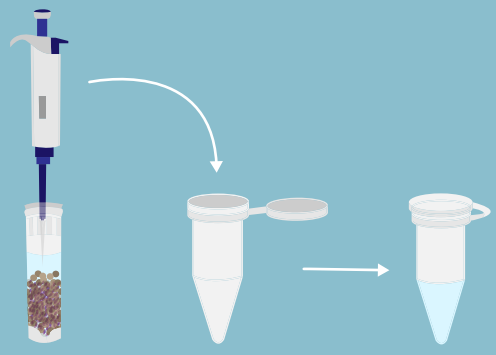
1. Spin the PowerBead Pro Tube briefly to ensure that the beads have settled at the bottom. Add up to 250 mg of soil and 800 µl of Solution CD1. Vortex briefly to mix.



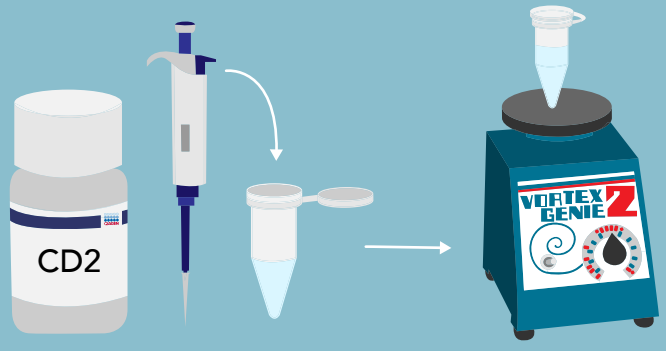
2. Secure the PowerBead Pro Tube horizontally on a Vortex Adapter for 1.5–2 ml tubes. Vortex at maximum speed for 10 min.



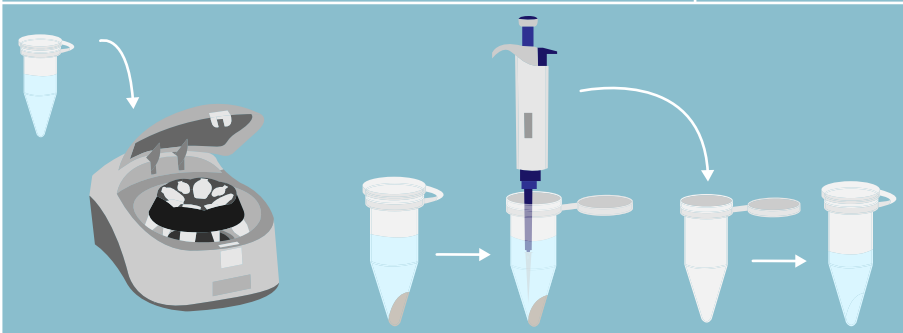
3. Centrifuge the PowerBead Pro Tube at 15,000 x g for 1 min.



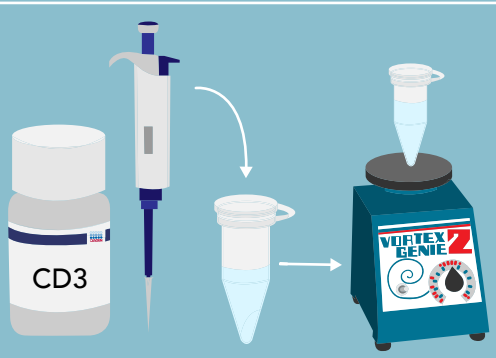
4. Transfer the supernatant to a clean 2 ml Microcentrifuge Tube (Expect 500–600 µl).



5. Add 200 µl of Solution CD2 and vortex for 5 sec.

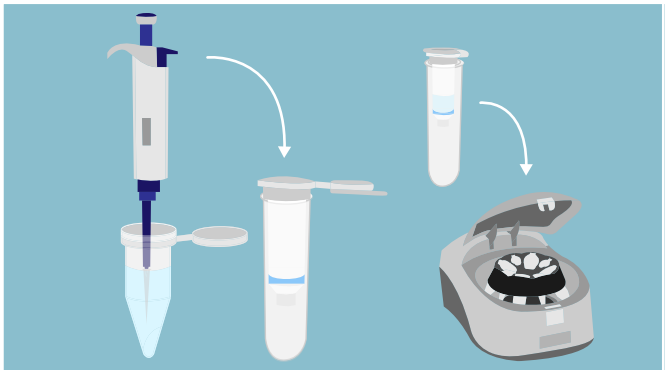


6. Centrifuge at 15,000 x g for 1 min at room temperature. Avoiding the pellet, transfer up to 700 µl of supernatant to a clean 2 ml Microcentrifuge Tube.

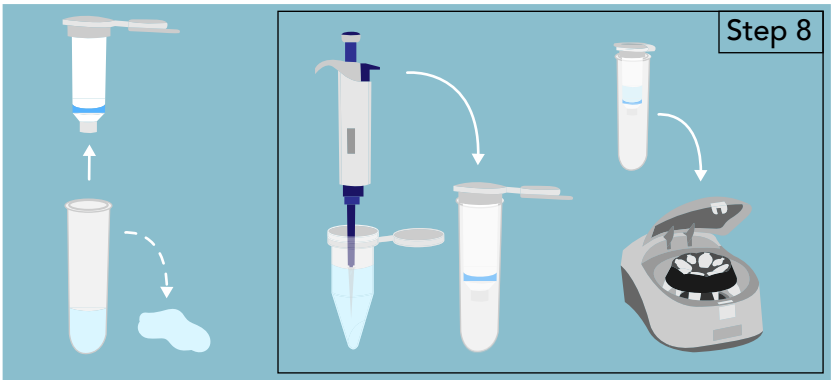


7. Add 600 µl of Solution CD3 and vortex for 5 sec.

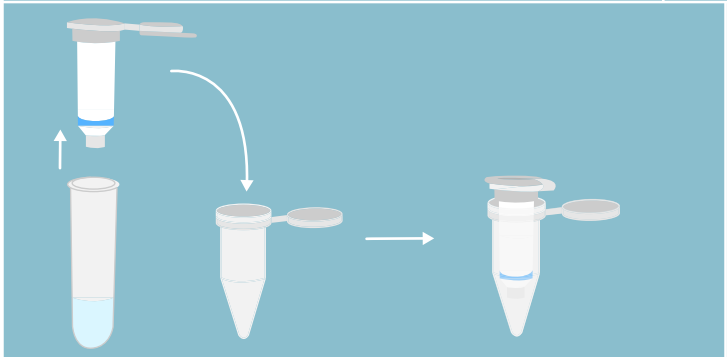
Created by Molly Metz



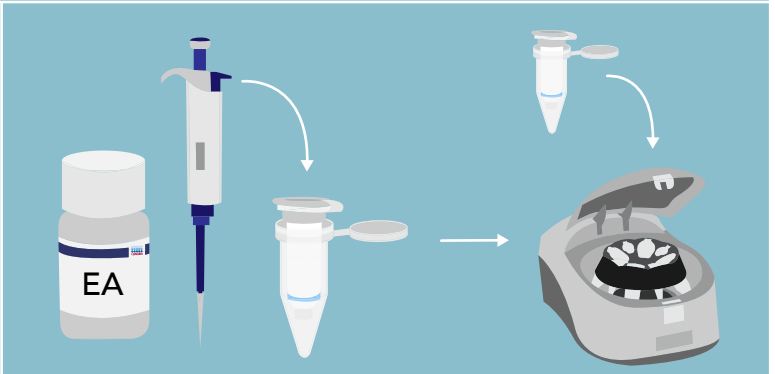
8. Load 650 µl of the lysate onto an MB Spin Column and centrifuge at 15,000 x g for 1 min.



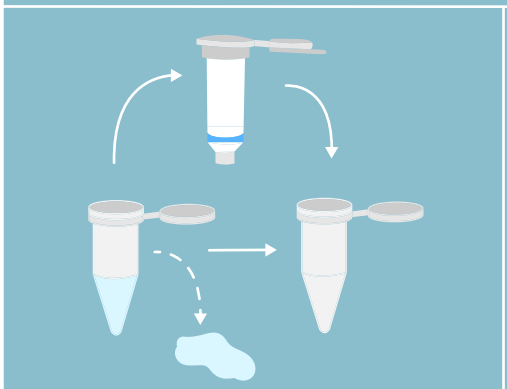
9. Discard the flow-through and repeat step 8 to ensure that all of the lysate has passed through the MB Spin Column.



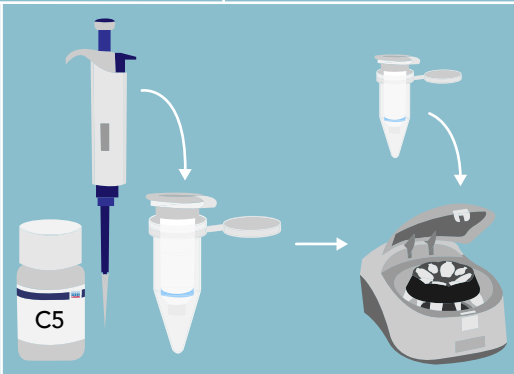
10. Carefully place the MB Spin Column into a clean 2 ml Collection Tube. Avoid splashing any flow-through onto the MB Spin Column.



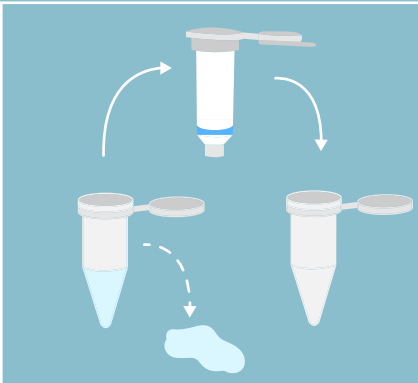
11. Add 500 µl of Solution EA to the MB Spin Column. Centrifuge at 15,000 x g for 1 min.



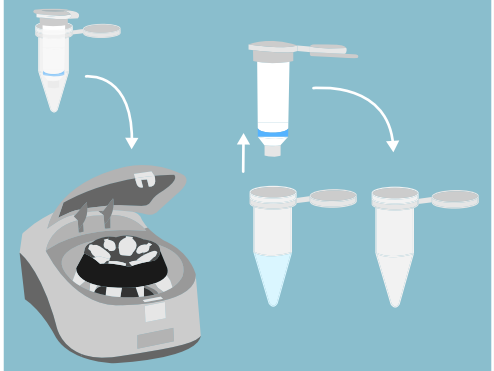
12. Discard the flow-through and place the MB Spin Column back into the same 2 ml Collection Tube.



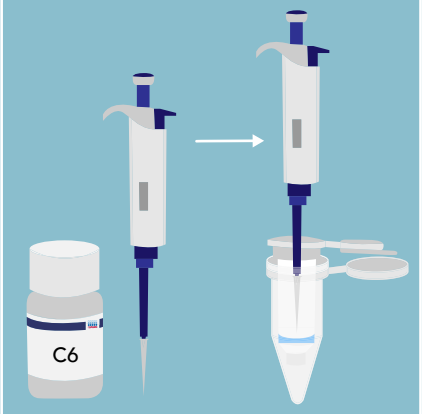
13. Add 500 µl of Solution C5 to the MB Spin Column. Centrifuge at 15,000 x g for 1 min.



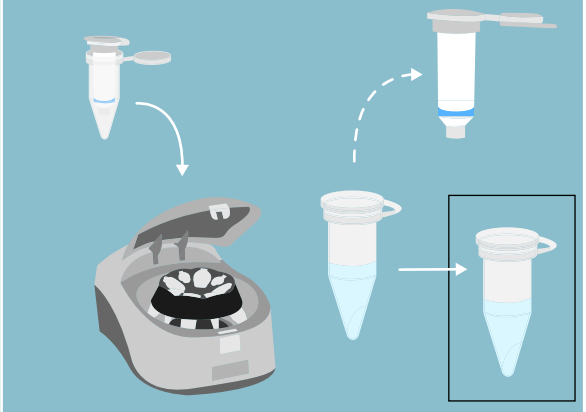
14. Discard the flow-through and place the MB Spin Column into a new 2 ml Collection Tube.



15. Centrifuge at up to 16,000 x g for 2 min. Carefully place the MB Spin Column into a new 1.5 ml Elution Tube.



16. Add 50–100 µl of Solution C6 to the center of the white filter membrane.

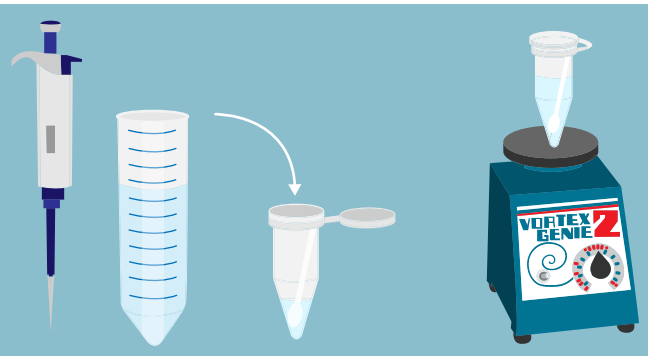


17. Centrifuge at 15,000 x g for 1 min. Discard the MB Spin Column. The DNA is now ready for downstream applications.

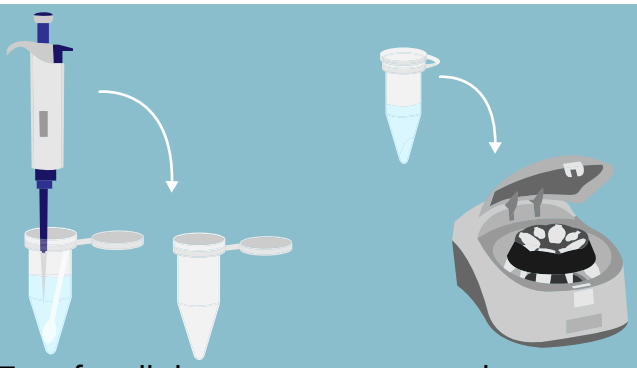
Note: Dotted line indicates discard.

Created by Molly Metz

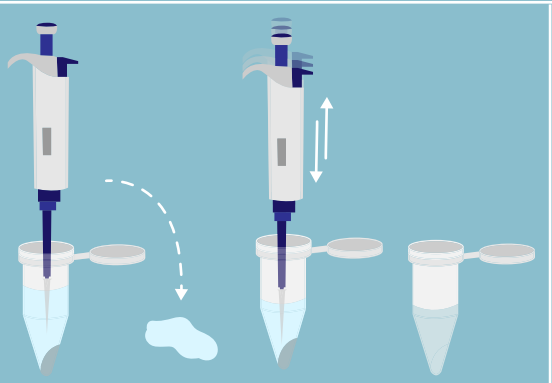
DNA Isolation from Swabs with the Qiagen DNEASY Powersoil Kit



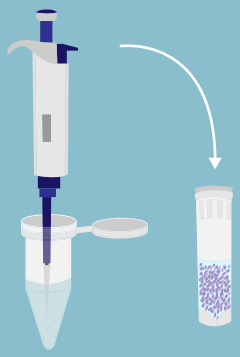
1. Add 500 µl of swab solution to microcentrifuge tube containing the swab. Vortex for 10 min.



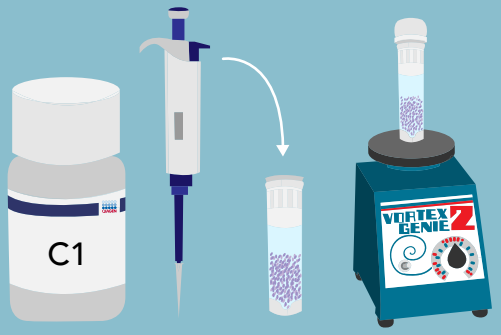
2. Transfer all the supernatant to a clean microcentrifuge tube. Centrifuge for 3 min.



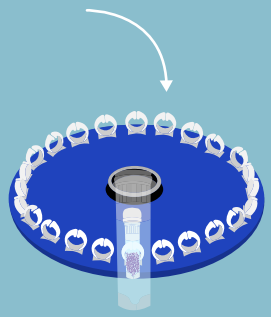
3. Remove 350 µl of supernatant. Then, resuspend the pellet.



4. Transfer 100 µl to PowerBead tube.

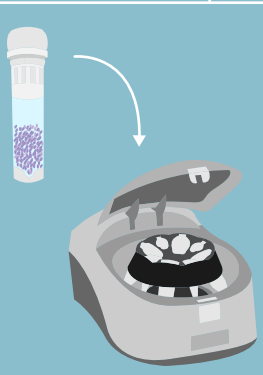


5. Add 60 µl of Solution C1 and vortex briefly.

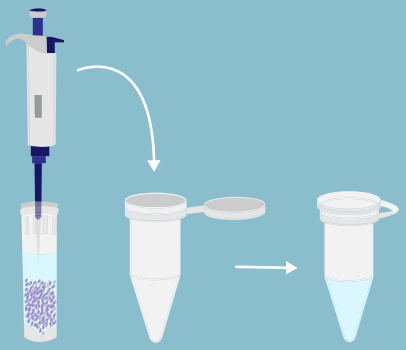


6. Secure Power-Bead Tubes to a Vortex Adapter.

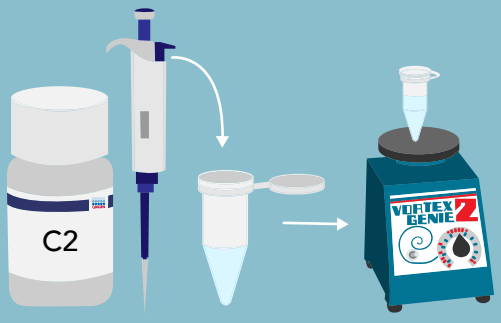
7. Vortex at maximum speed for 10 min.



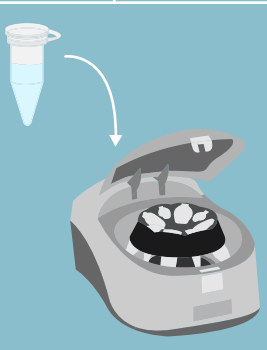
8. Centrifuge tubes for 30 sec.



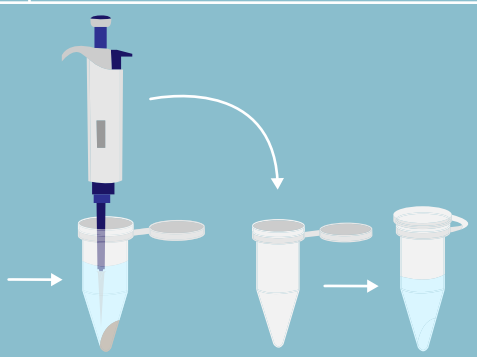
9. Transfer the supernatant to a clean 2 ml Collection Tube.



10. Add 250 µl of Solution C2 and vortex for 5 sec.



11. Centrifuge the tubes for 1 min.



12. Transfer up to 600 µl of supernatant to a clean 2 ml Collection Tube.

Note: Set centrifuge to 10,000 g. Dotted line indicates discard.

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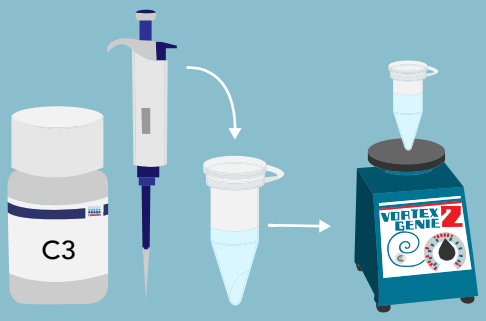
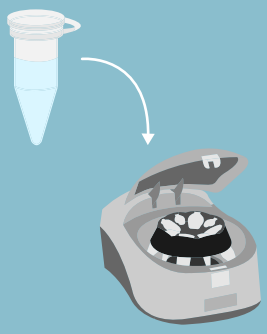
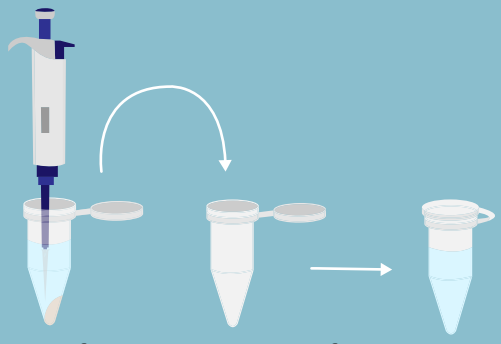


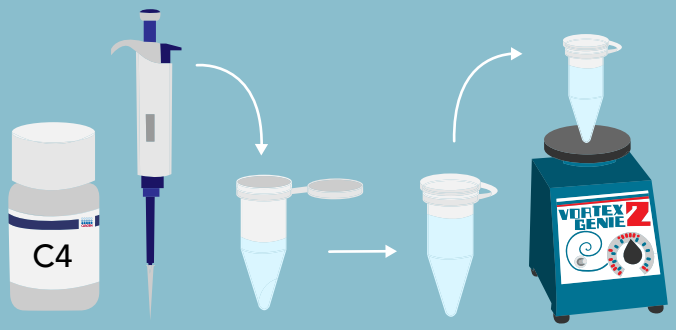
13. Add 200 µl of Solution C3 and vortex briefly.



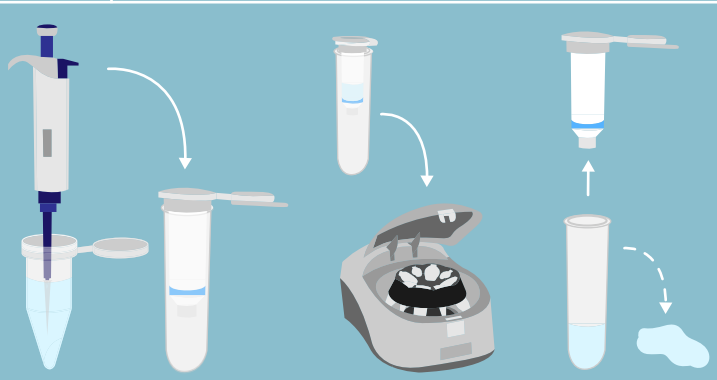
14. Centrifuge the tubes for 1 min.



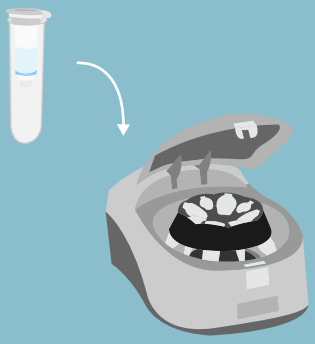
15. Transfer up to 750 µl of supernatant to a clean 2 ml Collection Tube.



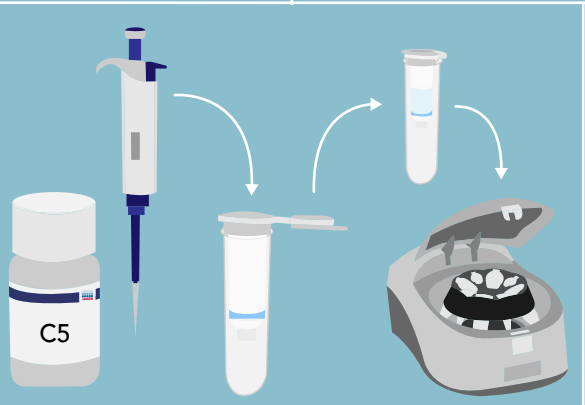
16. Shake to mix Solution C4 and add 1200 µl to the supernatant. Vortex for 5 sec.



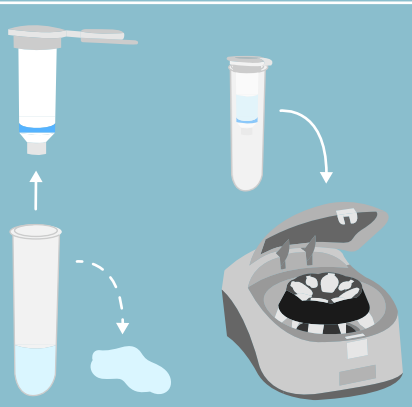
17. Load 675 µl onto an MB Spin Column. Centrifuge for 1 min. Discard flow-through.



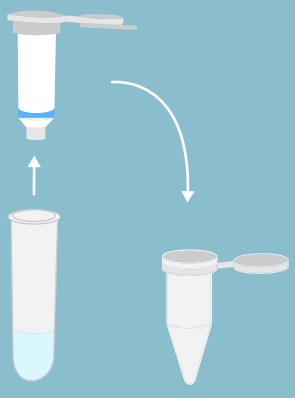
18. Repeat step 14 twice, until all of the sample has been processed.



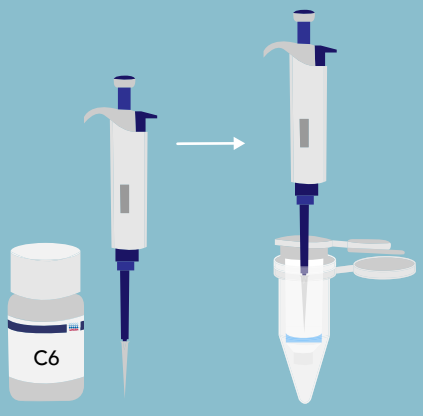
19. Add 500 µl of Solution C5. Centrifuge for 30 sec.



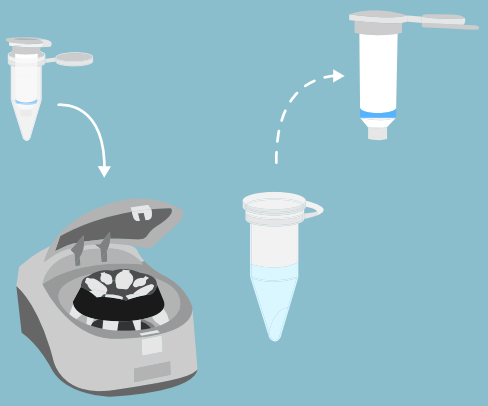
20. Discard the flow-through. Centrifuge again for 1 min.



21. Carefully place the MB Spin Column into a clean 2 ml Collection Tube.



22. Add 100 µl of Solution C6 to the center of the white filter membrane.



23. Centrifuge for 30 sec. Discard the MB Spin Column.

Part 4: DNA quantification by NanoDrop

Once you have eluted your final purified metagenomic DNA sample, quantify the sample using the NanoDrop and/or by running your sample on a 1% agarose gel.

1. In order to use the NanoDrop instrument you will need a P2 or P10 pipette and tips, a small volume of elution buffer and control lambda DNA of known concentration, in addition to your PowerSoil DNA sample.
2. Your instructor will demonstrate the use of the NanoDrop instrument.
3. Add 1µl of sterile water to the pedestal, move the arm into place, and the open again and use a Kim wipe to gently wipe away the drop of water.
4. Place 1µl of the appropriate blank (the same solution you eluted your DNA with – most likely C6 buffer from the PowerSoil kit) on the pedestal and close the arm. Blank the instrument. Then open the arm and gently wipe away the blank with a Kim wipe.
5. Place 1µl of a known concentration of lambda DNA control DNA or your sample on the pedestal and close the arm. Type in the control or sample name into the sample field on the computer. Now click on the "measure" icon to measure your sample. After measuring, lift the arm and wipe away your sample.
6. Repeat step 5 with any additional samples.

Part 5: Sample Sequencing

Sample DNA is sent for sequencing. Ideal quantity of DNA is at least 10ng/µL and quality of 1.8 (measured by the wavelength ratio 260/280, which indicates the absorbance of light from non-DNA elements such as: proteins, ethanol, and salts). Sequencing still requires a few weeks (mostly the lab facility sample que and about a day on the actual sequencing machine) to complete, therefore we will continue with our own validation procedures of PCR and gel electrophoresis.

Part 6: PCR amplification

Using universal primers that recognize conserved regions of the bacterial 16S rRNA gene you will amplify a variable region from the 16S rRNA gene. The DNA samples contain 16S rRNA genes from all the different bacteria present – therefore the PCR product will represent a mix of 16S rRNA sequences.

The following list shows and the set-up for a **25 µl** reaction (Qiagen HotStart kit (#203445)):

	Single 25 µL reaction
Forward primer	1 µl
Reverse primer	1 µl
2X master mix	12.5 µl
dH_2O	8.5 µl
DNA Template	*2 µl*

Prepare your template DNA for **two** PCR reactions**.

Reaction A –

1. 23 µl Master mix (HotStart 2x mix, water, and primers (Forward & Reverse))
2. Use 2 µl of your undiluted sample as template

Reaction B – Need to make a 1:10 dilution of sample DNA

1. Pipette 18 µl of sterile, molecular grade water into a clean microcentrifuge tube.
2. Add 2 µl of your isolated metagenomic DNA sample to the tube to make a 1:10 dilution of your template. Make sure your tube containing the 1:10 dilution is clearly labeled.
3. With diluted DNA set up second PCR reaction:
    a. 23 µl Master mix
    b. 2 µl of the 1:10 dilution of your DNA sample as template.

*Remember that one of your PCR tubes will receive 2 µl of undiluted template DNA and the other tube will receive 2 µl of the 1:10 dilution of the template DNA that you prepared above.

**Your instructor will work with you to prepare a positive and negative control PCR reaction to be used by the lab as a group.

Your instructor will introduce you to the working of the thermal cycler. The thermal-cycler should be set as follows (according to the HotStart basic protocol):

Step 1	Initial heat activation	15 min		95°C
Step 2	Denaturation/melting	30 sec	95°C	
Step 3	Annealing	45 sec	55°C	
Step 4	Extension	45 sec	72°C	
Step 5	Cycle to step 2 for 34 times			
Step 6	Final extension	10 min	72°C	
Step 7	Hold/Pause	Indefinitely	10°C	

On the graphical display you can watch the how the instruments runs through the cycles and an estimate of when your samples will have finished their run. After the PCR run, make sure your instructor has your samples and that they have been places in a box or rack for storage at -20C.

Part 7: Gel electrophoresis

In order to check whether or not a PCR product of the expected size was amplified you will run a fraction of your PCR reactions on a 1% agarose gel to visualize the DNA bands that are present in your two PCR reactions.

A 1% agarose gel was made as follows:

1. Make sure you have gloves and safety glasses on.
2. In a ~250 ml Erlenmeyer flask measure out 100 ml of 1x TAE buffer.
3. Weigh out 1 gram of agarose powder and add it to the Erlenmeyer flask with the buffer.
4. Carefully microwave the agarose in the buffer at ~70% power in a microwave for 2-4 minutes. Do this in 30 second increments to avoid having the liquid boil over. All the agarose powder should go into solution and the buffer should be clear with no agarose floating.
5. Allow the melted agarose to cool for 5 min. Then carefully add 2 drops of ethidium bromide (this is a carcinogen, so nitrile gloves must be worn). Gently swirl to mix.
6. Pour the melted agarose into the casting cassette (make sure it is balanced) and insert the appropriate comb to create the wells.
7. Let the agarose cool and gel for ~30 minutes.

**The gel box and gel are arranged with the wells of the gel closest to the side with the negative electrode (black). DNA is negatively charged and will move towards the positive electrode (red).

Procedure: Loading the Gel with PCR product –

1. In each 25 µL PCR reaction tube add 5 µL of loading dye.
2. Flick the tubes to mix the sample with the loading dye.
3. Load 10 µL of sample into assigned gel lane.

The gel should be run at ~80-100 volts for 45-75 minutes. Make sure not to run the gel so long that the dye front runs off the gel or at such a high voltage that the gel box heats up. The higher the voltage will cause the DNA to move faster so the run time should be shorter. Smaller DNA sequences will move farther through the gel than larger sequences.

When the gel has finished running, DNA bands can be seen using a UV light box (transilluminator). Check for clear ladder bands, no band in the negative lane and a band in the positive lane. Know that a reasonable amount of *primer dimer* (extra primers and when primers bind to themselves) will show up as a band at the smallest end of the ladder. Compare the size of any bands in your sample lanes with the size standard and record your results.

Part 8: DNA sequence data analysis

Your instructor will provide more information and details related to the data analysis component of the experiment on soil microbiome diversity