Molecular Methods in Microbial Ecology

Kristin Gribble 508-289-7194 kgribble@mbl.edu Lillie 305

Tuesday	10/26/21	Introduction, Extraction of DNA from Winogradsky columns Run DNA products on gel
Thursday	10/28/21	Lecture on PCR, Prepare PCR reactions
Tuesday	11/02/21	Analyze PCR results and Lecture

Day 1, Part I: Introduction, DNA Extraction, and Run DNA products on gel

We'll sample sediment from your Winogradsky columns and extract the genomic DNA from all of the organisms in the sample. To do this, we'll use a common DNA extraction kit that lyses the cells by mechanical "bead beating" (exactly what it sounds like) and further goes on to separate the nucleic acids from the other cell constituents using a spin column. Look at your GC results and think about the genes we are going to try to amplify (methanogenesis, sulfate reduction) to guide your decision on where in the column you want to sample from. For example, did you have a large peak of methane somewhere? Perhaps you could choose that to target methanogens. Remember to label all your tubes since you will be sharing the microcentrifuges!

1. Pick a depth section of your Winogradsky column.

2. Remove ~1.0 gram of sediment from your chosen depth section with a 5 ml pipet and put it into a labeled microcentrifuge tube (between the 0.1 and 0.5 ml marks). Do this by flipping the pipet over, tip up, and sticking it in to the depth of interest. Then place you finger over the back end (wearing gloves, of course) and remove.

3. Spin for 2 minutes at 8,000 x g. Carefully remove the surface water from the sample via pouring.

4. Transfer the remaining sediment to the Qiagen **PowerBead** Tube (labeled) using a spatula.

5. Follow Qiagen PowerSoil DNA Kit Extraction protocol.

Day 1, Part II: Qiagen PowerSoil DNA Kit Extraction protocol
Please wear gloves at all times!

1. To the **PowerBead Tubes** provided, add 0.25 - 1gm of soil sample (you already did this).

2. Gently vortex to mix.

3. Check **Solution C1**. If precipitated, heat solution to 60°C until dissolved before use.

4. Add 60µl of **Solution C1** and invert several times or vortex briefly.

5. Secure **PowerBead** tubes horizontally on a flat-bed vortex and secure with electrical tape. Vortex at maximum speed for 10 minutes. Make sure your vortexer doesn't walk away!

6. Centrifuge tubes at 10,000 x g for 30 seconds. DO NOT EXCEED THIS SPEED.

7. Transfer the supernatant to a clean 2 ml collection tube (label). Expect between 400 to 500µl of supernatant. Supernatant may still contain some particles.

8. Add 250µl of **Solution C2** and vortex for 5 sec. Incubate 4°C for 5 min (in the fridge).

9. Centrifuge the tubes for 1 minute at 10,000 x g.

10. Avoiding the pellet, transfer up to, but no more than, 600µl of supernatant to a clean 2 ml collection tube (label).

11. Add 200µl of **Solution C3** to the supernatant and vortex for 5 seconds. Incubate 4°C for 5 min (in the fridge).

12. Centrifuge at 10,000 x g for 1 minute.

13. Avoiding the pellet, transfer up to, but no more than, 750µl of supernatant to a clean 2 ml collection tube (label).

14. Shake to mix **Solution C4** before using. Add 1200µl Solution C4 to the supernatant and vortex for 5 seconds

15. Load approximately 675µl onto a Spin Filter (label) and centrifuge at 10,000 x g for 1 minute at room temperature.
    Discard the flow through and add an additional 675ul of supernant to the Spin Filter and centrifuge at 10,000 x g for 1 minute.
    Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x g for 1 minute.

16. Add 500µl of **Solution C5** and centrifuge for 30 seconds at 10,000 x g.
17. Discard the flow through.
18. Centrifuge again at 10,000 x g for 1 minute.
19. Carefully place spin filter in a new clean 2 ml tube (label). Avoid splashing any Solution C5 onto the spin filter.
20. Add 100µl of **Solution C6** to the center of the white filter membrane without touching it.
21. Centrifuge at 10,000 x g for 30 seconds.
22. Discard the spin filter. DNA in the tube is now ready for next steps. Transfer 10 ul into another (labeled) microcentrifuge tube for the gel, as described below. Remaining 90 µl goes in freezer for PCR.

Day 1, Part II: Visualize DNA by Electrophoresis

When you extract DNA from a community of microbes, you will ideally have a representation of the genomic DNA from all of the microbes living in the sample community. What "good" genomic DNA looks like on a gel is a blobby smear – but a pretty specific kind of blobby smear: one that remains near the wells of the agarose gel and is darkest on top, lightest as it trails down the gel. This means the majority of the DNA is still in large, long strings and we didn't shear it much in the extraction process. You MUST wear gloves at all times during this procedure.

1. Add 2µl of 6x loading buffer to your tube with 10 µl of your DNA. The loading buffer contains glycerol to keep the DNA sample in the well, rather than floating up in the buffer, as well as a blue dye to make tracking the progress of the sample easier as it moves through the gel.

2. Load the sample DNA/dye mixture into a well of a 1% agarose gel made with a salt TAE buffer (Tris Acetic acid-EDTA).

3. The agarose gel also contains ethidium bromide, which intercalates into the DNA and fluoresces under UV light. This allows you to see your DNA within the gel. It is a carcinogen, so do not touch this with any exposed skin.

4. I will load 4µl of nucleic acid marker to the first well of the gel. This marker contains DNA fragments of known sizes and is a "ladder" (or ruler) against which you can compare your DNA to estimate size.

5. We'll place the gel in a gel-running bed containing more TAE buffer. Cover goes on, wires are attached to power supply. When an electric current is run through the buffer (and hence through the porous gel), the negatively charged DNA will migrate from the negative (black) side towards the positive (red) side. If you were to run a gel long enough, the smaller DNA would eventually exit the gel and end up in the buffer on the positive side of the gel bed. Our gels will run at 100mV for ~45 minutes.

Day 2: Polymerase Chain Reaction Protocol for 16S rRNA and functional genes
Thursday, October 24, 2019

Set-up PCR

Next we are going to amplify specific fragments of genes in the DNA that we extracted. We will target the small subunit ribosomal RNA (rRNA) gene for two of the three domains of life, Bacteria and Archaea, as well as the functional genes involved in sulfate reduction (Dissimilatory Sulfite Reductase, *dsr*) and methanogenesis (Methyl Coenzyme Reductase). In addition to running what we hope are PCR products from our DNA samples, we will also run a positive control (in which we add DNA that we know belongs to the target organism) and a negative control (in which we don't add any DNA, but just water and the PCR cocktail).

In all, we will have 6 reactions but you should make up enough master mix for 7 reactions. This accounts for small pipette errors. Since it is very important to keep primers and reagents as cold as possible, we will first create a "master mix" that contains sterile water, OneTaq 2X Master Mix, and Bovine Serum Albumin (BSA). BSA is added to minimize PCR inhibition.

Tip: When working with DNA, keeping it cold and keeping time short work in your favor.

PCR is extremely susceptible to contamination. Wear your gloves at all times- they help protect your sample from you! In addition – you must be very, very careful with pipettes to avoid contamination. Be sure to change your tips *every* time so that you don't cross-contaminate anything (When in doubt, throw it out (the tip, that is)!). Please remember that the PCR reaction will amplify most all DNA present so even trace amounts of contaminant DNA in your master mix can be amplified and interfere with your results. This is why we run a negative control with just the master mix and no added DNA – if you see a PCR product in the negative control it will be because you contaminated your reaction so be careful with your reagents and with your pipette.

PCR Protocol

Wear your gloves at all times and be sure to change your pipet tip every time you add a new reagent/sample to your tube.

1. Thaw reagents on ice. Vortex each reagent gently to mix.

    Forward primers at 10µM concentration
    Reverse primers at 10µM concentration
    Master Mix
        Molecular-grade water
        0.4% BSA
        OneTaq 2X Master Mix with Standard Buffer

2. Place your DNA extract on ice as well.
3. Label each tube in the strip with your initials and number, eg. KG-1, KG-2
4. To save time, the master mix below has been prepared for you, and you have received an aliquot with enough for 7 reactions.

Reagent (1X)	**1 reaction (25 µl)**
Molecular-grade water	5.5 µL
OneTaq 2X Master Mix	12.5 µL
0.4% BSA	4 µL
Total	22 µL total

5. Pipet 22 µl of master mix into each of your 6 strip tubes.

6. Then add 1 µl of each of the following of the tubes. Be sure and mark which tube #1 is. *Use the chart below to help you keep track of your additions. Use a new pipet tip for every addition.*

    #1 Your DNA template plus *dsr* specific primers dsr1F and dsr4R

    #2 Your DNA template plus *mcr* specific primers ME1 and ME2

    #3 Your DNA template plus Bacteria specific primers 8F and 1492R

    #4 Your DNA template plus Archaea specific primers 21F and 958R

    #5 Positive control- Methanogen plus Archaea specific primers 21F and 958R

    #6 Negative control- no template plus Archaea specific primers 21F and 958R

Tube	Target	Master Mix (µl)	Template	Vol (µl)	F primer	Vol (µl)	R primer	Vol (µl)
1	Sulfate reducers	22	DNA	1	dsr1F	1	ds4R	1
2	Methanogens	22	DNA	1	ME1	1	ME2	1
3	Bacteria	22	DNA	1	8F	1	1492R	1
4	Archaea	22	DNA	1	21F	1	958R	1
5	Archaea	22	+ control	1	21F	1	958R	1
6	Water	22	Water	1	21F	1	958R	1

7. Firmly close the tubes when you are done adding all reagents. Once they are closed, put your strip on ice. Make sure you have labeled the end of the strip cap with something so that you know what strip of tubes is yours. We'll use a thermocycler in the Bay Paul Center Lab.

8. Each primer set has a slightly different program based on primer properties and optimizations carried out in our lab. Here is what we will use:

	Bacteria	**Archaea**	**dsr**	**mcr**
Polymerase activation	94 °C, 3 min	94 °C, 3 min	96 °C, 5 min	95 °C, 5 min
Number of cycles	35	35	35	40
DNA denaturing	94 °C, 40 s	94 °C, 40 s	94 °C, 1 m	95 °C, 45 s
Primer annealing	55 °C, 1:30	55 °C, 1:30	56 °C, 1 m	50 °C, 45 s
Extension	72 °C, 2 m	72 °C, 2 m	72 °C, 1 m	72 °C, 1:30
Final extension	72 °C, 10 m	72 °C, 10 m	72 °C, 10 m	72 °C, 10 m

9. Emma will run gels of your PCR reactions, and we will share results with you next week.