Colony-based PCR and Electrophoresis Protocol Instructor Notes

The method below is based on "Effect of Diet on Bean Beetle Microbial Communities" by Cole et al. to be published in *Tested Studies for Laboratory Teaching, Proceedings of the Association for Biology Laboratory Education,* Volume 39. Vinny Ciavetta at Emory University helped to optimize the protocol.

Students will be working in the same teams as they did to plate-out the bean beetle microbiomes. There are three main objectives for this activity:

1. Observe and record bacterial colony phenotypes and abundance data for the colony plates students previously prepared (see the handout: Bean Beetle Microbiome Culturing Protocol).
2. Pick a bacterial colony from one of the agar plates to prepare a PCR reaction of the 16S rRNA gene.
3. Conduct gel electrophoresis of the PCR reactions to identify successful amplification.

Notes to Instructors

Positive and Negative Controls
The positive and negative controls used in the PCR reactions are for the purpose of assessing whether the PCR reactions worked as expected and to clearly assess the difference between amplified 16S rRNA from the picked bacterial colony and a failed PCR reaction in the electrophoresis of the PCR products.

A dilution of an *Escherishia coli* culture is ideal for use as a positive control. A laboratory strain of *E. coli* (such as K-12) grown on a nutrient agar plate may be picked (see steps 2 and 3 in **Specific Instructions for Each Step**) and diluted in 100μL of sterile molecular grade water to prepare the positive control.

Preparing PCR primers
The PCR primers described in the components of the PCR Master Mix (below) are delivered in lyophilized form and must be diluted to prepare the solutions needed for the PCR Master Mix. Faculty active in the Bean Beetle Microbiome Project will request and receive primers, but for others primers may be ordered from the same vendor that conducts your Sanger sequencing (such as Azenta-GeneWiz). Ordering primers at 25nmol scale will result in a de-salted yield of at least 5.0nmol of primer DNA. The actual amount of primer DNA delivered will vary and will not be the same for each primer or even different orders of the same primer. The amount of DNA delivered (in nmol) will be printed on the tube of lyophilized primer or on printed documentation with the tube of primer.

Prepare 100μM primer by adding a μL volume of sterile molecular grade water to each tube of lyophilized primer equal to 10x the nmol value for that primer. For example, if your tube of 27F primer contains 44.9nmol of DNA, you would add 449μL of water to that tube, vortex, and then briefly centrifuge to get all the primer solution to the bottom of the tube. Primers are delivered in 1.5mL microfuge tubes so there is plenty of room in tube for this solution to be prepared. Using TE buffer to dilute the primers will better ensure the stability of the DNA but is not necessary. The 100μM primer solution may be frozen and held at -20°C for future use.

Use the 100μM primer solution to make the volume of 10μM primer that you will need for immediate use. Make the volume of 10μM primer needed for your PCR Master Mix by making a 1:9 dilution of the 100μM primer using molecular grade water. The PCR Master Mix containing the primers should be kept refrigerated until use. The minimum yield of PCR primer, 5.0nmol, would provide a total of 500μL of 10μM primer. Since each PCR reaction requires 0.5μL of each primer (see Preparing PCR Master Mix),

500μL of 10μM primer is enough for 1000 reactions. Faculty active in the Bean Beetle Microbiome Project will not need a primer to include with their samples sent for Sanger sequencing (See Preparing Samples for Sanger Sequencing on page 5).

Preparing PCR Master Mix

For a class of 24 students, you will need PCR Master Mix for each student plus one negative control and one positive control for each group of 4 students, so a total of 36 PCR reactions. We advise instructors to add one additional reaction volume of Master Mix to the aloquot for each group of 4 students to ensure there is sufficient volume, so a total volume of Master Mix for 42 PCR reactions would be needed. The table below lists the three components in the Master Mix, the total volume needed for 42 reactions, and the volume to aloquot for each group of 4 students.

Reagent	Per Rx	42 Rx	Per Aloquot
50X or 10 μM Forward Primer (27F)	0.5μL	21.0μL	
50X or 10 μM Reverse Primer (1492R)	0.5μL	21.0μL	
OneTaq Hot Start Quick-Load 2X Master Mix with Standard Buffer	12.5μL	525.0μL	
Total Volume of complete Master Mix	13.5μL	567.0μL	94.5μL

Procedure Overview

Each group consisting of 4 students will:

1. Obtain a microfuge tube of PCR Master Mix with primers (MM), a microfuge tube of molecular grade water (H_2O), 4 sterile microfuge tubes, one strip of 8 PCR tubes, a microfuge tube of a known bacterial suspension for a positive control (PC).
2. Aliquot 13.5μL of the PCR Master Mix to 6 tubes of a labeled PCR strip (leave the other 2 tubes empty).
3. Each student will pick one bacterial colony and immediately mix bacteria in sterile molecular-grade water.
4. The bacterial colony mixed in sterile, molecular-grade water is your **colony suspension**.
5. Each student then adds 11.5μL of their colony suspension to one of the first four PCR tubes, add 11.5μL of the positive control to the fifth PCR tube, and 11.5μL of molecular grade water to the sixth PCR tube that contains the PCR Master Mix. What is the purpose of the sixth PCR tube?

The PCR Master Mix (MM) contains the following components:

Reagent	
50X or 10 μM Forward Primer (27F)	0.5μL
50X or 10 μM Reverse Primer (1492R)	0.5μL
OneTaq Hot Start Quick-Load 2X Master Mix with Standard Buffer	12.5μL

0.2μM (final concentration) forward primer – 27F (5' – AGA GTT TGA TCC TGG CTC AG)
0.2μM (final concentration) reverse primer – 1492R (5' – GGT TAC CTT GTT ACG ACT T)

The OneTaq Hot Start Quick-Load Master Mix (New England Biolabs, M0488S) contains the DNA polymerase necessary to conduct the polymerase chain reaction, it also contains the four triphosphate nucleotides (adenine, thymine, guanine, and cytosine), and a buffer and an electrophoresis dye. This reagent (and DNA ladder for use in electrophoresis) may be requested directly from New England Biolabs https://www.neb.com/en-us/forms/educational-course-support-program-request

Specific Instructions for Each Step:

1. **Aliquot 13.5μL of the PCR Master Mix to 6 tubes of a labeled PCR strip.**
   a. Label the PCR strips. When you get the DNA sequencing results, you need to have all the information about the colony you sequenced. In your notebook, write down the order of reactions on your PCR strip including – plate type and colony morphology. Also note the location of your positive and negative controls.
   b. Add 13.5μL of the PCR Master Mix to the bottom of each tube.
   c. Cap the tubes.
2. **Pick a bacterial colony.**
   a. Get 4 sterile 1.5mL microcentrifuge tubes (one for each student in your group) and label each.
   b. Add 100μL of sterile molecular grade H_2O to each tube.
   c. Set a P20 micropipetter to 11.5μL.
   d. Attach a sterile yellow tip to the end of the P20 micropipetter.
   e. Gently touch the pipette tip to a well-separated colony from one of the bacterial growth plates. (Bacteria will stick to the pipette tip.) **Do not scoop a glob of bacteria.**
3. **Mix the bacterial colony in water.**
   Submerge the pipette tip in the appropriate tube of 100μL water and pipette up and down vigorously for about 5 seconds to make the colony suspension.
4. **Add 11.5μL of the colony suspension to a tube that contains the PCR Master Mix.**
   a. Using the same pipette tip that was used for picking and mixing, transfer 11.5μL of the colony suspension to the PCR Master Mix in the appropriate PCR tube.
   b. Pipette up and down 3 times to be sure the colony suspension and PCR Master Mix are well-mixed.
   c. Go back to step 2d (above) and repeat until one colony for each student in your group has been picked, suspended, and the suspension added to the correct PCR tube.

d. For the positive control, add 11.5µL of the positive control DNA (*E. coli* suspension) to the PCR tube labeled for positive control. For the negative control, add 11.5µL of sterile molecular-grade water to the PCR tube labeled for negative control.
e. Cap the PCR strip so all tubes are sealed and press to ensure the cap is fully sealed.
f. Tell the laboratory instructor you are finished assembling your PCR reactions.

PCR Program

95°C, 10 min (purpose is to help disrupt bacterial cell walls/membranes to release DNA)

Then 36 cycles of:

95°C, 30 sec

55°C, 30 sec

72°C, 1.5 min

Lastly,

72°C, 4 min,

4°C, hold

Visualizing and sequencing of DNA

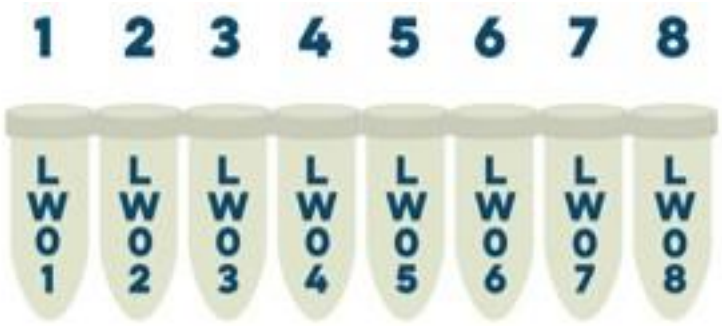
1. **Pour an agarose gel**
   a. Assemble your gel box and comb – make sure gaskets are in place
   b. If it has not already been added, bring your agarose to the instructor to have 5.0µL GelRed added. GelRed added to melted gel at 0.5µL per 10mL of gel, so 5.0µL for 50mL of gel. Gel is 1% agarose in TAE buffer.
   c. Swirl to mix well
   d. Pour in your gel box
   e. Pop any bubbles
2. **Load gel**
   a. Load 7.0µL premixed DNA ladder into the leftmost lane
   b. Load 5.0µL of your prepared samples into lanes (SAVE the rest of your PCR product to send out for Sanger sequencing).
3. **Run your gel** at ~135v until dye front is more than halfway through the gel. The negative electrode should be nearest the gel wells. DNA will migrate in the electrical field toward the positive electrode.
4. **Visualize** your PCR reactions using the UV box
5. **Save PCR sample for Sanger sequencing**
   Inform your instructor of the samples that were successfully amplified by PCR. Those samples will be sent for Sanger sequencing so save your PCR tube strip and give it to your instructor.

Preparing Samples for Sanger Sequencing

Only the PCR reactions that are shown to have resulted in the successful amplification of the 16S rRNA gene should be sent for Sanger sequencing. The results of electrophoresis will indicate which samples should be sent for Sanger sequencing. **Do not send** the positive or negative control PCR samples for Sanger sequencing. You **do not need to send** primers with your samples if you are a supported participant in the Bean Beetle Microbiome Project.

Each sample to be sent for Sanger sequencing should be placed in a new PCR tube (0.2 mL) in an 8-tube strip clearly marked using a fine tip Sharpie with two letters (indicating your college or university) and sample number marked on the side of each tube (as shown below, Marking Tubes for Sanger Sequencing). **Samples must be numbered sequentially** and should correspond to a sample manifest that details the source of each sample. Put at least 10μL of each successfully amplified PCR sample in each of the new PCR tubes. If a successful sample has less than 10μL of volume, you may add sterile molecular grade water to the original PCR tube to increase the volume and then transfer 10μL to the new PCR tube. Firmly close each tube after pipetting sample. PCR tubes that are not used may be cut-off the strip.

Marking Tubes for Sanger Sequencing: The ordering system of our Sanger sequencing vendor (Azenta-GeneWiz) assigns tube ID codes using your initials and the sample number (see below). Please write these codes on the sides of your tubes using indelible marker. These should match your Sample Manifest (see below) (and will be used in the Azenta order receipt we prepare).



Fill only the PCR tubes that are needed. Tubes are in strips of 8, but if you do not fill all the tubes in a strip, cut off the empty tubes.

The new strips of PCR tubes containing the successfully amplified 16S rRNA gene should be wrapped in parafilm and placed in one or more 50mL conical tubes with a screw caps and a small amount of tissue to prevent excessive movement. Write the sample letters and number range on the outside of the 50mL conical tube indicating the samples contained in them.

Primer for Sanger Sequencing

Faculty active in the Bean Beetle Microbiome Project will not need a primer to include with their samples sent for Sanger sequencing. We have primer stored by our Sanger sequencing vendor to use with the samples sent to them as part of the Bean Beetle Microbiome Project. If you are not or no longer an active participant in the Bean Beetle Microbiome Project you must include a quantity of one primer (either primer in molecular grade water) with your samples being sent for Sanger sequencing. Azenta GeneWiz requires 5.0μL of 5μM primer (this is one-half the concentration used in the preparation of PCR Master

Mix) for each sample that is sent or you would need to order primer to be stored by the vendor for use in Sanger sequencing (note that Azenta only stores primer for one year). When sending primer with the amplified PCR samples, you would include an additional PCR tube with your samples labelled "primer" containing the total volume needed. If every student in a class of 24 successfully amplified their picked colony, then you would need to send 120μL of 5μM of one primer (either 27F or 1492R) in a separate PCR tube.

Refrigerate your samples until they are sent to the Sanger sequencing lab.

Sample Manifest

Faculty in the Bean Beetle Microbiome Project should send a Sample Manifest to us so we may provide a prepaid order form. The 50mL conical tubes containing your PCR products should be sent directly to our Sanger sequencing vendor with a print out of our prepaid order form included. No special packaging is required (no insulated packaging, no ice packs).

Our current Sanger sequencing vendor is Azenta (GeneWiz). Azenta cleans our PCR samples and then Sanger sequences them with a turn-around time of 24-48-hours. The sequence data will be provided to you via email.

You should record the following information in a spreadsheet format for the samples that were successfully amplified by PCR. Faculty in the Bean Beetle Microbiome Project, email this spreadsheet to us as soon as you know which samples are to be sent for Sanger sequencing.

- Sample ID written on the PCR tube to be sent for sequencing (two letters and a number).
- Beetle ID # (provided to you by your instructor)
- Beetle life cycle stage (egg, larva, pupa, adult)
- Beetle sex (if adult)
- Beetle host bean species
- Experimental Treatment Group
- Media (nutrient agar, EMB, PEA, blood, other) from which the bacterial colony came
- Colony phenotype (color, gloss, form, elevation)

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