Student Handout – Colony-based PCR and Electrophoresis Protocol

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.

You will be working in the same teams as you 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 you previously prepared (see the handout: Bean Beetle Microbiome Culturing Protocol).
2. Pick a bacterial colony from one of your agar plates and prepare a stab culture.
3. Prepare a PCR reaction on the same bacterial colony that you used for your stab culture.

**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 13.5 µL 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, make a stab culture and immediately mix bacteria in sterile molecular-grade water.
4. The bacterial colony mixed in sterile, molecular-grade water is your colony suspension.
5. Add 11.5 µL of each colony suspension in one of the first four PCR tubes, add 11.5 µL of the positive control to the fifth PCR tube, and 11.5 µL of molecular grade water to the sixth PCR tube that contains the PCR master mix.

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 (1429R) | 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 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.

**Specific instructions for each step:**

1. **Aliquot 13.5 µL 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 13.5 µL of the PCR master mix to the bottom of each tube.
    c. Cap the tubes.
2. **Pick a bacterial colony.**
    a. Get 4 sterile 1.5 mL microcentrifuge tubes (one for each student in your group) and label each.
    b. Add 100 µL 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.**
    a. 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 11.5 µL 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 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.
    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. Bring your agarose to the instructor to have 5.0μL GelRed added. GelRed added to melted gel at 0.5 μL per 10 mL of gel, so 5.0 μL for 50 mL 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 7μL premixed DNA ladder into the leftmost lane
   b. Load 5μL 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**
   Successful PCR reactions (samples that were successfully amplified) should be reserved for Sanger sequencing. Put at least 10μL of your PCR sample in a sterile locking microfuge tube. Clearly mark the tube with identifying letters (indicating your college or university) and sample number corresponding to a detailed sample list prepared by your instructor.

<u>Stabs for culture collection</u>

Each of you will prepare one stab culture of the same colony that you sampled for colony-based PCR.

Using a flame sterilized wire inoculating loop, pick the colony and repeatedly stab the loop into the agar of a sterile stab culture tube. Record the bar code number on your tube. The stabs should be incubated at 37C for 8-12 hours until they become cloudy. Afterwards, they can be stored in the dark at room temperature. The bacteria in a stab can be recultured by using an inoculating loop to streak some of the stab culture out on an LB plate.

After incubation, your stab culture will be sent to Emory University for archiving.

You should record the following information in your laboratory notes and on the data sheet your instructor has prepared for your class:

- 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)
- Barcode # for stab associated with the colony (Note: this is not the same as the Beetle ID #, but the number on the barcode sticker on the stab vial)
- Tube number on tube of PCR products that will be sent to sequencing center