Independent project guidelines:

Students using nucleotide sequencing as a means of identifying unknown microbial cultures in conjunction with their independent projects must complete the following steps. Specific information about materials and methods is provided below.

1. **Obtain a pure culture of the organisms being investigated.** This may require samples to be taken from well-isolated colonies and streaked on new media multiple times, but is essential to the identification process. A mixed culture will yield unusable data.

2. **Determine the cell wall type of your organisms by preparing a Gram stain and by conducting a KOH test**. To conduct a KOH test, mix a loopful of cells with one drop of 3% KOH on a clean glass slide, stir and look of changes in consistency. Gram-negative cultures will become viscous (snot-like) while Gram-positive cultures will not. Gram-positive bacteria in the genus *Microbacterium* will often stain pink in a Gram-stain, but have thick peptidoglycan cell walls typical of Gram-positive cells.

2. **Extract chromosomal DNA from cells taken from solid media or from cells grown in a broth culture. Note that the methods used are different for different cell types.** You will obtain the best results if your culture is fresh, i.e., no more than 48-72 hours old depending on the organism type. Many Gram-negative bacteria will form cultures suitable for extraction procedures within 24 hours if maintained under optimal conditions. If your culture is Gram-negative you can extract chromosomal DNA by boiling cells in 10mM Tris, pH 8.5 as described in the lab syllabus (Application of the PCR in Bacterial Identification). If your culture is Gram-positive or has Gram-positive type cell walls, as indicated by the KOH test, chromosomal DNA extraction will require an extraction kit/procedure. The extraction kit/procedure currently available in this laboratory is the MoBio – Ultra Clean Microbial DNA Extraction Kit. Completion of a kit extraction procedure typically takes about one hour and is most efficient with two or more participants (3, 6, 8) because the centrifuge rotor must be balanced.

   If your DNA extract will not be used immediately after preparation, store it in the refrigerator (overnight) or in the freezer (for longer periods). Make certain that your tube is clearly labeled with your name (last name in full) and the extraction date.

3. **Amplify 16S Ribosomal-DNA using the polymerase chain reaction**. Make certain the Gene cycler is available for use, and ask an instructor or laboratory assistant to provide you with the materials necessary. It is most efficient to run multiple tubes at one time, so make a list of participant names by number and label the tubes with the appropriate numbers. Clearly label each PCR tube with a permanent marker, on the flat portion of the cap. Prepare an ice bucket with shaved ice, thaw the required solutions and place these on ice. The reaction mixture currently being used in this laboratory contains the following materials (Use a yellow pipette and yellow tips for the larger volumes and a white pipette and white tips for the smaller volumes).

Reaction mixture:
25µl Taq-polymerase master mix (Qiagen)
0.5µl primer mix – 10 µM each of bacteria 8-forward and universal 1492-reverse
0.5µl template DNA – Boiled cell suspension or kit extract
15µl Sterile distilled water pH 8.0

Make certain the correct volumes of primer mix and template DNA are added by placing these on the inside wall of each tube where they are clearly visible. The total volume required for each reaction is 50 µl and it is important that this volume be at the tube bottom when the tubes are loaded into the thermal cycler. If liquid is clinging to the sidewall, carefully place the tubes into a microfuge and gently press the momentary button to force the liquid to the tube bottom. **Use extreme caution** with the microfuge, because PCR tubes are fragile, and will often break if thrown to the bottom of rotor wells. Following centrifugation, return the tubes to the ice.

Ask an instructor or laboratory assistant to turn on the thermal cycler and to set the program for the PCR. In this laboratory we are using Program No. 1 for all PCRs run to amplify 16S ribosomal DNA. Load the PCR tubes into the block after the temperature has begun to increase (when the block feels hot). Program parameters are as follows:
Cycle 1
94º C for 4 min – to denature template DNA (load tubes into heated block)
55º C for 45 seconds – to anneal primer DNA to the template
72º C for 2 min – to extend DNA (Taq polymerase adds nucleotides to the primers)
Cycle 2 through 34
94º C for 30 seconds – to denature template DNA
55º C for 45 seconds – to anneal primer DNA to the template
72º C for 2 min – to extend DNA (Taq polymerase adds nucleotides to primers)
Cycle 35
94º C for 30 seconds – to denature template DNA
55º C for 45 seconds – to anneal primer DNA to the template
72º C for 5 min – to extend all DNA segments generated to maximum length

The PCR will require about 3 hours and 20 minutes for completion, so make arrangements for someone to remove the tubes when cycle 35 has ended (block temperature drops to below 40º C). Remove the tubes from the thermal cycler and place them on ice.

4. **Run your PCR product DNA into an electrophoresis gel for visualization and extraction**. If time permits it is useful to run a single 5µl sample (several different student samples in one gel) to check the quality of your PCR product before running a gel for extraction purposes. Ultimately you will run your entire PCR product DNA in one gel (45µl if you have run a sample gel first, and 50µl if you have not).

   Ask an instructor or laboratory assistant to provide you with a premade, agarose gel, or the materials required to make gels (depending on what is currently available) as

well as the materials necessary for running the gel (electrophoresis chambers and power supplies, buffer solution, parafilm and loading dye. Mix your DNA sample with loading dye (5µl dye with each DNA sample) prior to loading the wells of your agarose gel as described in the "Electrophoresis of DNA Samples" section of the laboratory syllabus. If you are using a six-well gel (thin wells), load 10µl maximum volume into the two outside wells and load extra DNA in the four center wells (10µl DNA plus 5µl dye in each). If you are using a two-well gel (thin wells) you will be loading approximately 25µl of DNA in each well and can mix the DNA with the dye in a 1:1 ratio. Be sure to load your entire volume of PCR product DNA into the gel. Label your sample by placing a section of white tape on the gel box or counter top. Include your name (last in full) and the date. Secure the lid to the electrophoresis chamber, turn on the power supply, set the voltage to 46, and allow the gel to run for at least one hour to insure that the DNA sample has moved into the agarose.

Carefully remove the gel from the electrophoresis chamber and rinse it with water. Make arrangements for the gel to be stained with ethidium bromide.

5. **Stain your PCR product gel with ethidium bromide to make your DNA samples visible. <u>Caution</u> – Ethidium bromide is recognized as a carcinogenic substance, and must be handled with extreme caution. Wear gloves at all times.** Place your gel in the staining tray provided and add enough ethidium bormide solution to cover the gel with liquid. If staining solution is already in the staining tray, carefully add your gel to the tray. Move your label tape to the stain tray so your gel will not become mixed up with those of other students. Allow the stain to penetrate the gel for about 10 minutes, then remove the gel from the tray and carefully rinse the gel with water. Place your stained gel on a foil-covered pallet (1x1' wooden board), lay a section of plastic wrap over the gel and place it into the refrigerator.

   Note – If your stained gel shows your PCR product to be weak (poorly amplified), you will need to repeat steps 2 through 5 before proceeding to step #6.

6. **Carefully dissect your PCR product DNA from the stained gel, weigh it and place it into a microfuge tube for purification**. Note – To insure the purity of your PCR product DNA, this step should be completed as soon after running the gel as possible, and must be completed within 24 hours. DO NOT COMPLETE THIS STEP IF YOUR PCR PRODUCT DNA IS OF POOR QUALITY.

   Caution! – **Ethidium bromide is present, please wear gloves for this procedure**. Place a section of plastic wrap over the work surface you intend to use (the platform of the transluminator or foil-covered pallet if an overhead UV source is available). Carefully position your gel so the stained DNA bands are clearly visible. Use a clean razor blade to carefully cut the gel on both sides of the clearly visible (bright orange) bands containing your PCR product DNA. Smeared DNA above and/or below the bright bands will be removed during this procedure. Remove the excess gel from the workstation and place it into a discard container (zip lock bag). Lay the brightly stained strip on its side and trim away the excess gel from the ends and the section

forming the floor of the gel. It is not necessary to trim away the clear agarose sections between the wells. Place all discard gel material in the waste container.

Place a clean weight boat on the balance available and press tare to zero the balance with the boat in place. Carefully transfer the thin section of brightly stained gel (your PCR product DNA) to the weight boat and record the weight. Further processing will be easier if your gel section weighs between 0.90 and 0.200g. Obtain a clean, sterile microfuge tube, label it clearly with your name (last in full), the date, and the weight of your PCR product DNA using a permanent marker. Carefully transfer the gel slice from the weight boat into the microfuge tube, close the tube and proceed with step #7 or place the tube in the freezer provided. PCR product DNA frozen in gel sections can remain frozen for several days without damage.

7. **Purify your PCR product DNA for sequencing with a QIAquick gel extraction kit/procedure (Qiagen).** Please wear gloves during this procedure and process an even number of PCR product tubes to balance the centrifuge rotor. Ask an instructor or laboratory assistant to provide you with the materials necessary to complete this purification step, and with the waste container required. Follow the procedure as indicated in the booklet provided.

   Note – for best results, allow the final solution MD5 (10 mM Tris pH 8.5) to stand on the filter basket for 2-5 minutes in the warm centrifuge before completing the final 1-minute spin.

   Make certain your final collection tube is clearly labeled with your name (last name in full) and the date before performing the final spin. The DNA collected should be ready for sequencing following the purification procedure, but may remain in the freezer for two weeks or more while other students are completing their preparations. Place all collection tubes in the freezer provided in the box indicated. Notify an instructor when you have completed your purification.

8. **Run your PCR product DNA in an electrophoresis gel to check the quality of your purified sample**. Sequencing is expensive, and we cannot afford to send poor quality DNA samples to the sequencing laboratory.

   Ask an instructor or laboratory assistant to provide you with a premade, agarose gel, or the materials required to make gels (depending on what is currently available) as well as the materials necessary for running the gel (electrophoresis chambers and power supplies, buffer solution, parafilm and loading dye. Mix 5µl of your purified DNA sample with 5µl of loading dye and load your sample into one well of a thin-well agarose gel. To increase efficiency, arrange to have four other students use other wells in the gel to fill the gel to capacity. Run 5µl of Hind III cut bacteriophage lambda DNA in one well of the gel to serve as a calibration standard. Label the gel box indicating which DNA samples are located in which wells. Secure the lid on the electrophoresis chamber, turn on the power supply, set the voltage to 46, and allow the gel to run for 2.5 to 3 hours. Carefully remove the gel from the electrophoresis

chamber and rinse it with water. Make arrangements for the gel to be stained with ethidium bromide.

9. **Stain your PCR product gel with ethidium bromide to make your DNA samples visible. <u>Caution</u> – Ethidium bromide is recognized as a carcinogenic substance, and must be handled with extreme caution. Wear gloves at all times.** Place your gel in the staining tray provided and add enough ethidium bormide solution to cover the gel with liquid. If staining solution is already in the staining tray, carefully add your gel to the tray. Move your label tape to the stain tray so your gel will not become mixed up with those of other students. Allow the stain to penetrate the gel for about 10 minutes, then remove the gel from the tray and carefully rinse the gel with water. Place your stained gel on a foil-covered pallet (1x1' wooden board), lay a section of plastic wrap over the gel and place it into the refrigerator.

10. **Observe the stained gel on the transluminator and determine the quality and quantity of your PCR product.** The band visible in your lane of the gel should be clean and bright with little or no smearing. Determine the approximate concentration of DNA present in your sample by comparing the brightness of your DNA band to those of the bacteriophage lambda standard. The concentration of DNA required for sequencing is 30 ng/µl. One 50µl volume of PCR product DNA will usually yield DNA of sufficient concentration for sequencing.

11. **Make certain your purified DNA is placed in the freezer in the box indicated for sequencing reactions.** Since the cost of sequencing is significantly lower if we take 48 or more samples to the laboratory at one time, we will wait until we have multiple samples to run before any are taken to the sequencing lab. You will be notified when your samples are taken to the lab, and when the electropherogram data is returned.

12. **Streak a fresh sample of your pure culture on a new plate of the appropriate medium, seal the plate with parafilm and turn it in to the instructor.** It is imperative that the organisms being identified with DNA sequencing be maintained. **Do not allow your culture to die!** New organism types, i.e., those not previously cultured in this laboratory will be added to our collection and maintained for future use. Photomicrographs of these organisms, and their 16S ribosomal DNA sequence data will eventually be displayed on the microbiology website.

Thank you for your participation in this project.