

**PCR-ID: IDENTIFICATION OF MICROORGANISMS
USING POLYMERASE CHAIN REACTION**

I. OBJECTIVES

- To get familiar with the common methodology and instrumentation used in molecular biology today.
- To be able to quickly identify a microorganism based on the polymerase chain reaction (PCR) and sequences of nucleotides of a particular gene.

II. INTRODUCTION

In order to determine the relatedness of organisms from all domains of life (bacteria, archaea, and eukaryotes), it is important to find a trait that would be present in all living organisms. In the 1980s Carl Woese suggested the use of DNA sequences of certain common genes. Such a molecular chronometer not only needed to be present in all organisms, but also needed to have retained the same function. Woese proposed using a gene that encoded one of the RNA molecules found in ribosomes, the protein-RNA complexes on which proteins are synthesized in all prokaryotes and eukaryotes. Although there are differences in size between the ribosomes of prokaryotes and eukaryotes, the sequences of the rRNA molecules (and their corresponding rDNA genes) from all sources contain regions that are very similar, allowing the alignment and comparison of these sequences. Further, the gene is small enough to be easily sequenced and large enough to contain enough information for genetic comparisons.

Using extensive computer analyses of rRNA gene sequences, it has been possible to identify the so-called signature sequences, which are short oligonucleotides that are unique to certain groups of organisms. Not only are these signature sequences specific for each of the three domains of life, but they can also be specific within a domain or, a particular genus or even a single species. The specificity of the signature sequences is useful for many purposes. For example, signatures can be used for quickly placing a newly isolated or misclassified microorganism into its correct phylogenetic group. Using the sequence of 16S rRNA genes isolated from DNA of environmental samples allows us to define the composition of a microbial community without relying on the ability to culture all organisms present in these environments.

SESSION I

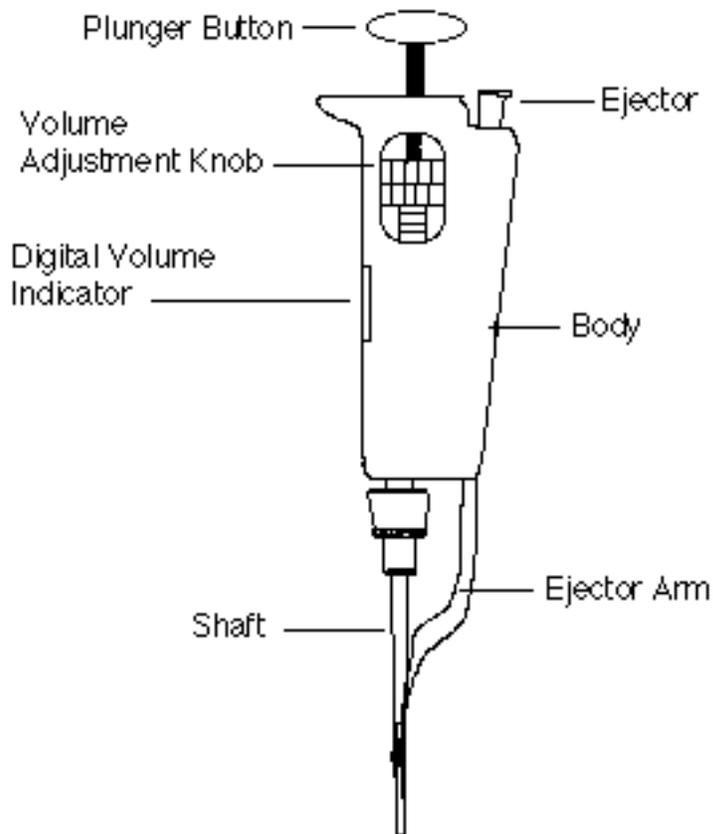
Thus far you have been using pipets to measure and dispense a specific volume of a liquid. Pipets are good for quantities in the range of 0.1 ml and up. However, in molecular biology, we may need much smaller volumes of a liquid. Digital micropipettors are specifically designed for such small and accurate amounts. In our lab, we use the Pipetman or the “Continuously Adjustable Digital Microlite Pipets” to measure and transfer volumes of liquid in the range of 0.1 to 1000 μl . Each microliter (μl) is a thousandth of a milliliter (ml).

Digital Micropipettors: Let us first familiarize ourselves with the different parts of a Pipetman. Each Pipetman is composed of a body that houses a Digital Volume Indicator or DVI. The numeric value of DVI changes with the turning of the Volume Adjustment Knob. This knob should not be turned below or over the volume capacity of the Pipetman. Attached to the body are the Plunger Button and Tip Ejector Button on one side and Plastic Shaft and stainless steel Ejector Arm on the other side.

Each group will receive a set of 3 Pipetmans, namely P-20, P-200 and P-1000. P-20 is adjustable from 0.1 to 20 μl in 0.1 μl increments. The fractional volumes (0.1-0.9) are shown in red as the lowermost tier of numbers while the whole numbers are shown in black in the DVI. P-200 is adjustable from 1 to 200 μl in 1 μl increments and is recommended for volumes 20-200 μl . And finally, P-1000 is used for volumes from 10 μl to 1000 μl (1 ml) in 2 μl increments. For this Pipetman, the uppermost tier of numbers is in red and can be either 0 or 1. The figures should be multiplied by 10 to obtain the actual volume dispensed. For example, for a P-1000, if the DVI shows 41, the volume of the liquid withdrawn will be 410 μl and if DVI shows 100, you are transferring exactly 1 ml. The following is the procedure that you should use when employing any Pipetman:

1. Set the desired volume by holding the Pipetman body in one hand and turning the volume adjustment knob until the digital indicator shows the correct volume.
2. Attach a new disposable tip to the shaft of the pipet. Press on firmly with a slight twisting motion to ensure an airtight seal.
3. Depress the plunger to the first positive stop. This part of the stroke is the calibrated volume displayed on the micrometer.
4. Holding the Pipetman vertically immerse the disposable tip into the sample liquid to a depth of 1-2 mm.
5. Allow the push-button to return slowly to up position. Never permit it to snap up.
6. Wait 1 to 2 seconds to ensure that the full volume of sample is drawn into the tip. Then withdraw the tip from the sample liquid.

7. To dispense the sample, place the tip end against the side wall of the receiving vessel and depress the plunger slowly to the first stop. Wait about 1-2 seconds and then depress the plunger to the second stop (bottom stroke), expelling any residual liquid in the tip.
8. With the plunger fully depressed, withdraw the Pipetman from the vessel carefully with the tip sliding along the wall of the vessel.
9. Let the plunger return to the up position.
10. Discard the tip by depressing the tip ejector button smartly. A fresh tip should be fitted for each sample to prevent carryover between samples.



Microfuging Samples: In many cases you may have just a single tube or an odd number of tubes that you want to microfuge. You should make sure that every tube has another counterpart of the same weight opposite it in the machine. If you have only a single sample, it is easier to let another group spin their sample opposite and along with yours so that your tube would act as their balance and their tube would act as your balance.

Samples: Your lab instructor will determine what samples you will be working on. If your sample comes from an environment where more than one species of microorganism may be present (e.g., the inside of your mouth, a water sample from the BioPond, a stool sample from a patient, a soil sample, etc.), you should inoculate (quadrant method) or spread a known volume of the sample on an agar plate to obtain individual colonies. You can then see how many types of organisms are growing and take a sample of each type from a small amount of each colony type and then proceed with “Setting Up and Running the PCR”. Make sure to describe the characteristics of the colony of interest and include this description in your report (Refer to the Pure Culture Techniques exercise).

- Note: Your lab instructor may decide to let you try the PCR-ID methods your EU or OU species. In this case you need to just hand in a single report for EU+PCR-ID or OU+PCR-ID projects. However, this single report should incorporate all the points that are relevant for each of the reports, as described in their Results Sheets at the end of their respective chapters.

Labeling: The label you use on all your materials in this part should consist of the 3 digits of your section and then 3 capital letters of your choosing. For example 110-EBY shows that the sample belongs to the group EBY of section 110 of the course. Use this code on all your tubes and plates for your PCR-ID unknown. Make sure all the members of your group know the label code because the sequencing data that will be posted on the course BlackBoard later will bear this code.

SESSION II

Setting Up and Running the PCR

PCR was developed by the Nobel Prize winner Kary Mullis and associates in 1985 and is essentially a method that uses the smallest amounts of samples and by which one can replicate a specific segment of DNA (a gene or a smaller segment) millions of times while the rest of the DNA is not replicated. The reagents needed for amplification are: the original sample, two oligonucleotide primers that can complement the base pairing at the opposite ends of the target DNA, a suitable DNA polymerase, the four deoxyribonucleoside triphosphates (dATP, dTTP, dGTP and dCTP) and Mg⁺⁺ ions.

To perform the PCR, the reagents are mixed in a thin-walled tube and placed in an instrument called a thermocycler. What this machine does is that it changes the temperature very quickly according to a set of instructions. The changes in the temperature cause denaturation of the target DNA strands, annealing of primers to the separated strands and primer extension. These 3 actions constitute a cycle. Many such cycles can be performed quickly to obtain a large number of copies of the target DNA.

Depending on the goal of the analysis, different primers may be needed for the amplification of the 16S rRNA gene. To determine the presence of a specific organism in an environmental sample, one can choose primers that will only bind to signature sequences of this organism. However, if the question is rather how diverse the bacterial community is (for example, from a swab sample of your mouth), one should use universal primers that will bind to conserved sequences of the 16S ribosomal-RNA gene of the domain *Bacteria*. Depending on the primer sequences and the length of the expected product, the conditions for the PCR will vary.

LABORATORY SUPPLIES

Heat block set at 100°C	1/lab
Sterile distilled water	1 bottle/table
Eppendorf tubes, different sizes	1 beaker of each/table
PCRID1 primer, 1.1 μM	1.0 μl/sample
PCRID2 primer, 1.1 μM	1.0 μl/sample
FailSafe PCR Enzyme Mix	1 vial/lab
FailSafe PCR 2X PreMix A	1 vial/lab
PCR machine	1/lab
Microfuge adapters	6/lab

PROCEDURES

1. Get ice.
2. Add 1 ml of sterile water in an Eppendorf tube. Going to your sample plate, choose a well-isolated colony that seems to be interesting to you. Write extensive notes as to the colony morphology. This description of the colony would help as a confirmatory test after you have identified the species. Pick about a quarter of the colony with a sterile cool loop and transfer to the water in the Eppendorf. Vortex the tube until all cells are completely dispersed. You should not see any cloudiness; if you do, your sample is not dilute enough. [Remember that only a few cells are necessary for this procedure. Too many cells produce much debris that will interfere with the chemicals used for the PCR].
3. Place the tube in a heat block at 100°C for 10 minutes to lyse the cells, then move the cells to ice for 5 minutes.
4. Obtain a 0.5 ml Eppendorf, label it with your group name and place it on ice. Transfer the following into this tube in the following order:
 - 2.5 μ l of your boiled sample
 - 20.0 μ l sterile water
 - 1 μ l of PCRID1 primer
 - 1 μ l of PCRID2 primer
 - 25 μ l of FailSafe PCR 2X PreMix A
 - 0.5 μ l of FailSafe PCR Enzyme Mix

Note: The FailSafe PCR Enzyme mix contains mainly buffers and Taq polymerase and the FailSafe PCR 2X PreMix A contains MgCl₂ and dNTPs. PCRID1 and PCRID2 are universal primers and each has a concentration of 1.1 μ M.

5. Vortex the tube for 5 seconds and microfuge for 3 seconds using a special adapter supplied for these smaller tubes. Don't forget a balance tube. Tap the tube to mix and microfuge for another 3 seconds. Place your tube on ice.
6. When all groups have prepared their tubes, it is time to use the PCR machine. The machine has already been programmed to run as is shown in the following table.

Cycles	Action	Temperature	Duration
1	Denaturation	94	4 min
	Annealing	55	45 sec
	Extension	72	1 min
2-34	Denaturation	94	1 min
	Annealing	55	45 sec
	Extension	72	1 min
35	Denaturation	94	1 min
	Annealing	55	45 sec
	Extension	72	10 min
	Hold	4	-

7. Switch the machine on and open the lid.
8. Place the samples in the wells and also place 4 empty tubes at the corners. Make sure the lids of all tubes are well pushed down and the tubes are in good contact with the wells. Also make sure the entire class' tubes are in the machine before starting the program. Close the lid.
9. Select the program PCRID and select Start Current Program. Make sure the name of the current program appears at the bottom of the screen when the program starts.
10. The program will now run according to the following designated denaturation, primer annealing and primer extension temperatures and durations and will stop after it has gone through all the cycles (~2.5 hrs.). At that point, a temperature of 4°C will be maintained indefinitely. Your TA will store your PCR DNA at -20°C till needed.

SESSION III

Cleaning Up the PCR Product

ExoSAP-IT is a product developed by the USB Corporation for cleaning up the PCR DNA product. It essentially makes use of two hydrolytic enzymes (Exonuclease I and Shrimp Alkaline Phosphatase) to digest all the unconsumed dNTPs and primers remaining in the PCR reaction tube that may interfere with the sequencing process. These enzymes are active at 37°C but get quickly deactivated at 80°C. After ExoSAP-IT treatment, the PCR product is pure enough for any sequencing or digestion work.

LABORATORY SUPPLIES

Eppendorf tubes (0.5 and 0.2 ml capacities)	1/sample
ExoSAP-IT	2 μ l/sample
Microfuge	2/lab
PCR machine	1/lab
PCRID1 primer, 1.1 μ M	3 μ l/sample
PCRID2 primer, 1.1 μ M	3 μ l/sample
UPSF forms	1/section

PROCEDURES

1. Take your PCR tube out of the freezer and thaw at room temperature, then microfuge for 1 minute.
2. Transfer 5 μ l from the top of the supernatant into a fresh Eppendorf tube (0.5 ml capacity) and label this new tube. Make sure no cell debris is transferred. Return the original tube to the freezer as we will use it for electrophoresis.
3. Add 2 μ l of ExoSAP-IT to the tube, tap the tube with your fingers to mix, pulse 3 seconds in a microfuge and place back into the PCR machine. Start the ExoSAP? program. This program is just one cycle of 15 minutes at 37°C and 15 minutes at 80°C and a hold at 4°C. This should take about 30 minutes to finish.
4. Add 3 μ l of either 1.1 μ M PCRID1 or PCRID2 to each tube. Tap gently to mix, microfuge 3 seconds, transfer to a fresh Eppendorf (0.2 ml capacity), label clearly and give to your instructor to be sent to the UPSF.
5. Within 3-4 days, the results will be back from UPSF and will be posted as individual files on the course BlackBoard Website. You need to download the file that has the DNA sequence of your sample and use BLAST to determine the name of your unknown species as described below.

SESSION IV

Agarose Gel Electrophoresis of the PCR Product

Suppose we take a long piece of a DNA molecule and digest it with a restriction enzyme. It is important for us to know how many fragments are obtained and what their sizes are. Fragments can be separated on the basis of their size, shape and charge by the technique of gel electrophoresis. The cut DNA is placed at one end of an agarose gel and subjected to an electrical current. Small fragments will move fast in the agarose, while large fragments move at a slower rate. In this way fragments are separated into distinct bands, the approximate sizes of which can be estimated if a sample of fragments of known sizes is run alongside the unknown sample.

In general, electrophoresis is a method used to separate cell fractions, proteins, nucleic acids or even intact chromosomes. There are many different protocols used in electrophoresis but all take advantage of an inert support matrix (e.g. starch, agarose, polyacrylamide) and an electrical field. Molecules move in this electric field.

The solid support that we will be using here is agarose, supplied as a polysaccharide powder. Agarose bed serves as a molecular sieve to separate the molecules according to their size, shape and net ionic charge. DNA is negatively charged at neutral pH and thus moves towards the anode. When mixed with water, boiled and then cooled, agarose will turn into a gel-like substance. This gel is a complex network of fibrils (of polysaccharide bonded together by hydrogen bonds) through which the DNA fragments must pass. The distance between the fibrils or the pore size is determined by the concentration of agarose used; i.e., a 20% agarose would hardly let even very small DNA fragments to pass through while a 0.2% agarose is rather flimsy and can let very large DNA fragments travel through rather easily. So, controlling the concentration of agarose based on the sizes of the DNA molecules is important.

The shape of the molecules also plays an important role in its relative migration distance in an electric field. Compact molecules naturally move more rapidly than loose molecules. For example covalently closed circular twisted plasmid DNA migrates faster than open linear or nicked circular DNA.

Two other important factors are the amperage of the electrical field and the ionic strength of the electrophoresis buffer. With higher amperage and/or buffers of high ionic strengths, the molecules move faster in the field. However, too high a voltage, amperage, or ionic strength of the buffer would cause heating of agarose and loss of its natural matrix and even denaturation of the DNA.

Electrophoresis is conducted in a Plexiglas apparatus composed of a middle section where the agarose gel is placed and two buffer compartments or tanks at either end, connected by platinum wires to the current. To prevent damage to the Plexiglas, the molten agarose should be cooled to 50°C before pouring. Many sizes and shapes of the

apparatus are commercially available or can be constructed. One should make sure that, when a gel is running, the apparatus is well shielded and there are no exposed parts that could impart an electric shock.

We add a marker dye (bromophenol blue) to the samples to be able to visually follow the front. After the front has traveled the length of the field, the gel is taken out and stained with an intercalating dye such as ethidium bromide (EtBr) or SYBR Gold.

EtBr and SYBR Gold are dyes that fluoresce under ultraviolet radiation and have a very high affinity for the double-stranded DNA molecule. For example, if we place the gel in a solution of EtBr for a short time, take it out, wash it with water and then irradiate it with ultraviolet, the DNA molecules in the gel fluoresce and we can photograph the gel to obtain a permanent record. The positions of the DNA fragments appear as clear bands in the gel and the photograph. By comparing each band with a standard, run in parallel in the gel, we can approximate the size of each fragment.

Unfortunately EtBr is a highly mutagenic and carcinogenic agent and extreme care should be practiced in its handling. Its waste disposal is another problem that many molecular biology laboratories have to deal with. Recently new dyes have been produced by chemical companies that are quite good in resolving electrophoretic bands while they are not as toxic as EtBr. Of course, they may be much more expensive. Since student safety is one of our priorities in this lab, we have replaced EtBr with SYBR Gold in all our exercises.

WARNING: Although the Ames test has shown very low toxicity for SYBR Gold, we still caution you to wear gloves and pay special attention to safety methods and regulations since any intercalating agent can, by nature, be quite hazardous.

LABORATORY SUPPLIES

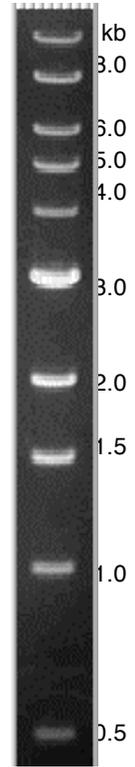
Standard DNA (1 Kb ladder--100 ng/ μ l)	2 μ l/table
Eppendorf tubes	1 beaker/table
Microfuge	2/lab
Loading dye	20 μ l/group
Bio-Rad gel apparatus + 8-tooth comb	1/table
Sterile distilled water	40 μ l/group
Power supply	1/table
Agarose	1.2 g/table
Balance	1/lab
Heat-resistant gloves	1 pair/lab
Electrophoresis buffer, TAE	720 ml/table
Flask, 250 ml	2/table
Spatula	1/lab
Graduated cylinder, 100 ml	1/table
Graduated cylinder, 1 L	1/table
Microwave oven	1/lab
SYBR Gold	8 μ l/table
Tape, roll	1/table

Photography equipment	1/lab
Pipetman, one set of P20 and P200	1/group
Pipet tips, large and small	1 box of each/group
Pipetman holder	1/group

PROCEDURES

1. Thaw out the original PCR tube on ice and microfuge for 10 seconds. Transfer 2 μl of the top supernatant to a fresh Eppendorf. Also add 11 μl of water and 2 μl of loading dye. Mix and pulse and place on ice.
2. Prepare a standard marker as follows: add 1 μl of 1 Kb DNA marker, 12 μl water and 2 μl of loading dye in a fresh Eppendorf, mix and pulse. Place on ice.
3. The students at every table will prepare a single gel as described here. Obtain a complete Bio-Rad gel apparatus and power supply and make sure the parts are dry and clean. Watch the demonstration by your T.A.
4. The gel apparatus is composed of a gel base and a gel caster. Set up the gel caster by placing the UV-transparent gel tray into it and tightening the cam lever. Place an 8-tooth comb into its slot at one end of tray and make sure the gel caster is on a level surface.
5. Weigh out 0.56 g agarose in a 250 ml flask and add 35 ml of electrophoresis buffer (TAE). Place in a microwave oven and heat to a boil (about 1 minute). Take the flask out --BE CAREFUL: the flask is very hot; use the heat-resistant gloves provided. Mix well without making bubbles. Make sure no agarose particles are visible and all have completely dissolved. If you see undissolved agarose, heat for a few more seconds in the microwave again. No boiling over should occur.
6. Add 35 ml more TAE to the flask to bring its temperature to about 50°C. Let your TA add 4 μl SYBR Gold to your flask and mix gently. Pour the agarose into the tray. Do not move or touch the tray until the agarose has completely hardened.
7. When the agarose has solidified (about 15 min), wear gloves, loosen the cam lever and transfer the gel tray with the comb into the gel base. Pour in 1X TAE to fill the buffer compartments and cover about 1 mm over the gel surface. Gently remove the comb. The gel is now ready to be loaded with your samples.
8. Learn how to load the gel slots from the demonstration by your T.A. Load your samples into the gel (15 μl per lane=total volume of each tube), taking note as to the sequence of samples in the gel slots. Start the electrophoresis at about 100 volts and watch the migration of the front blue band. Note: It is a good idea to load the 1 Kb standard onto the first well.
9. When the blue band has reached halfway to the end of the gel (about 40 min), turn off the power supply, wear rubber gloves and place the gel over a UV

illuminator and let your TA take a photograph. The photograph can then be photocopied for the members of your group.
The 1 kb standard ladder is given below. This is used to determine the size of a band on a gel.



SESSION V

Using BLAST to Identify the Species Based on its rRNA Gene Nucleotide Sequence

BLAST stands for Basic Local Alignment Search Tool. It is essentially a search engine that searches a database of DNA sequences at very high speed. You can visit:

- <http://ncbi.nlm.nih.gov/Education/blasttutorial.html>
-

For a thorough tutorial on how to use BLAST. An overview of BLAST is found at:

- http://www.ncbi.nlm.nih.gov/BLAST/blast_overview.html

It should be mentioned that BLAST is as effective as the data it contains. For example, if the sequence of a specific species is not contained in the database, BLAST cannot produce an accurate result. In such a case, many close relatives would be shown at the end of the search. However, most of the time, BLAST results in correct identification of the genus and the species. There are occasions where some species are extremely close genetically (at least for the 16 S rRNA gene) to give a 100% definite outcome. There are also times when the genus is given and the species name is not completely certain. So, combining the search results with other diagnostic test (such as the colony morphology) may become very important in deducing the correct answer. What follows is just a short summary of the steps that are required for identifying your unknown.

1. Download your unknown 16 S rRNA gene sequence from the BlackBoard Website to your computer. If you did not get a good sequence, there would be some extra files on the BlackBoard that you may download instead of yours. Your TA will give you more instructions in this regard.
2. Open your browser (Netscape, Internet Explorer, etc.) to:
<http://www.ncbi.nlm.nih.gov/BLAST>
3. Under “Basic BLAST” click on “nucleotide blast”. A new page will open up.
4. This page has several components. These components and the parameters that we choose are as follows:
 - Enter Query Sequence: Either paste your sequence directly in the box given or press the “Browse” button and find your file on your computer to be used in the search. You can also give a name to your search in the “Job Title” box.
 - Choose Search Set: Since our search does not involve human or mouse databases, click on the “Others (nr etc.)” button. Click on the drop-down menu and choose “16S ribosomal RNA sequences (Bacteria and Archaea)”.

- Next in the “Exclude” option, place a checkmark in the box for “Uncultured/environmental sample sequences”. Leave the optional “Organism” and “Entrez Query” as they are.
 - Program Selection: Click on the radio button next to “Highly similar sequences (megablast)”.
 - Finally press the big blue BLAST button on the left panel to get to the results. However, if you find that you need to find more similar species, first press “Algorithm parameters” located underneath the BLAST button and increase the number of results from the default 100 to 250 or even 500, then press the BLAST button.
5. After pressing the BLAST, a new page appears that may get refreshed a few times. Finally a final page will appear that will give you an ID # (Query ID), the number of nucleotides present in your sequence (Query Length), and some other information. Underneath you will see red bars each of which represents a matching species. Scrolling down further, you can see the species names that match your unknown. The best match is at the top and the matching becomes less accurate as you go down. Pressing on the “Accession” brings up a rather comprehensive description of the matching species, while pressing the “Max Score” shows the actual matching of nucleotides between your sample and the matching species. The species with the highest Max Score is usually the species that should be taken as your unknown result. However, sometimes a few species have the same Max Score, and then you may need to read about the differences among them by consulting the Bergey’s Manual and checking the described characteristics against your unknown. Make a printout of the first 2-4 pages of these results and include it in your report.

Use of any section of this Lab Manual without the written consent of Dr. Eby Bassiri, Dept. of Biology, University of Pennsylvania is strictly prohibited.

Results of PCR Identification of Microorganisms Lab Exercise

Write a short typed paper and include the following points in it. This counts as two weekly reports.

- **Cover Sheet:** This must include a title for your paper, your name, unknown number and the name of your lab instructor.
- **Introduction:** This should include the nature of the problem and why you are using these specific techniques to solve the problem.
- **Materials and Methods:** This part should include
 - The label you use for your sample (This is the label used on the tube sent to the Sequencing Facility and the name of your file). If you could not use your own file, what was the name of the substitute file you used?
 - An overall flow diagram of the whole experiment and some short descriptions of what was done.
- **Results and Discussions:** This part should include
 - Description of the colony morphology of the colony that you took your sample from.
 - The printout of the first 2-4 pages of the BLAST results.
 - The organism of best alignment according to the sequencing results you obtained.
 - Discuss whether you could or could not unambiguously identify your organism based on your 16S rRNA PCR sequence? Also discuss if other species may be possibilities as well. Explain how certain you are of the correct I.D. of your unknown and why this organism does (or does not) make sense; i.e., whether the species of best alignment was what you expected or not.
 - Write out the species names (scientific names) of 10 other species that show good alignment to your unknown species.
 - Describe pathogenic capabilities, characteristics, habitats, economic value, etc. of your previously unknown but now identified organism.
 - Attach a photograph of your gel and discuss the bands obtained and what they signify.
- **Summary:** This is a statement that gives the species name of your unknown.

- **References:** Use the scientific method of acknowledging the citations in your paper, as posted on the course BlackBoard.