

PCR TECHNOLOGY

Adapted from "DNA and Chromosomes" and "Genetic Alleles"¹, BioG 1110 Lab Manual. The staff of BIOG 1106 gratefully acknowledges the assistance of M. Howland, C. Eberhard in the preparation of this laboratory exercise.

INTRODUCTION

What You Will Do In This Lab:

In this lab, you will isolate a sample of your DNA from your cheek cells, specifically the D1S80 locus. You will then prepare your sample for amplification via polymerase chain reaction (PCR). Once this reaction is complete, you will use gel electrophoresis to separate your DNA. Finally, you will be asked to describe your genotype for D1S80.

To Prepare For This Lab:

1. Enroll in a lab sections at: <http://fit.cit.cornell.edu/biog105/student/>

NOTE: THIS IS A TWO-PART LAB. The "A" session is 2 hours; the "B" session, a week later, is 1.5 hours. Make sure that you have enrolled in BOTH Parts "A" and "B." Ideally, your "B" lab session should be exactly one week after your "A" lab, at the same time and with the same TA. But if you absolutely can't do that, it is OK to enroll in different "A" and "B" lab sections.
2. Read and review pp. 403-404 and 419-420 and Figs. 20.8-20.10 (pp. 404-406) of your textbook.
3. Answer the Pre-Lab Questions found on the previous page. They must be handed in at the beginning of lab (Part A).
4. Read this lab guide carefully, paying particular attention to the procedural instructions. Because the thermal cycler and gel take a long time to run, you will have to begin the amplification and make the gel with minimum introduction by the TA. If you have read over the instructions, it will make it easier for you, and we will all finish on time.
5. Don't brush your teeth or chew gum immediately prior to lab (Part A only).

To Do This Lab:

1. Attend the laboratory sessions for which you signed up. In Part A of the lab you will isolate and amplify part of your DNA using the polymerase chain reaction. The following week, you will return to lab to perform Part B of the lab where you will carry out gel electrophoresis on your amplified DNA sample. Follow the

¹ Portions of these labs were adapted from "Forensic DNA Amplification", a publication of the Cornell Institute for Biology Teachers by Jim Blankenship and Glenn Simpson © 1992.

- instructions of your lab instructor concerning when and where you will receive an electronic image of your gel for analysis.
2. Attend the mandatory lecture on lab report writing on February 3.
 3. This lab is worth 7% of the 35 percentage points for your laboratory work this semester. Your grade for this lab will be determined by your lab instructor's evaluation of the following written assignments:
 - a) Your answers to the Pre-Lab Questions (to be turned in at the beginning of lab): 15 points. Late submissions will lose 5 points.
 - b) Your lab report. You will write a partial lab report including an abstract, your results, and a discussion of your results, answering the specific questions asked (see p. L13).
 4. The report is due exactly **one week** after your second laboratory session (Part B). Deposit it in the wooden "mailbox" in the front room of the Study Center. Late reports will receive a 5% deduction from the original total percentage score, for each day they are late up to a maximum 50% deduction. Be sure you know the Study Center hours for each day of the week: reports are due 15 minutes prior to closing. Lab extensions can be used. (Note: You will have a total of 3 lab report extension days this semester.)

BACKGROUND:

Polymerase chain reaction (PCR) is a technique used to amplify the number of copies of a specific region of DNA, in order to produce quantities of DNA that can be adequately tested. Its inventor, Kary Mullis, received the Nobel Prize for his discovery in 1993 after pioneering the technique 10 years earlier. The PCR technique produces copies of DNA sequences in an exponential fashion, until millions of copies are made. The copying is performed in a specialized machine called a **thermal cycler**. The PCR technique is used to amplify small amounts of DNA into amounts that are large enough to allow scientists to identify, with a very high probability, things such as disease-causing viruses and/or bacteria, a criminal suspect, or information about populations of endangered species. These are only a few applications of PCR, can you think of others?

The PCR technique can only be used if the exact sequences that flank both ends of a given region of interest in the DNA are known. These flanking sequences are the complements to primers and must be known even if the DNA sequence being investigated is not known. While some genes may be the same, or very similar among organisms, there will always be genes whose DNA sequences differ among different organisms. The PCR technique uses the structural characteristics of DNA (namely, its two anti-parallel nucleotide chains and complimentary pairing) to form multiple copies of a selected gene sequence. The first step of PCR is to synthesize **oligonucleotide primers** (known sequences of typically 20 – 30 bases that attach to flank sequences of DNA that proceed and follow the gene of interest). The discovery of restriction endonucleases (enzymes that recognize a specific sequence of DNA and cleaves its sugar-phosphate background) made it possible to construct the primers. The oligonucleotide primers initiate the amplification process by restricting the location and sequence of DNA replication. The thermal cycler facilitates the exponential cycling to create millions of copies of the desired nucleotide sequence (gene). The machine does this by using different temperature cycles to: 1) open the DNA molecule and expose the bases; 2) bond the oligonucleotide primers to the flanking regions that come before and after the

desired gene; and 3) activate **Taq polymerase** to implement the building of complimentary DNA molecules (polymerization). Taq polymerase is heat stable and allows many cycles of DNA synthesis to be carried out in the same PCR tube. This discovery eliminated the need to add enzyme after each round. Each cycle runs through the three steps of the process in the thermal cycler, as the DNA in the reaction tubes is heated and cooled. The amount of DNA is doubled after each cycle. After 31 cycles, the amount of DNA produced can be visualized.

Summary of Polymerase Chain Reaction Events

Round 1

Step 1: Denature by heat (94°C). DNA is heated to break the hydrogen bonds and separate the strands of every helix, making single-stranded DNA.

Step 2: Anneal the primers (65°C). Primers are short, single-stranded oligonucleotides that flank the DNA sequence of interest. Two different primers will complement and bond the two flanking regions (one to the left and the other to the right) of the sequence of interest.

Step 3: Extend the primers (74°C). Taq DNA polymerase will attach and begin DNA synthesis wherever there is double stranded DNA provided by the annealed primers. A supply of nucleotides triphosphate subunits and magnesium is also necessary for this step.

Using PCR, you will amplify a small region of DNA located on the tip of Human Chromosome 1 and is known as a **variable number tandem repeat (VNTR)**. This locus has no known genetic function. It is of interest to us here because of its polymorphism; it is a good example of a gene with multiple alleles, at least 29 have been identified so far. Each allele consists of a sequence of 16 bases, repeated over and over. The sequence is the same for all alleles. It is the *number of repeats* that differs between alleles. As you would expect, the alleles with more repeats are longer. D1S80 alleles are inherited just like your other genes. Although there are approximately 29 different alleles (each containing a different number of repeats), each of your chromosomes has only one D1S80 allele, resulting in a total of two alleles (one inherited from mom and one from dad). If you received different alleles from each parent, you are heterozygous; if you received the same allele from each parent, you are homozygous.

You will determine which D1S80 allele(s) you have by separating the alleles on the basis of their size and weight by **agarose gel electrophoresis**. DNA has a negative charge and will move toward the positive pole in an electrical field. An *agarose* gel is like a complex maze with very tiny channels. When placed in an electrical field, short pieces of DNA navigate more easily through the maze and therefore move a greater distance. A tracking (loading) dye is added to the DNA prior to injecting it into the gel so that the researcher can tell when the small fragments have run the length of the gel. Otherwise the DNA would either be pulled right off the positive end of the gel or not moved far enough into the gel. (In our procedure the loading dye is much smaller than the shortest DNA pieces.) In order to then visualize the DNA, several techniques may be employed. In our procedure, we will label the sequence of interest with a compound that will fluoresce under ultraviolet light. You will then observe the distribution of the bands on the gel from which you will be able to determine your genotype.

EXPERIMENTAL PROCEDURES: PART A AMPLIFICATION OF D1S80

Isolation of DNA (Each student prepares his/her own sample)

1. Locate the items you need for the DNA isolation. Once you begin, you need to work steadily. These items include:
 - one yellow-capped tube of 10 mL of sterile saline
 - two 1.5 mL microfuge tubes
 - one microfuge tube with 500 μ L of 10% Chelex
 - distilled water
 - one plastic transfer pipette.

The TA will give you a numbered PCR tube containing a bead and primers at the appropriate time. Make note of the locations of the following equipment: clinical centrifuge, microfuge, boiling water bath, and ice bath.
2. Label all of your tubes with your initials before you begin: 1 yellow-capped tube, 2 small microfuge tubes (label on top), and 1 Chelex tube.
3. Swallow all your saliva, and then rub your tongue around the inside of your cheeks for about 30 seconds. Gently swirl the 10 mL of 0.9% isotonic saline from the yellow-capped tube around in your mouth and return it to the tube. Recap the tube.
4. Spin the sample in a clinical centrifuge for 10 minutes to pellet your cheek epithelium at the bottom of the tube.
5. Carefully decant (pour off into the sink) and discard the supernatant, saving the visible white pellet in the tube.
6. Using a sterile plastic pipette, suck up and transfer all (500 μ L) of the 10% Chelex suspension from the microfuge tube to the tube containing your cheek pellet.
7. Resuspend the cells, including as many of the Chelex beads as possible, by pipetting up and down several times using the transfer pipette. *The Chelex will bind free ions (e.g. Mg⁺⁺) to protect from degradation by DNase enzymes.*
8. Transfer the cell suspension to your fresh, labeled microfuge tube.
9. Cap the tube tightly. Put it in the boiling water bath rack and boil for 10 minutes. *Boiling releases the DNA from the cheek cells and separates the two strands from the helix.*
10. Transfer your tube to ice for 1 minute. *Icing keeps the strands separate.*
11. Centrifuge your tube in the microfuge for 30 seconds to pellet the insoluble debris. *Your DNA released from the cells will stay suspended in the supernatant as single strands.*
12. Using a micropipettor with a fresh tip, transfer 20 μ L of the DNA suspension (liquid supernatant) to your second, fresh, labeled 1.5 mL microfuge tube (avoiding the Chelex pellet). Place the tube on ice. This tube contains your DNA and you are now ready to begin PCR preparation.

PCR Preparation

1. Sign out PCR tube with bead. The bead contains: buffer, KCl, MgCl₂, heat stable Taq polymerase, and four deoxynucleotide triphosphates (dATP, dTTP, dCTP, dGTP).
2. Record the number of your PCR tube.
3. Using a micropipettor, add the following reagents to your numbered tube (don't lose the bead!) using a fresh tip for each transfer:
 - a. 10 μ L distilled water
 - b. 10 μ L primers
 - c. 5 μ L of your DNA
4. Cap the tube and tap for 10 seconds to mix the contents, then microfuge for 5 seconds.
5. Place the tube in the iced collection rack at the thermocycler station. Your TA will place all tubes in the thermal cycler
6. Your TA will prepare the following tubes to include in the thermal cycler:
 - a. Positive control: Steps 3-5 using 5 μ L of previously tested heterozygous control DNA. This control will show that the PCR worked correctly with no problems with the reagents or thermal cycler.
 - b. Negative control: Steps 3-5 using 15 μ L of distilled water and **no** DNA. This control will show that there was not any contamination of the PCR by extraneous DNA.
7. Run the PCR. The thermal cycler will be preprogrammed for 31 cycles, taking approximately 2.5 hours. It will be programmed to run as follows:

30 cycles at: Denature at 94°C for 1 minute
Anneal at 65°C for 1 minute
Extend at 72°C for 1 minute

1 cycle at: Denature at 94°C for 1 minute
Anneal at 65°C for 1 minute
Extend at 72°C for 10 minutes

Store at 4°C overnight or until sample is rescued

There will be 31 cycles of DNA synthesis, so each original DNA sequence will produce billions of copies of itself. The exact amount is not important, we are only interested in producing enough to visualize on the gel you will prepare next week. It is, however, quite an impressive amount – 31 cycles will produce 2,147,483,648 copies!

EXPERIMENTAL PROCEDURES: PART B GEL ELECTROPHORESIS

Preparation of Agarose Gel (Work in Groups of Five)

1.
 - a) Obtain an Erlenmeyer flask containing 0.75 gram of agarose and add to it 50 ml of 1X TBE buffer. (Why add buffer? Why couldn't this system be run in water?)
 - b) Place the flask in the microwave oven and heat for 1 minutes and 15 seconds. This should dissolve the agarose. Check to make sure the agarose is dissolved.
 - c) Remove the flask carefully with gloves. To minimize the risk of being scalded by the hot solution, hold the flask away from your body and avoid shaking it.
 - d) Examine the flask. If all the agarose is dissolved, insert a thermometer in the flask and allow the solution to cool to 65°C.
2.
 - a) While the agarose is cooling, place colored tape across the ends of a gel tray as demonstrated by your instructor and insert the comb into the slots. The tray should not be in the electrophoresis apparatus set up at this point.
 - b) Once the solution is cooled, add 3 μL of GelStar to the flask and gently swirl. *GelStar is a nucleic acid gel stain that will bind to the DNA and make it visible under UV light.*
 - c) **After** ensuring agarose is cooled, pour the agarose solution into the tray. Tip the tray gently to spread the agarose across the entire surface. If large air bubbles are present in the gel, use a pipet tip to move them to the edge of the gel.
 - d) Leave the gel undisturbed on the tabletop for approximately 15 minutes until the agarose becomes cloudy and firm.
3.
 - a) Meanwhile, if the gel box has not been filled, add 250 ml of 1X TBE to the gel apparatus.
 - b) When the gel is firm, remove the tape from the ends of the tray, cut the corner as indicated on the "Gel Record Sheet and lower the tray containing the gel into the running chamber. The gel should be fully submerged by about 2-3 mm of buffer.
 - c) Remove the comb by gently **pulling it straight upward**. You will load your samples into the slots created by the comb. *The comb will create the slots (wells) into which you will load your samples.*

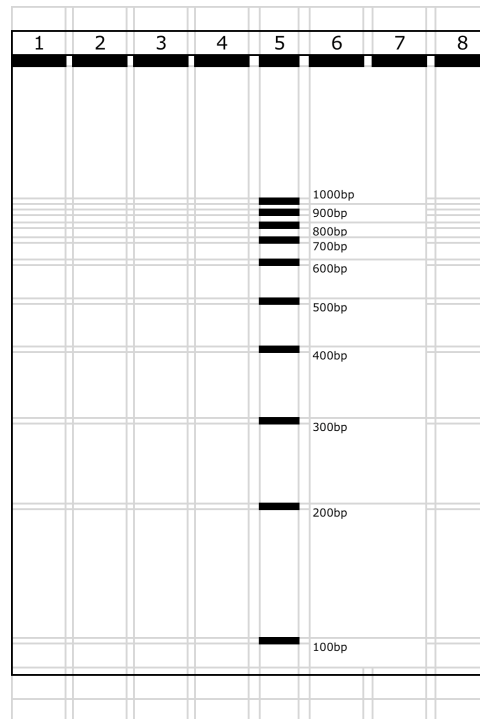
Loading of Gel and Electrophoresis

1. Obtain your DNA sample in the numbered PCR tube that you prepared last week from your TA. Microfuge the tube for 5 seconds.
2. Remove 20 μL of your sample and transfer it to a fresh microfuge tube that contains 3 μL of loading dye. Be sure to label the top of this tube with your PCR tube number.
3. Microfuge this tube for 5 seconds.

4. Load 15 μL of your sample (with loading dye) into the gel using the methods below. Record your gel box number here: _____ Record the lane number in which you load your sample on the gel record sheet and here: _____ (Your TA will help you determine which lane to load your sample in.)
 - a. Set a micropipettor at 15.0 (15 μL)
 - b. Depress the plunger to the first STOP, then lower the tip into your sample and slowly release the plunger to draw up the sample.
 - c. Stand directly over the gel and locate the wells. Steady the pipettor with 2 hands. Lower the tip into the well. Be careful not to puncture the bottom of the well.
 - d. To load the sample, slowly depress the plunger to the first STOP and keep it pressed. Do not press to the second STOP.
 - e. Remove the tip from the well before releasing the plunger.
 - f. Do not take too long when loading the gel or your sample will begin to diffuse out of its well.
5. Your TA will load the positive and negative controls into your gel, as well as a 100 base pair ladder. *The ladder is a mixture of fragments with known sizes to which you will compare your PCR fragments in order to determine their sizes.*
6. Place the cover on the gel electrophoresis apparatus and connect the leads to the power supply, remembering that the black lead goes to the negative pole and the red to the positive pole. Make sure your gel is oriented properly. Electrophorese at 190 volts for 30 minutes. You will be able to observe the *tracking* dye as it moves towards the positive pole. You will turn off the apparatus just before the *tracking* dye reaches the end of the gel.
7. Lift your gel in its tray out of the gel box and slide it into the tray provided. Gently rinse the gel off with distilled water and allow it to cool for a few minutes. Bring the tray to the photographic apparatus and with gloves, place it directly on the UV transilluminator. Once the camera shade is placed on the transilluminator, it will be turned on and a digital image of your gel will be captured. **DO NOT LOOK AT THE UV LIGHT WITHOUT THE CAMERA SHADE. THIS SHADE BLOCKS OUT THE DAMAGING UV RAYS.**
8. Within 24 hours, retrieve the image of your gel on the course Blackboard site (<http://www.blackboard.cornell.edu>). Under PCR GELS, you will find a folder labeled with your "B Lab" date and time. This folder will contain your gel image.

Interpretation of Electrophoresis Results: Use of the Standard Curve

To determine the size of each DNA fragment visible as a band on your gel, first construct a **standard curve** based on the bands produced by the ladder. To construct a standard curve, you need to have a number of DNA fragments of known sizes (i.e., size standards), and measure how far each of the known standards migrates on the gel. These measurements are then used to prepare a graph of size versus distance migrated. The bands in the center of figure below represent the expected banding pattern for the fragments in the ladder.



a) Set up graph paper (supplied by your TA) with distance migrated (in mm) on the X-axis and # of base pairs (bp) as the Y-axis. Then, plot base pairs versus distance migrated for each band of the ladder. Connect the points with a linear curve. If the points do not fall in a straight line, estimate the best fitting line. This is the standard curve you will use to determine the size of the fragments in your amplified DNA sample.

b) In the lane containing your sample, you should see one (if you are homozygous) or two (if you are heterozygous) bands. These bands may or may not match the bands of the ladder. Human beings have 23 pairs of chromosomes. Each band corresponds to the same region in each of the homologous chromosomes in a pair. A baby receives one member of each chromosome pair from each parent. You therefore inherited one allele from your mother and one from your father.

c) To determine your genotype for the D1S80 locus, use the standard curve you constructed. Once you determine the size of your fragments, you can convert this size to the number of tandem repeats you have in each of your D1S80 alleles as follows:

$$((\# \text{ of bp}) - K) / 16 = \text{approximate number of tandem repeats}$$

by nonspecific binding of the primer to locations other than the D1S80 locus. You can ignore these bands.

The 29 alleles for D1S80 could appear in an individual's DNA in any one of 435 possible combinations. In addition to describing your own genotype by comparing the results of your DNA with the DNA ladder, you will also be able to compare genotypes within your lab section. It is likely that everyone in a lab section will have a unique genotype. However, it is pretty likely that a certain genotype will appear more than once among the approximately 170 BioG106 students. Each allele occurs with a certain frequency in the population (see Table 1). For example, suppose allele 18 occurs with an allelic frequency of 0.174. This means that in a sample of 50 people (100 alleles), you would expect to find 17 individuals carrying this allele. The allelic frequency may differ among different ethnic groups, i.e., the frequency (f) of allele 18 = 0.073 for African Americans and 0.152 for Asian Americans.

Table 1. D1S80 allele frequencies in five sample populations*
(N values are number of individuals typed)

Allele	African American	Caucasian	SE Hispanic	SW Hispanic	Asian American	Total Population	Maximum Frequency
N	606	718	247	162	204	1937	1937
15	0	0	0	0.003	0	0	0.003
16	0.002	0.001	0.004	0.019	0.034	0.007	0.034
17	0.028	0.002	0.012	0.003	0.025	0.014	0.028
18	0.073	0.237	0.225	0.222	0.152	0.174	0.237
19	0.003	0.003	0.004	0.006	0.022	0.005	0.022
20	0.032	0.018	0.01	0.019	0.007	0.02	0.032
21	0.115	0.021	0.03	0.025	0.034	0.053	0.115
22	0.081	0.038	0.028	0.019	0.017	0.046	0.081
23	0.014	0.012	0.014	0	0.017	0.012	0.017
24	0.234	0.378	0.316	0.315	0.23	0.304	0.378
25	0.045	0.046	0.059	0.093	0.027	0.049	0.093
26	0.006	0.02	0.008	0.006	0	0.011	0.02
27	0.008	0.007	0.012	0.022	0.047	0.013	0.047
28	0.13	0.063	0.081	0.074	0.076	0.089	0.081
29	0.053	0.052	0.079	0.019	0.042	0.052	0.079
30	0.009	0.008	0.018	0.071	0.123	0.027	0.123
31	0.054	0.072	0.051	0.056	0.093	0.065	0.093
32	0.007	0.006	0.006	0.003	0.012	0.007	0.012
33	0.004	0.003	0.004	0.003	0.005	0.004	0.005
34	0.086	0.001	0.008	0.003	0.005	0.029	0.086
35	0.002	0.003	0	0	0.005	0.002	0.005
36	0.001	0.004	0.014	0.006	0.005	0.005	0.014
37	0	0.001	0.006	0	0.007	0.002	0.007
38	0	0	0	0	0	0	0
39	0.003	0.003	0.002	0.006	0.005	0.003	0.006
40	0	0	0	0	0	0	0
41	0.002	0	0	0.006	0.007	0.002	0.007
>41	0.007	0.001	0.008	0.003	0.002	0.004	0.008

* After Budlowe, B. et al., "D1S80 – Population data in African Americans, Caucasians, Southeastern Hispanics, Southwestern Hispanics, and Orientals", *J. Forensic Sciences*. 40(1), 1995, 38-44.

The Lab Report

For this lab procedure we are asking you to write an abstract and write up and interpret your results in a partial lab report, with formal Abstract, Results and Discussion sections. Limit your paper to 3-4 typed, double-spaced pages. If you cite any references a Literature Cited section must be included as well.

DO NOT INCLUDE A TITLE PAGE since this is not a complete lab report. Instead, you should include the following information in this format at the top of your first page:

Name _____ Date _____

Lab TA (Part A) _____ Lab TA (Part B) _____

Write an **Abstract** for this experiment. Remember that an abstract is a brief summary of all parts of the experiment: the problem investigated, the main methods employed, the major results, and all-important conclusions. Review the information on abstracts in Appendix A in your Survival Manual.

Your **Results** section should include the results from your procedure, including the following: (See also Appendix A.)

1) A diagram of your gel and the band(s) that you observed. You need only include in your diagram the lanes corresponding to the ladder, the positive and negative controls, and your sample. You do not need to include the lanes containing your classmates' samples. Note: If your results turned out poorly, you may use someone else's sample, but be sure to explain what was wrong with your own and why you suspect it occurred (in the Discussion section).

2) List the distances traveled in millimeters for each of the bands found in the allelic ladder and the distances that each traveled in a table.

3) Your standard curve, correctly titled and labeled on semi-log paper.

4) The estimated sizes (based on your standard curve and calculations) of your D1S80 alleles.

The Results section must also contain text providing a brief description of the data.

In the **Discussion** section you must interpret your results. There should be a discussion of all data presented in results. If your data deviate from the expected values or was inconclusive, analyze the reasons for the difference. Other questions to answer in your discussion are:

1) What is the frequency for your genotype in the population?

2) Discuss one use of PCR technology.

Note: You should be able to confine your report to four pages of text (not counting tables, figures). Your TA will stop reading after four pages.

**PCR Technology Lab
BIOG 1106 Laboratory Evaluation Form**

Pre-lab Exercise: 25%²**Abstract: 17%**

- Does it give a good, clear, concise summary of the problem investigated, expectations, major methods employed, major results obtained, and major conclusions reached?
- Is it single-spaced and separated from the main body of the text?

Results: 23%

- Are raw data suitably transformed and summarized, and then clearly and precisely presented in figures and/or tables?
- Are the required tables and figures (standard curve and diagram of gel) logically organized and fully labeled (see Appendix B)?
- Do tables and figures have adequate and correct titles/legends?
- Does the text of the results section completely but concisely present the tables and figures?
- Are all tables and figures in this section referred to in the description of the results?

Discussion: 27%

- How well are the reported results interpreted?
- Are all the results discussed including the standard curve?
- In light of the reported results, are the conclusions valid?
- When appropriate, are tables and figures in the Results section referred to in the discussion?
- Are reasons given to explain any discrepancy between these results and expected results?
- Are the questions answered?

General Form: 8%

- Is the grammar, language, and writing style of good quality?
- Overall, is the paper clear, concise, and complete?
- Have irrelevant digressions been excluded?
- Is the format correct, including the heading?
- Is the length of the paper within the prescribed page limit (reasonably close to 3-4 double-spaced pages)?
- If literature was cited is there a "Literature Cited" section correctly presented?

² Percentages for each section of the checklist represent the approximate "worth" of that section. Pay attention to these numbers as a similar scheme will be used in grading your papers.

PCR TECHNOLOGY LAB EVALUATION

Please fill out this evaluation sheet after you have completed the lab, and place it in the wooden box in the Study Center. These forms will provide feedback necessary for improving the laboratory.

Who taught your lab? Part A _____ Part B _____

Do you feel your TA(s) did a good job in organizing and teaching the lab? (Please feel free to comment.)

Was your TA(s) knowledgeable and helpful?

Were the prelab instructions clear? Please indicate any problem areas.

Did you find the prelab helpful in preparing for lab?

Were the lab instructions clear? Please indicate any problem areas.

Overall, how much do you feel you learned from this lab?

A. very little B. a moderate amount C. a great deal

How can we improve this lab?