

Texas A&M University-Corpus Christi
CHEM4402 Biochemistry II Laboratory
Laboratory 4 - Polymerase Chain Reaction (PCR)
Please bring a laptop or portable memory device to laboratory

Progressing with the sequence of experiments, we are now ready to amplify the green fluorescent protein gene from *Aequorea victoria* DNA. The technique we will be using is the Polymerase Chain Reaction, better known as its acronym, PCR. This procedure was first developed in the 1980's by Kary Mullis and a group of scientists at a biotechnology company in California. The Polymerase Chain Reaction is based on the biochemical mechanism behind natural DNA replication but is performed in an artificial environment, where conditions are manipulated to promote the copying ("amplification") of a specific DNA target sequence (figure 1).

PCR involves formation of base pairs between oligonucleotide DNA "primers" and their complementary target sequence in the DNA (annealing). Once bound, the primer/DNA target pair serves as a starting point for attachment of DNA polymerase, the enzyme responsible for DNA replication. By controlling the temperature and time of these steps (a "cycle") a portion of DNA can be copied millions of times in a relatively short period of time (2-4 hours). The procedure has revolutionized biochemistry, molecular biology, genetics and the related fields of forensics, clinical chemistry and practically all other life science disciplines. For their work, Mullis and his associates received the Nobel Prize in medicine in 1993.

Today, we will be using primers designed from the *Aequorea victoria* DNA sequence to make multiple copies of the green fluorescent protein gene. We will use this DNA for a cloning experiment, where we insert the PCR product into a bacterial plasmid vector and then place it into a specific strain of bacteria. The amplified PCR product will then be in an environment where it has all the molecular machinery necessary (enzymes, amino acids, ATP, coenzymes, etc.) for the expression of the gene. In addition, we have the ability to biochemically activate the genes in bacterial plasmids, enabling us to make lots of GFP protein for characterization and analysis.

PCR, and cloning of PCR products into bacterial plasmids are two of the primary techniques used in the various "genome" projects, including the recently completed human genome project that recorded every single nucleotide from all 48 human chromosomes (albeit from a single individual). Relatively speaking, that was the easy part. The real work will come in deciphering this information: determining which DNA sequences code for which genes, identifying the function of these genes, and determining how, why and when they are expressed. Hopefully, this information will enable us to better understand human physiology and disease, and help in the design of new drug or genetic therapy treatments.

PCR is very powerful, but the individual reaction components are very sensitive. Therefore, we will be setting up our reactions on ice. We will also be transferring volumes that are quite small (1-5ul), so pay careful attention to your pipetting technique.

Keep your eye on the tip of your pipet to ensure that it is actually drawing up solution. When you are through making up your sample, return it to your instructor, who will demonstrate how to load, program and run the PCR. Since the cycling reactions will take a few hours, your instructor will remove your samples after the PCR is finished and store them in the freezer until next week. Be sure you have identified your sample with at least one lab partner's initials.

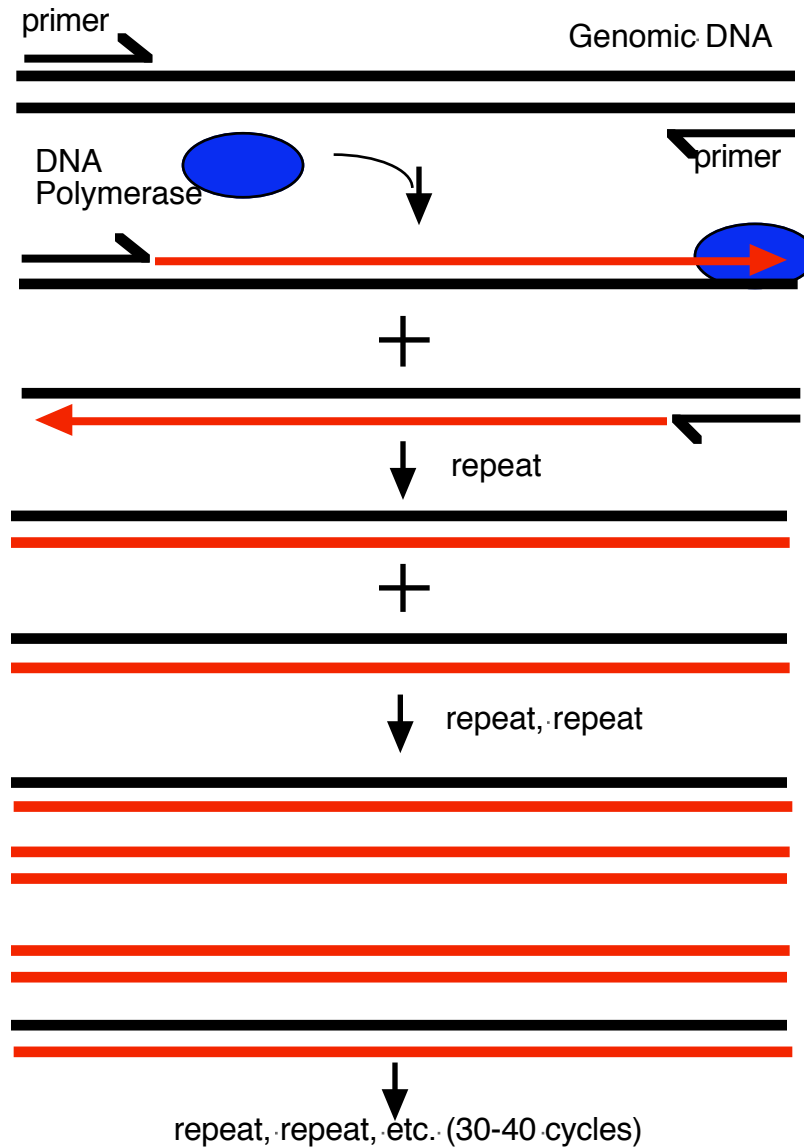


Figure 1. Amplification of target DNA sequence during PCR procedure

Materials

Forward and Reverse Primers
Master Mix (Green Solution)
0.2 ml PCR tube

Aequorea Victoria DNA Sample
Sterile H₂O
PCR machine (thermal cycler)

Procedure

1. Obtain a cup of ice. Obtain aliquots of the following from your instructor:

- (W) Sterile H₂O
- (G) Green Master Mix (contains enzyme, buffer and nucleotides)
- (F) Forward Primer (20 uM)
- (R) Reverse Primer (20 uM)
- (D) AV DNA

3. Thaw the reagents as needed (place in water bath or roll between fingertips). Mix gently by tapping tube with your index finger. If necessary, centrifuge in a tabletop microcentrifuge to bring contents down to bottom of tube.

4. Set up your PCR reaction in a 0.2 ml PCR tube as shown below. Add reagents to the tube in order (top to bottom). To avoid pipetting errors, follow these tips:

- a. hold the reagent tube with one hand while using the other to gently draw the desired volume into the pipet
- b. Be sure that your pipet tip actually contains sample before expelling it into your PCR reaction (hold pipet/tip up to the light for best contrast)
- c. Insert the tip of your pipet INTO the PCR reaction BEFORE you expel a reagent. GENTLY depress plunger.

<u>Reagent</u>	<u>Volume (ul)</u>
Sterile H ₂ O	16
Green Master Mix	25
Forward Primer	2
Reverse Primer	2
AV DNA	5
Total Volume	50

5. When everyone has finished setting up their PCR reaction we will collect the samples for thermal cycling.

Operation of the MJ Research PTC-200 thermal cycler.

1. Turn the machine on by flipping the toggle switch on the back of the instrument. The machine will perform a self-check for a several seconds.
2. A screen will appear with several options, including: RUN, FILE, EDIT etc. Select RUN (scroll through options with arrow keys to highlight and press the PROCEED button to enter your choice).
3. You will be brought to a screen which contains two electronic “folders”: MAIN and LARKIN. Select LARKIN.
4. Select the desired program for PCR cycling (GFP in our case). Note: PCR cycling programs are designed according to the melting temperature of the primer (T_m) and the number of cycles desired for amplification. Instructions on how to set up a thermal cycling program and store it in a personal folder are outlined in the PTC-200 instruction manual in the drawer beneath the instrument.
5. A new screen will ask which type of vessels are to be used: TUBES or PLATE. Select TUBES.
6. A new screen will ask you to enter the volume of the PCR reaction in the tubes. Enter “50”.
7. A new screen will ask you whether you wish to use the heated lid. Select YES.
8. The thermal cycling program will now begin. It will start by heating the lid and then heating the tube block. It is always a good idea to wait until the block has reached the initial denaturation temperature until placing your samples in. To load your samples, lift up on the hinged lid and unscrew the blue flywheel all the way (loosen). Place your samples in the block and follow the instructions inside the lid for tightening the cover.

Thermocycler Program GFP

Process	Temperature	Time	No. of Cycles
Initial denaturation of DNA	95 ^o .C	2.min.	1
Denaturation	95 ^o .C	30 s	35
Annealing of primers	56 ^o .C	1.min.	35
Copying of DNA target	72 ^o .C	1.min.	35
Final extension	72 ^o .C	15.min.	1

When the samples have finished running, your instructor will retrieve and store them in the freezer until we can analyze our results by electrophoresis (next week)

Assignment

Find an article from the primary literature (i.e not a review article) that uses the PCR technique as part of its experimental procedure.

1. Go to the Mary & Jeff Bell Library home page (<http://rattler.tamucc.edu/>). Select the ***Find Articles*** icon
2. Selecting the ***Find Articles*** icon will bring you to the ***QuickSearch*** page. Be sure that the ***Advanced*** tab at the top has been selected. Enter a search term in the first search box. Select ***Subject*** in the drop-down box next to it. Enter any other relevant term in the second search box to narrow your results to a region of interest (e.g. “insects”, “vibrio”, “disease”, “dentistry”, etc.). Be sure to check an appropriate place to search for the second term (all fields, subject, author, etc.) in the associated drop-down box.
3. Below the search boxes you will find a number of subject databases that serve as potential resources for your search. Its advisable to select only one of these subject areas (e.g. “Chemistry” or “Biology”) to reduce the number of results returned. If your second search term doesn’t clearly fit one subject, go ahead and leave the default setting (*multiple subjects*) as the subject setting.
4. Select “Go”. The search engine will check a number of databases, such as *Science Direct* and *Web of Science*.
5. After your search has completed a ***QuickSearch Results*** page will appear. Note that not only does this page contain a list of articles that closely match your search terms but it also contains an organizer panel on the right-hand side of the screen. Here you restrict your results by associated topics, date of publication or journal title. Browse through the titles and examine the summary of those that look interesting. When you find one that you like, select the ***Search for FT*** (full text) link. Another page will open telling you whether the university has electronic access to the article. If we do, follow the link to the full text, online version. When you have found an online, full-text version of an appropriate article, save a PDF copy to a portable memory device or email the PDF file to yourself. Remember, the article needs to be from the primary literature (involves actual experiments).
6. If your search turns up too many results, restrict your search by limiting the subject databases searched, limiting the results to only one year, or by being more selective in your original search terms or fields where the term is to be found (subject, author, etc.)
7. As in the literature searching lab, give your instructor a sheet of paper with the following information:
 - a. Title of Article
 - b. Name of first author
 - c. Title of journal, volume and page numbers
 - d. Date of publication
8. review the article and answer the questions in the attached worksheet. Turn in your worksheet along with a copy of the first page of the article next week.

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Find article in lab. Turn in completed worksheet at your next laboratory period.

1. Laboratory performance (0-2 pt).
2. Copy of first page of article (1 pt).
3. Refer to the introduction of your paper. What was the motivation for this work? What were the specific goals? (2 pt)

4. List each of the individual techniques the authors had to perform to collect and analyze their data. You need not list the details of how each technique was performed. (2 pt)

5. What role did the Polymerase Chain Reaction have? (1 pt)

6. Choose two results (i.e. two figures, one figure and one table, two tables, etc). In your own words, describe what is presented and what it shows. (2 pt)

7. Refer to the discussion section. Based on their results, what conclusions do the authors draw? Do they postulate any broader significance for the work? If so, what? (2 pt)