Experiment #3
Polymerase Chain Reaction

Introduction

The polymerase chain reaction (PCR) was originally developed by the Nobel Prize winning Kerry Mullis and has dramatically changed recombinant DNA technology. PCR is extremely versatile and has been implemented in gene cloning and manipulation, gene mutagenesis, DNA sequencing, forensic DNA typing, and amplification of ancient DNA.

To amplify a sequence from DNA, two short oligonucleotide primers are annealed to denatured (strand-separated) DNA under conditions ensuring that only primers perfectly complementary (or nearly so) with the desired sequence will anneal. The two end primers are complementary to the two 3' ends of the DNA segment to be amplified. Advantageously, the primers may contain additional noncomplementary sequences at their 5' ends, such as sequences encoding sites for restriction enzyme cleavage. The primers are then extended using DNA polymerase and the four-deoxynucleotide triphosphates. After time sufficient enough for extending the DNA fragment of interest, the reaction is terminated by denaturing the DNA strands.

Three steps: denaturation, annealing, and elongation represent one cycle of PCR. The process is repeated with new primers (already in the reaction mixture) annealing and elongating to produce more DNA fragments. Multiple cycles are done to obtain a sufficient amount of the DNA sequence of interest.

The three steps of PCR are accomplished by alteration of the reaction temperature. The denaturation step is done at 95^{o}C. The annealing step should be done at a temperature that selects for efficient matching of only perfectly annealed DNA strands, commonly 55^{o}C. The temperature for the elongation phase is chosen to match the properties of the polymerase used. These thermostable polymerases usually work best at 68-72^{o}C. They are derived from bacterium that live in thermal vents and hot springs.

The primers in PCR are designed to anneal specifically to a known sequence. The part of primer that anneals to the target DNA is usually 16 or more nucleotides long. Because

there are four possible nucleotides in any position in a 16-mer sequence, a particular 16-mer sequence should only appear in any genome once in every 2.2×10^{10} base pairs. Additionally primers can be modified with a restriction site at their 5' end so that they can be cleaved and ligated to a plasmid of choice.

Experiment

You will amplify a 798 base fragment of DNA from a vector. The 3' end of the fragment to be modified will anneal with the primer (5'-CTG CCG CAA GCC GCC CCA GAG-3') and the 5' end of the fragment will anneal with (5'-ATG GTA GTG TTC AAT GGC CTT-3'). **Please be careful not to contaminate any of the solutions that you will use for this assay.** You should use a fresh tip whenever you change solutions to be used. Furthermore, add reagents in order indicated to insure stability of your DNA and efficient amplification of the target DNA. You can consult the additional handout for more detailed information on PCR reagents and frequently encountered problems.

Day 1: Add following reagents into a sterile 0.2 mL PCR tube:

31 μL of ddH_2O
5 μL of 10x polymerase buffer
5 μL of primer A stock solution
5 μL of primer B stock solution
1 μL of template DNA
2 μL of 10mM deoxynucleotide triphosphate mixture

Gently tap the tube to mix the reagents and finally add

1 μL of DNA polymerase **(always added last)**

It is important to add DNA polymerase last and just before the cycles are started because polymerase has exonuclease activity and can degrade the primers. Note that all reagents are sterile and proper precautions should be taken to keep them this way.

The protocol for amplifying this target DNA has been perfected and thus the PCR thermacycler has been preprogrammed for this method. When all groups are prepared to begin the PCR reaction, your TA will assist you in loading your samples into the apparatus. Before this step, be sure to gently mix and spin down your reaction mixture. Once all groups' samples are loaded, the program will be started. Any questions regarding programming the Perkin Elmer

GeneAmp PCR system 2400 can be answered by your TA or the instrument manual. The preprogrammed cycle is as follows:

23 cycles of

1 minute at 95^{o}C
1 minute at 50^{o}C
3 minutes at 72^{o}C

then 10 minutes of elongation at 72^{o}C
finally the mixtures are cooled to 4^{o}C

Because the time required to complete these cycles is greater than that of the class, your TA will remove your samples, and you will analyze them during the next class period.

Day 2: Your TA will direct you to the storage place of your sample and you should put it on ice. To your sample you will add 6 μL of the loading dye provided by your TA. You will load your sample into two wells of a 0.4% agarose gel and use a standard (control) that is the same size as your target DNA. After all samples are loaded, your TA will start the electrophoresis apparatus and run at 80 volts. Be aware of the properties of DNA and the electric field, which can be reviewed in most biochemistry textbooks. DNA has negatively charged phosphate groups and thus will migrate based upon its size towards the positive side of your gel box. After approximately 30 minutes the DNA band should have migrated sufficiently for observation. Take your gel to the UV viewing box to compare your PCR sample with the standard. **Always be sure to wear gloves when handling these gels!!!** They contain carcinogenic ethidium bromide that intercalates DNA. Furthermore, UV light is dangerous for your eyes and any viewing using this type of light should only be done in the safe box.

Analysis

You now should be able to explain the PCR principle and create primers for amplifying a known sequence of DNA. In addition, you should be able to explain why a PCR reaction may not have worked based upon your gel results.