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ModernBio SERIES
BY JOHN N. ANDERSON

IND-7• STUDENT GUIDE

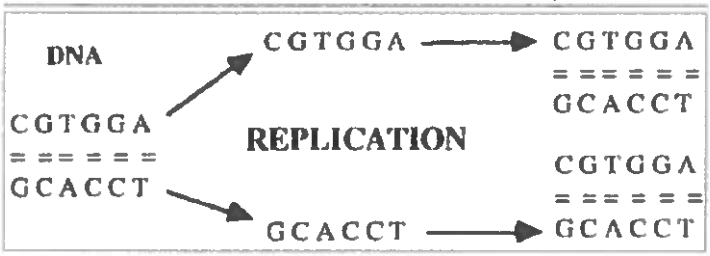
Amplification of a Hemoglobin Gene by the Polymerase Chain Reaction (PCR)

Background Information

I. The Polymerase Chain Reaction

Prior to the time of each cell division, the two strands of the DNA helix in a chromosome separate from one another and each serves as a pattern or template for the synthesis of a new, complementary chain as diagrammed in Figure 1. This process of DNA biosynthesis is known as replication. The major enzyme that catalyzes this reaction is called DNA Polymerase. The polymerase chain reaction (PCR) is used to amplify specific DNA segments in a test tube. The PCR uses a basic strategy for DNA replication that is similar to the mechanism of replication that is used in the cell. One major difference between the cellular and PCR reactions is that the *in vitro* method is carried out at elevated temperatures (as high as 95° C) as will be described below. At this temperature, DNA polymerase isolated from most cells is inactivated. Thus, a thermostable DNA polymerase purified from the thermophilic bacterium *Thermus aquaticus* is used in the PCR. This enzyme is referred to as *Taq* DNA polymerase.

Figure 1. A Simplified Diagram of DNA Replication



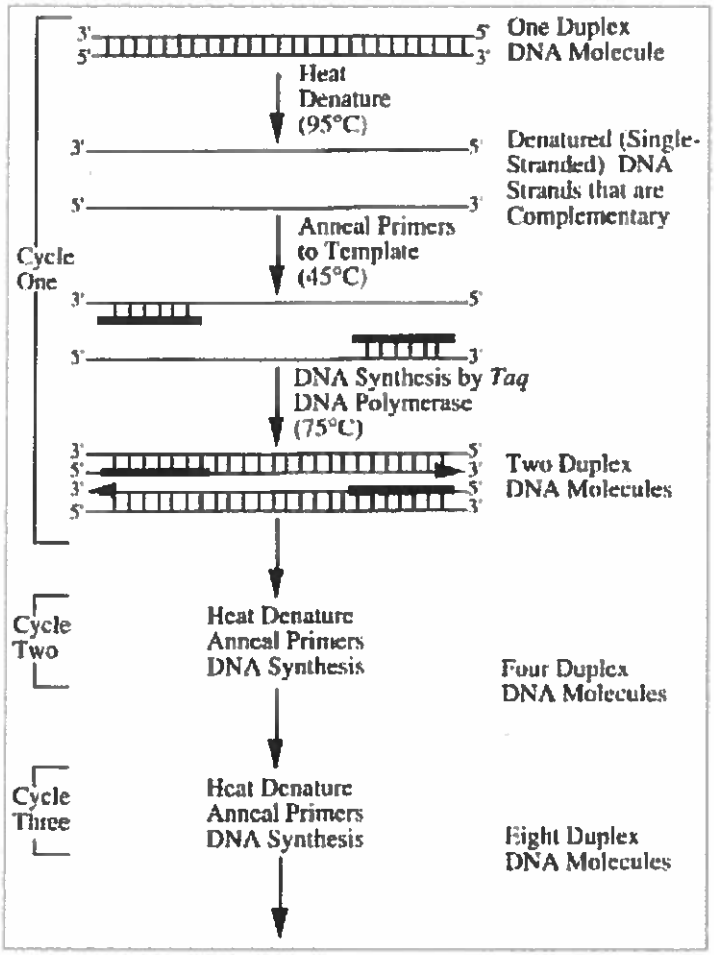
The basic steps in a typical PCR are outlined in Figure 2. These steps will be carried out in this laboratory. In the procedure, DNA isolated from a cell is first heated to 95° C in order to separate its complementary strands. The reaction mixture is then cooled to 45° C and the separated strands anneal with two synthetic oligonucleotide primers that are present in the mixture. The primers are designed to be complementary to sequences on opposite strands of the DNA template. These primers, which are usually 15-20 nucleotides in length, serve to specify the positions that DNA synthesis can be initiated by *Taq* polymerase. The annealed mixture is then incubated at 75°C which is the optimal temperature for *Taq* polymerase catalysis. In addition to this enzyme, the mixture contains the four deoxyribonucleotide triphosphates which are the raw materials for synthesis of DNA. During this synthesis phase, the regions adjacent to the primer sites are synthesized (5' -> 3') as indicated in Figure 2. The above three steps (denaturation, primer annealing, and synthesis) represent a single PCR cycle which takes about 5 minutes and doubles the amount of DNA. It should be noted that each cycle doubles the amount of DNA in the previous cycle. Thus, the amount of DNA after 1, 2, 3, and 4 cycles is 2, 4, 8, and 16 times the amount of original template. This exponential amplification of template DNA can result in amplification levels as high as 10^6 during the course of a typical PCR reaction of 20 to 30 cycles. A standard reaction of 20 - 30 cycles is usually performed in order to obtain sufficient quantities of DNA that can be detected following electrophoresis.

The PCR has been used extensively in studies of gene structure and function. The method is also becoming increasingly important in detecting nucleic acid sequences of pathogenic organisms in tissue samples. In addition, the PCR is now used in DNA typing procedures such as DNA fingerprinting and in the identification and characterization of mutations that cause human diseases. In this exercise, you will amplify the β globin gene from the rabbit by PCR and determine its length. This gene is described below.

II. The Hemoglobin Genes

Red blood cells, or erythrocytes, carry the protein hemoglobin in the circulation. This protein serves to transport oxygen from the lungs to the tissue. Hemoglobin is a globular protein made up of four subunits. Each subunit contains a polypeptide chain attached to an iron-containing component called heme. Each heme group contains one iron atom and each iron atom can bind one 0 molecule. The polypeptide chains of hemoglobin are referred to as the globin portion of the molecule. Normal adult hemoglobin (hemoglobin A) has four subunits made up of two alpha (α) and two beta(β) polypeptide chains. Each α-chain contains 141 amino acid residues and each β-chain contains 146 amino acid residues. The α and β globin chains are coded by different genetic loci that are called the α and β globin clusters. Each cluster contains a series of globin and globin-like genes and the two clusters are located on different chromosomes. Figure 3 shows the structure of the globin gene which is located on chromosome II in humans. This gene, like all globin genes, contains three exons which are interrupted by two introns. Most eukaryotic genes contain introns which are segments of DNA within genes that do not code for proteins. During transcription, the gene gives rise to an RNA species which is a precursor to the mRNA. The intron sequences are then removed from the precursor and the remaining segments (coded by the exons) are spliced together to form the mature mRNA. The mRNA then enters the cytoplasm and is translated into a polypeptide chain.

Figure 2. The PCR Procedure.



III. Description of this Laboratory Exercise

In this exercise, you will amplify the rabbit β globin by PCR using the basic procedure shown in Figure 2. The template for the reaction is a plasmid that contains the globin gene and flanking DNA as shown in Figure 3. The primers that are in the PCR mix were designed to anneal to this DNA at the very beginning and at the very end of the β globin gene as indicated in Figure 3. Thus, the PCR will amplify the globin gene but not the flanking DNA. Following the reaction, you will electrophorese the amplified β globin gene on an agarose gel and determine its length.

Materials Provided

DNA Template- The DNA contains the rabbit β globin gene and flanking rabbit

DNA shown in Figure 3 that has been inserted into the plasmid pUC18. PCR buffer - The buffer contains the following components after dilution: 50mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X 100, 10mM Tris-HCl, pH 8.3 at 25°C

Nucleotides - This tube contains the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, dTTP). The final concentration is 0.2mM each.

*_Taq_ DNA polymerase

Primers - The two oligonucleotide primers were designed to anneal to the beginning and to the end of the rabbit β globin gene as shown in Figure 3. The sequences of the primers are given as follows:
Primer 1-5' GCTGCTGGTTGTCTAC 3'
Primer 2-5' AGATCTCAGTGGTATT 3'

Mineral oil

PCR (0.5ml) tubes

Electrophoresis Sample Buffer

DNA Markers- the sizes of these markers are 3621, 2040, 1120, and 784 base pairs.

* Prepared as described in the Instructor Guide.

Materials Needed but not Provided

*Solutions and materials required for electrophoresis, sample handling and gel staining

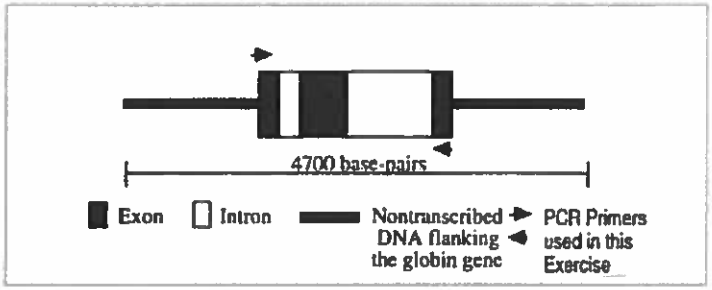
*Three water baths maintained at 95°C, 45°C, and 75°C and floating tube rack for eight 0.5ml tubes or an automated DNA Thermal Cycler

*See Instructor Guide

Laboratory Schedule

The PCR procedure can be completed during a single laboratory session of 2-3 hours. The electrophoretic analysis of the product can be completed during a second laboratory session.

Figure 3. Organization of the Globin Gene.



Procedures

Part A: The PCR

The exercise was designed for 8 groups of students. The procedure described below was designed so that the PCR can be done manually. Your instructor will assign either one student or a group of students to carry out the temperature transfer reactions. The procedure can also be performed in an automated manner using a DNA Thermal Cycler. If this option is chosen, your instructor will provide instructions for operation of the device.

1. Number three 0.5ml tubes #1, #2 and #3 on the tops with a pencil.

2. Place the following into tube #1:
   10µl of the primers
   10µl of the nucleotides
   10µl of the PCR buffer
   10µl of the DNA template
   10µl of the *Taq* DNA polymerase

3. Tap the tube with the tip of your index finger to mix the solution.

4. Transfer 15µl of the solution from tube #1 to tube #2.

5. Add 5µl of electrophoresis sample buffer to tube #2 and place this tube in the freezer until the next laboratory session.

6. Carefully overlay 50µl of mineral oil over the liquid in tube #1.

7. Transfer tube #1 to the tube rack that will be used during the PCR noting the position of your tube in the rack.

8. Perform cycle one of the PCR by transferring the tube rack to the following water baths for the indicated times:

Process/Water Bath	Temperature	Time
Denature	95°C	4 minutes
Anneal Primers	45°C	1 minute
DNA Synthesis	75°C	3 minutes

9. After completion of this first cycle, perform at least 20 additional cycles using the conditions listed below.

Process/Water Bath	Temperature	Time
Denature	95°C	1 minutes
Anneal Primers	45°C	1 minute
DNA Synthesis	75°C	3 minutes

10. Retrieve your tube from the tube rack after the last cycle. Remove 15µl of the PCR mixture and transfer the mixture to a 0.5ml tube that is labeled #3. Make sure your pipet tip is in the PCR mixture the mineral oil prior to this transfer.

11. Add 5µl of electrophoresis sample buffer to tube #3 and place the tube in the freezer until the next laboratory session.

Part B: Electrophoresis

1. Prepare 1.2% agarose gels as described in Part A (pages 15-17).

2. Load 15µl of the following samples into the sample wells of your agarose gel.

Sample Well		
1	Tube#2	
2	Tube#3	Group I
3	DNA markers	
4		
5	Tube#2	
6	Tube#3	Group 2
7	DNA markers	
8		

3. Electrophorese until the bromophenol blue has migrated to within 1-2mm of the positive end of the gel. At 170 volts. this should take about one hour.

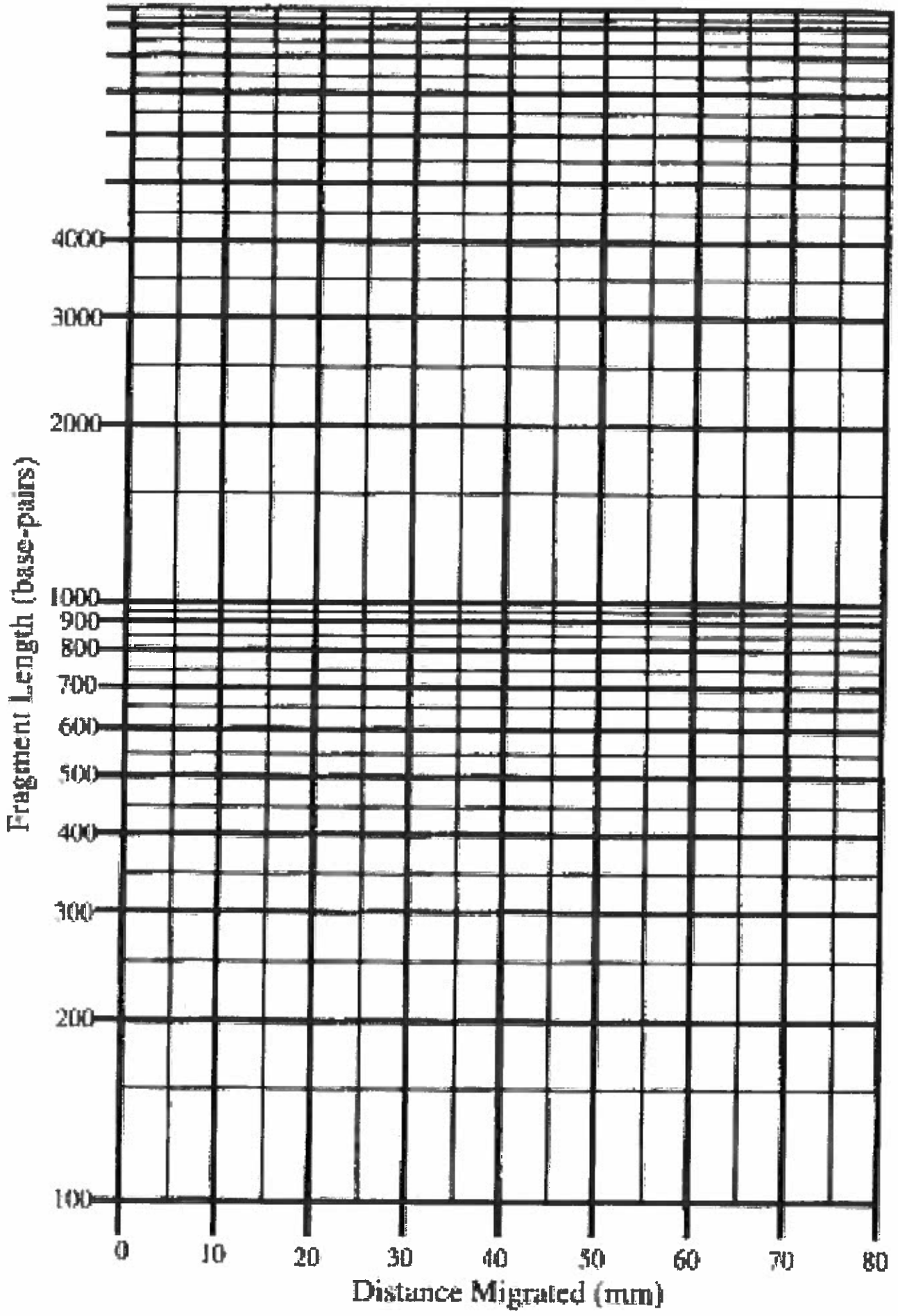
4. Stain the gels overnight with methylene blue as described in Part A (page 18).

Data Analysis and Study Questions

1. On the semilog paper provided on the following page, plot the electrophoretic mobilities of the DNA markers (lanes 3 and 7) as a function of their lengths. The lengths of these standards are 3621, 2040, 1120, and 784 base-pairs. Determine the length of the DNA band that was produced during the PCR (lanes 2 and 6). This band is the β globin gene from rabbit.

2. The rabbit β globin gene has a single BamH1 site that is located 200bp from the left (5') end of the gene. What would be the length of the DNA fragments produced if your PCR product was digested by BamH1.

3. β Thalassemia is a disease that is most often caused by a deletion of all or part of the β globin gene. Describe a PCR procedure that you would use to determine if a patient is suffering from β thalassemia.


Fragment Length (base-pairs)
4000
3000
2000
1000
900
800
700
600
500
400
300
200
100
0
10
20
30
40
50
60
70
80
Distance Migrated (mm)