Exercise 16
APPLICATION OF THE POLYMERASE CHAIN REACTION IN BACTERIAL IDENTIFICATION

Introduction

The **Polymerase Chain Reaction** (**PCR**), as conceived by **Kary Mullis** and his coworkers at Cetus Corporation (Emeryville, California) in **1986**, is a powerful diagnostic tool with multiple applications in genetic and molecular analysis. The procedure can be applied to the identification of unknown genes or microorganisms from a variety of samples and has revolutionized nucleotide sequencing. The polymerase chain reaction allows specific segments of DNA to be amplified (replicated over and over again) by utilizing some characteristic features of DNA and its replication process as follows:

1. The two nucleotide strands of a cellular DNA molecule (DNA double helix) are **complementary** to one another and **antiparallel.**
2. The **complementary** base pairs of a DNA double helix are held together by relatively weak **hydrogen bonds**, and can be induced to separate. Within cells, this involves enzymes, but can be accomplished in vitro by the application of heat.
3. During DNA replication, the enzyme complex known as **DNA-dependent DNA polymerase** uses the nucleotide sequence of an existing DNA strand as the **template** or pattern for building each new strand. This enzyme builds DNA by catalyzing the formation of **phosphodiester bonds** that attach nucleotides to the free 3' ends of existing nucleotide strands (i.e., builds from 5' to 3' and requires a primer).
4. Within cells, **primase** enzymes (**DNA-dependent RNA polymerases**) build the RNA primers required for replication. In vitro, single-stranded oligonucleotide sequences that are complementary to specific regions of DNA can hybridize with these (anneal to them) and can serve as primer sequences (providing a DNA duplex and free 3' ends) required for DNA-dependent DNA polymerase.
5. The energy required for DNA synthesis (phosphodiester bond formation) is provided by **nucleoside triphosphates** (**dNTPs**) and these can be supplied for in vitro replication.

The polymerase chain reaction can be initiated in vitro by mixing template DNA, forward and reverse primers, DNA polymerase, and dNTPs with reaction buffer in a thin-walled tube, and then modulating the temperature to initiate alternate cycles to denature the double helix, allow the primers to anneal and then promote extension of new nucleotide strands. A brief summary and animated version of the PCR can be observed by visiting **Principle of the PCR** (http://users.ugent.be/~avierstr/principles/pcr.html).

The **primers** used for the polymerase chain reaction are **oligonucleotide sequences** (single strands of DNA) usually around 18-20 nucleotides in length. Since nucleic acid sequences of this length can be artificially synthesized in vitro, it is possible to design and construct primers for a wide variety of genes, and numerous commercial sources are available. Primers are typically designed to contain around 50% C-G (give or take 15%) and should not contain complementary sequences rich in C or G at the ends (to avoid the formation of primer dimers). There must be a forward primer and a reverse primer designed to **anneal** (hybridize) with opposite ends of the DNA sequence to be amplified. These form short regions of DNA duplex and free 3’ ends (both required in order for DNA polymerase to bind the DNA and add nucleotides by forming phosphodiester bonds). The composition and length of the primers used will influence the temperature required for the annealing step in the PCR process. Primer pairs should be designed to have similar **melting temperatures** (T_m), i.e., temperature at which they will release the template DNA strand. A rough estimation of melting temperature can be calculated using the formula $T_m = 4(G+C) + 2(A+T)$ in degrees C. The anneal temperature is then typically set at 5^o C below the value determined.

Since the PCR involves alternately heating and cooling the preparation, the enzymes used to catalyze the building process must be able to remain functional after being subjected to high temperatures, i.e., must be **thermostable**. Although a variety of enzymes meet this requirement, ***Taq* polymerase** is the type most commonly used for standard PCR protocols. *Taq* polymerase (discovered in 1988) is DNA polymerase made by **hyperthermophilic** bacteria identified as ***Thermus aquaticus*** that live naturally in certain hot springs within Yellowstone National Park. Since these bacteria evolved in springs with water temperatures near boiling, their enzymes are able to function at high temperatures.

In a typical PCR protocol, a sample of **template DNA** is mixed with a buffer solution containing dNTPs, DNA polymerase, magnesium (a cofactor) and the primers necessary for amplification. The mixture is then placed into a **thermal cycler (aka, thermocycler)**, a device that can be programmed to alternately raise and lower temperatures to facilitate the reaction. High temperatures cause the two strands of template DNA molecules to separate (be **denatured**), cooling allows primers to **anneal** (hybridize), and then warming the sample to around 72^{o} C allows *Taq* polymerase to build new, complementary DNA strands (**extension**). By repeating this process over and over again, it is possible to amplify a single strand of DNA millions of times within a few hours.

In order to apply the PCR to the identification of bacteria isolated from random environmental samples, it is useful to amplify regions of DNA that are common to all organisms and that contain highly conserved regions, i.e., nucleotide sequences (base sequences) that have remained stable over time. Regions of DNA (genes) that encode ribosomal-RNA (rRNA) exhibit these features. All known cellular organisms contain ribosomes, structures essential for protein synthesis, and consequently all of these organisms contain rRNA. A bacterial ribosome (70S) contains one molecule each of three types of RNA identified as 23S, 16S and 5S (S=Svedberg unit – sedimentation coefficient). Since the function of ribosomes within cells has apparently remained constant over time, the nucleotide sequences of ribosomal-RNA molecules tend to be highly conserved. Certain regions of the16S rRNA have remained extremely highly conserved during evolution, so provide ideal material for identification and classification. Sequence homology studies involving 16S rRNA or the DNA encoding it (16S ribosomal DNA) have been used extensively to determine the evolutionary relationships between bacteria and archaea. Much of the data associated with these studies can be accessed, compared and used in bacterial identification.

For this exercise, we will use oligonucleotide primers identified as **Bacteria 8 forward** (5'-AGAGTTT GATCCTGGCTCAG-3') and **Enteric 1530 reverse** (5'-AGGAGGTGATCCAACCGCA-3'), to amplify a 1530 bp region of the bacterial chromosome known to encode 16S ribosomal RNA, i.e., **16S ribosomal DNA**. The thermal cycler will be set to complete 35 cycles preceded by a 4-minute denaturation at 94^{o} C. Each cycle will include 45 seconds at 55^{o} C (to anneal), 2 minutes at 72^{o} C (to extend), and 30 seconds at 94^{o} C (to denature). During the final cycle, the extension time will be lengthened to 20 minutes and the denaturing step will be omitted. Samples of DNA amplified by this method (**amplicons**), will be used to identify the source bacteria through nucleotide sequencing and RFLP analysis during later exercises.

Procedure:

1. Obtain a 400mL beaker (one per table), fill it about half full with water and set it on a ring stand above a lit Bunsen-Burner to establish a boiling water bath.

2. Follow the instructions given in class to obtain a slant culture of bacteria. This culture will serve as the source of template DNA for this exercise, and will also be assigned to you as Physiological Unknown #2 (PUNK2). **Be sure to record the number of this culture.**

3. Obtain a clean, sterile 1.5mL tube containing 500µL of Tris buffer. Record the number of your PUNK2 culture on the cap surface with a glass marker (do not use tape).

4. Examine your bacterial culture to verify its purity and using a clean, sterile loop, pick up a mass of cells about 2mm in diameter. If your loop is large, you can obtain the appropriate cell volume by gently touching the culture surface. Do not pick up excess cellular material, as this will inhibit the PCR.

5. Transfer the bacteria to the tube of Tris buffer and remove them from the loop. Gently rolling your loop handle between your thumb and forefinger will help to remove the cells from the wire.

6. If your culture contains Gram-positive cells, aseptically add 15-20 sterile glass beads to the centrifuge tube. If your culture is Gram-negative, this step is **not** required.

7. Close the tube securely and briefly vortex-mix the sample to make certain the bacteria are evenly distributed in the buffer solution. The liquid in your tube should appear slightly cloudy, but should not appear opaque (milky).

8. Place a cap lock on your tube and place it in a foam float such that the cap is pressed against the upper surface and the bottom extends below the lower surface of the foam.

9. When the water in the beaker is boiling gently, place the floating tubes (caps up) on the water surface. Allow the tube contents to be exposed to the boiling water for 10 minutes. This will release DNA from the cells and will also denature proteases and nucleases that might interfere with the PCR. **Caution** – Excessive agitation caused by a full, rolling boil may cause the float to tip over and the tubes to spill out. If the water is boiling too vigorously, turn down the heat.

10. After 10 minutes, use a pair of forceps to remove the float from the boiling water and then allow the tubes to rest on the lab bench until they are cool enough to handle. Remove the cooled tubes from the float and place them in the ice bucket provided.

11. If your culture contains Gram-positive cells, tape the tube to the surface of a vortex mixer (platform head) and subject the contents to maximum vibration for 10 minutes. The glass beads present will insure the Gram-positive cell walls are broken, releasing the DNA.

12. Obtain a 0.2mL PCR tube marked (top surface) with the number matching that of your bacterial culture (Physiological Unknown #2). **You will set up your reaction mixture in this tube.**

13. Using the appropriate pipettes (10-100µL = yellow top, or 0.5-10µL = white top), and tips (yellow or white), transfer specified quantities of the reagents provided to set up a Polymerase Chain Reaction mixture as indicated below. Place the *Taq* DNA Polymerase Master Mix in the tube bottom first. Add each additional reagent in the order indicated, as separate droplets on the inside wall of the tube (so they are visible and quantities can be compared).

    25µL Taq DNA Polymerase Master Mix (Qiagen)
    5.0µL primer mix (5µM each forward and reverse primer)
    5.0µL template DNA (boiled cell suspension as prepared above)
    15µL sterile, distilled water pH 8.0
    50µL total volume

Important note – Be careful to keep all reagents and your PCR tubes **on ice** when they are not in use. Do not allow any of these materials to rest on the counter top, in wood blocks, or in tube racks.

14. If your group is asked to set up control reactions, complete the following: In one tube add all components of the reaction mixture except the template DNA. Label this tube negative control. To a second tube add all components of the reaction mixture plus a known DNA sample. Label this tube positive control. **Note** – Only one control set is required for each thermal cycler, so not everyone will be asked to complete this step.

15. When you have completed the preparation of your PCR mixture, gently tap the tube to move all components to the tube bottom and then place your tube in the shaved-ice container provided.

Note – Each thermal cycler must be set to run the appropriate program, and all tubes must be loaded together (in their appropriate positions) into the heated block. Coordination of this procedure is essential for successful completion of the PCR. The amplification process will require about 3 hours, so cannot be completed within the regularly scheduled laboratory period. The instructor will remove your DNA samples from the thermal cycler and make arrangements for their storage.

Stop point – Amplified DNA samples will be frozen until the next lab period.

Questions:

1. What is the PCR and what is it being used for in this exercise?

2. What are oligonucleotide primers, and why are they necessary in the PCR, i.e., what function do they serve?

3. What is *Taq* polymerase and where does it come from?

4. Why must the DNA polymerase used in PCRs be thermostable?

5. When the polymerase chain reaction is used to amplify DNA in vitro, the components of the reaction mixture are subjected to three different temperature settings during each reaction cycle. These temperatures cause DNA to be denatured, to anneal and then to extend. Explain what is occurring during each of these steps.

6. Why is amplification of 16S ribosomal DNA likely to yield a product that can be used in the identification/classification of unknown bacteria?