**EXERCISE 7**

**AMPLIFICATION OF DNA BY POLYMERASE CHAIN REACTION (PCR)**

**AND ANALYSIS OF PCR PRODUCTS**

**Introduction**

**Overview of PCR**

Polymerase chain reaction (PCR) is a technique widely used in molecular biology, microbiology, genetics, diagnostics, clinical laboratories, forensic science, environmental science, hereditary studies, paternity testing, and many other applications. The name, polymerase chain reaction, comes from the DNA polymerase used to amplify (replicate many times) a piece of DNA by *in vitro* enzymatic replication. The original molecule or molecules of DNA are replicated by the DNA polymerase enzyme, thus doubling the number of DNA molecules. Then each of these molecules is replicated in a second "cycle" of replication, resulting in four times the number of the original molecules. Again, each of these molecules is replicated in a third cycle of replication. This process is known as a "chain reaction" in which the original DNA template is exponentially amplified. With PCR it is possible to amplify a single piece of DNA over many cycles, generating and extraordinary number of copies of the original DNA molecule. For example, 10 cycles can produce 1000 copies, 20 cycles over 1 million, 30 cycles about 1 billion and 40 cycles more than 1 trillion. PCR has been extensively modified to perform a wide array of genetic manipulations, diagnostic tests and other functions.

The polymerase chain reaction serves to copy DNA. The reaction solution contains DNA molecules (to be copied), DNA polymerase (which catalyzes copying of the DNA), primers (these are oligonucleotides (often about 20 base pairs in length) that are complementary to DNA sequences to be amplified) and nucleotides (which are attached to the primers and further added to extend the DNA sequence. This process uses repeated cycles, each of which consists of three steps (see Fig. 1 below).

1. Denaturation (melting): The solution is first heated to about 95°. This causes the two complementary strands to separate, a process known as denaturing or melting.

2. Hybridization (annealing): The temperature is lowered (in the 50°-60° range) allowing the primers to bind to the DNA. The resulting bonds are stable only if the primer and DNA segment are complementary, i.e. if the base pairs of the primer and DNA segment match in a complementary way.

3. Extension: The temperature is again increased, this time to 72°C. This is the ideal working temperature for the DNA polymerases which catalyze addition of further nucleotides to the developing DNA strand. At the same time, any loose bonds that have formed between the primers and DNA segments that are not fully complementary are broken. Each time these three steps are repeated the number of copied DNA molecules doubles. As noted above, after 20 cycles more than a million molecules are generated from a single segment of double-stranded DNA. The temperatures and duration of the individual steps described above refer to the most commonly used protocol. Modifications are often made to give better results to meet specific requirements.

PCR is carried out in an instrument called a thermal cycler which can be programmed to change temperatures as described above in a sequential fashion.

In this laboratory exercise, we will amplify and analyze a portion of the Zip1 gene from the legume plant, *Medicago truncatula*. In one case we will amplify a portion of genomic DNA that includes an intron. In another, we will amplify a cDNA gene region that has been cloned into a plasmid. Since cDNA is copied from mRNA the intron should be removed during RNA splicing. Thus, the PCR amplification product of genomic DNA should be larger than the PCR product of cloned DNA gene region.

Materials and Methods

**Materials**

*Template DNA*

PCR requires template DNA. *M. truncatula* genomic DNA and cloned cDNA will be used in this laboratory; DNA will be provided the instructor.

*Primers*

PCR requires both a forward and a reverse primer. The primers sequences to be used in this study are shown below. A primer mix was prepared by the instructor (see below for details of preparation.

MtZIP1 F: CTCTTACTACTACCTTAAAGCTT.

MtZIP1 B; CAGTTCCTAAAGATATTCCA

*Qiagen HotStarTaq Master Mix:*

This is a commercially available premixed solution that contains HotStarTaq DNA Polymerase, PCR Buffer, and dNTPs that is provides at 2X concentration (see below for further detail).

**Methods**

*PCR protocol*

1. Defrost DNA to be amplified.
2. Obtain 3 individual PCR tubes.
3. Put tube on ice and to each tube add the following:
   1. DNA minus control:
      1. 10 L of HotStart Qiagen Readymix Taq (or other readymix taq)
      2. 8 L HPLC water
      3. 2 L diluted primer mix (see below for preparation)
   2. PCR with genomic DNA template
      1. 10 L of HotStart Qiagen Readymix Taq (or other readymix taq)
      2. 6 L HPLC water
      3. 2 L diluted primer mix
      4. 2 L *M. truncatula* genomic DNA
   3. PCR with Zip 1 clone DNA template
      1. 10 L of HotStart Qiagen Readymix Taq (or other readymix taq)
      2. 6 L HPLC water
      3. 2 L diluted primer mix
      4. 2 L *M. truncatula* genomic DNA
4. Centrifuge tubes for 5 seconds to mix components.
5. Place sample tubes in the thermal cycler and carry out PCR amplification (for each experiment the thermal cycler has been programmed for appropriate conditions). The following protocol has been programmed:
6. PCR Thermal Cycler Program
   1. Step 1: 10 min. 95°C (Hot start)
   2. Step 2: 1 min. 95°C
   3. Step 3: 1 min. 53°C
   4. Step 4: 1 min. 72°C
   5. Step 5: Go to Step 2 34x
   6. Step 6: 5 min. 72°C
   7. Step 7: hold 10°C
7. After amplification store samples at -20o.

*Preparation of Agarose Gel*

Set the gel casting tray on a level surface and insert the running plate. Before pouring the gel, insert the comb into the slot of the casting tray. There should be a space of about 2 mm between the bottom of the comb teeth and the surface of the running plate. Prepare 0.8% high melting point agarose in a 500 mL flask. Add 0.64 g of powdered agarose to 80 mL of 0.5 TBE buffer. Heat for 1 to 2 minutes in microwave; allow agarose solution comes to a boil for about 20 seconds then remove from the microwave. Put on protective gloves. Then, add 2L ethidium bromide (from a 10 mg/mL stock) to give a final concentration of 0.25 µg/mL Allow the solution to cool to about 45o-50o; do not pour agarose at temperatures above 50o or you risk warping the casting or running trays. Pour the agarose into the casting tray on top of the running plate. The agarose solution will flow around the teeth of the comb Allow the agarose to gel for at least 1 hour. Remove the comb carefully by lifting it gently at one end, thus tilting the comb as it comes free. Pulling the comb straight up may create a vacuum in the wells, which tends to lift the entire gel and may tear the wells.

*Agarose Gel Electrophoresis*

Remove the running plate and gel from the casting tray and scrape off any gel adhering to the underside of the plate. Remember to wear gloves. Place the running tray with gel on the center platform of the submarine gel unit. Fill the gel unit with buffer until there is approximately a 2-3 mm layer of buffer over the entire surface of the gel. . Caution: Always wear gloves when using ethidium bromide it is a powerful intercalating mutagen and readily crosses cell membranes.

For each PCR tube, remove 2 µL of sample and mix with 2 µL of gel loading buffer. Load 3 µL of DNA standard in the first well using a micropipet (Pipetman P20). This is done by carefully placing the pipet tip below the buffer surface and just above the well. Since the gel loading buffer contains 50% glycerol, the samples are more dense than the running buffer and should sink into the wells. Be careful not to puncture the gel with the pipet tip. Next load, all (about 4 µL) of the PCR samples into the next 3 wells. Well will be the negative control, well 3 the PCR product of the cDNA clone amplification and well 4 the PCR product from amplification of genomic DNA.

Place the lid on the gel unit so that the cathode (black lead) is at the end nearest the sample wells; the negatively charged DNA will migrate toward the positive pole (red lead). Connect the cables to the power supply. Run at 150 volts for approximately 45 minutes. Caution: The power unit can generate high voltages; make sure that the lid is closed before turning on the power. At the end of the run, turn off the power and unplug the power unit. Carefully remove the lid from the unit and then lift up the running plate and gel. Some words of caution; remember to always wear gloves when handling solutions or gels containing ethidium bromide. Keep the plate horizontal, since if it is tipped, the gel may slide off the running plate and break.

Carefully transfer the running plant and gel to the transilluminator. Caution: Put on UV absorbing goggles at this point. Place a UV absorbing cover over the gel and turn on the transilluminator. DNA containing samples should be fluorescent and now be visible as orange bands. Your instructor will then take a digital photograph of the gel as a permanent record.

**Results and Discussion**

The results section should include the photograph of the agarose gel labeled properly with a figure caption. Discuss the results of gel analysis of PCR products in the Discussion section. What were some of the problems encountered if any and how do the fragments compare to the banding of the DNA standard?

Appendix 7-1.

*Reagents and biological samples*

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|  | *Qiagen HotStarTaq Master Mix:* This is a commercially available premixed solution that contains HotStarTaq DNA Polymerase, PCR Buffer, and dNTPs. The solution is provides at 2x, and when diluted 1:2, gives a final concentration of 1.5 mM MgCl2 and 200 µM each dNTP as well as appropriate amount of DNA polymerase. |

What is a Master Mix? As noted above, PCR requires DNA polymerase, dNTPs, MgCl2 and buffer in addition to template DNA and sequence specific primers. When some or all of these components are mixed together (before placing in a PCR tube or plate) it is called a Master Mix. The master mix can be prepared by the researcher or obtained commercially. There are pros and cons for each. The researcher can alter the concentration of buffer, MgCl2 and dNTPs (in addition to DNA and primers) allowing optimization of reaction conditions. However, this requires several components to be added separately thus increasing the likelihood of error as well as taking more time. Using a commercially prepared taq master mix is simpler, quicker and minimizes error, but limits the ability to optimize reaction conditions.

What is Hot Start Taq? Taq is the name of thermally stable DNA polymerase that may be heated to 95~~o~~ to denature template DNA over many cycles with on modest lost of catalytic activity.Hot start Taq refers a DNA polymerase that must be activated by heating to 95o for a period of time before having catalytic activity. The advantage of this is that polymerase activity does not begin until DNA is heated to denaturizing temperatures this give greater specificity with less background from nonspecific PCR products including primer-dimers.

Different companies produce hot start enzymes based on different mechanisms. Many hot start systems employ polymerases mixture that also have antibodies that bind to and inhibit the enzyme until heated to 95o where the antibody is denatured. By contrast Qiagen HotStarTaq DNA Polymerase utilizes a chemically-mediated hot-start that, unlike antibody-mediated systems, leads to complete inactivation of the polymerase until the initial heat activation step.

*Preparation of primer stocks*

Most research groups routinely prepare primer stocks solutions at 100 M and then dilute the stocks to make a primer mix solution. Primers usually come from the manufacturer as undiluted dry samples. Both forward (usually marked F or alternatively as A) and reverse primers (marked R or B) are dilutee with TE buffer to give a final concentration of 100 M. The number of nmol of samples is always show and the vile containing the primer. For initial dilution, add 10 L of TE for each nmol of dried primer (for example, if the vial contains 6.8 nmol of primer add 68 L of TE vortex to dissolve). The stock solution for forward and reverse primers and stored separately (not mixed) at -20o. This was done by the instructor.

*Prepare diluted primer mixes for setting up PCR reactions*

The forward and reverse primers are then mixed together and diluted to give a working primer mix. In this case, add 5 L of primer F from stocks and 5 mL of primer B to 90 L HPLC water. The primer mix solution is then further diluted 1:10 for the PCR reactions (e.g. 1 L for each 10 L of final PCR reaction volume). Again, this was prepared by the instructor.

*Tris-Borate-EDTA (TBE) electrophoresis buffer*:

This buffer is usually made at a 5x concentrated stock and then diluted 1:5 before use. To prepare 5x stock the following are dissolved in 1 liter: 54 g Tris base, 27.5 boric acid (pH should be checked and adjusted if necessary), 3.8 g EDTA. The 1x buffer is comprised of the following: 89 mM Tris, 89 mM boric acid (pH 8), and 2 mM EDTA.

*Gel-loading buffer:*

50% glycerol, 100 mM EDTA, 1% SDS and 0.1% bromphenol blue.

*DNA storage buffer (TE buffer):*

10 m,M Tris-HCl (pH 8), and 1 mM EDTA.

*Ethidium bromide:*

10 mg/mL. Warning: Ethidium bromide is a powerful mutagen! Always wear gloves while handling solutions or gels that contain this dye.