RAMA UNIVERSITY

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FACULTY OF AGRICULTURE SCIENCE

ALLIED INDUSTRIES

(Principles of Biotechnology)

For

M.Sc. Ag (GPB)

Course Instructor

Dr Shiv Prakash Shrivastav

FASAI(Genetics and Plant Breeding)

Rama University, Kanpur

Polymerase Chain Reaction (PCR)

MATERIALS INCLUDED

This kit has enough materials and reagents for 24 students (six groups of four students).

- 3 bottles DNA Release Buffer
- 2 bottles Precipitation Solution
- 1 bottle DNA Salt Solution
- 1 vial Protease: Dry Protease
- 30 Cytology Brushes
- 120 Centrifuge Tubes (1.5ml)
- 1 vial PCR: 5' Genomic Primer
- 1 vial PCR: 3' Genomic Primer
- 1 vial 10X PCR Buffer (Mg^{2+} plus)
- 2 vials PCR: Deoxynucleotides (dNTPs)
- 1 vial *Taq* DNA polymerase
- 2 vials Sterile Water
- 2 vials PCR: Mineral Oil
- 24 PCR: PCR tubes

SPECIAL HANDLING INSTRUCTIONS

- Store 5' and 3' Genomic Primers, *Taq* Reaction Buffer, dNTPs, and *Taq* DNA polymerase at -20°C
- All other reagents can be stored at room temperature.

ADDITIONAL EQUIPMENT REQUIRED

- 15ml Centrifuge Tube
- Waterbath or beaker and thermometer
- PCR Machine (Thermocycler)
- Agarose Electrophoresis Equipment

TIME REQUIRED

- **Day 1**: 3 hours
- **Day 2**: 2-3 hours

OBJECTIVES

- Isolate your own DNA genome.
- Amplify a specific gene from your genome.
- Understand the principles of the polymerase chain reaction.

BACKGROUND

The Polymerase chain reaction (PCR), first envisaged in 1984 by Kary Mullis, has revolutionized life sciences and has become an essential technique in many aspects of science, including clinical diagnostics, forensics and genetic engineering. Kary Mullis eventually received the Nobel Prize in Chemistry in 1993.

PCR allows scientist to make unlimited copies of DNA fragments and genes from a single copy of initial DNA. Each cycle of the polymerase chain reaction doubles the number of copies of the gene of interest, so for this experiment, which has 33 cycles, over 17 billion copies of your gene of interest will be made for each starting template (see figure 1).

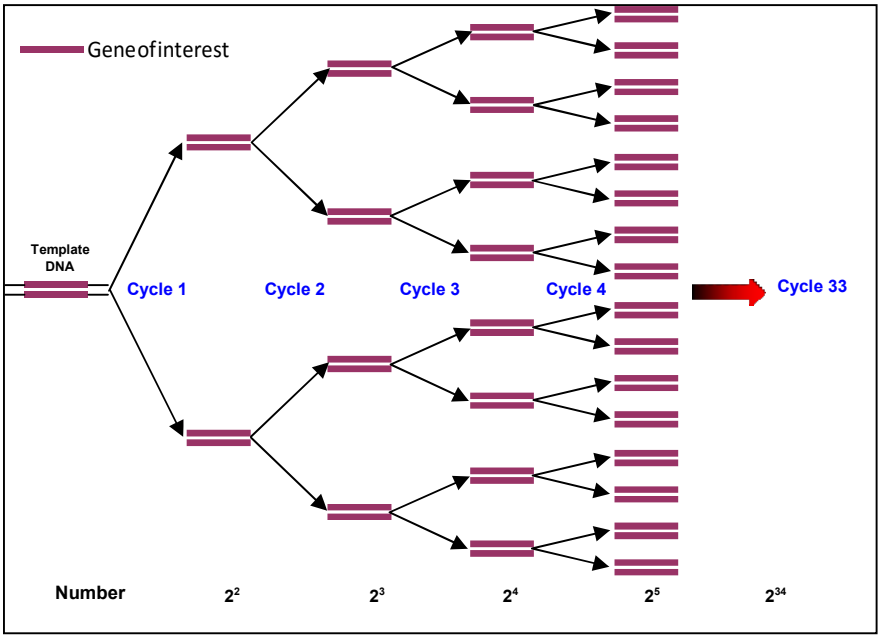


Figure 1: The exponential copying of a gene of interest during the polymerase chain reaction.

PCR utilizes the natural function of polymerase enzymes. In a normal dividing cell, the copying of the genes requires a series of enzyme mediated reactions:

1. The DNA strands are unwound (denatured) by enzymes to form two single strands.

2. A RNA polymerase binds and synthesizes a short complementary piece of RNA on the DNA strand at the initiation site of replication.

3. This DNA/RNA heteroduplex acts as a priming site for the DNA polymerase that binds and produces the complementary strand.

The key to the polymerase chain reaction was first discovered in 1976. The key is the *Taq* polymerase that was purified from the thermophile *Thermus aquaticus*. A thermophile is an organism that grows at extreme temperature (>100°C). The importance of the *Taq* polymerase being purified from a thermophile is that the enzyme will not be destroyed at high temperatures required to denature the DNA and allow PCR to begin.

A schematic of the PCR reaction is shown in figure 2 and a representation of the critical temperature cycles is shown in the graph in figure 3.

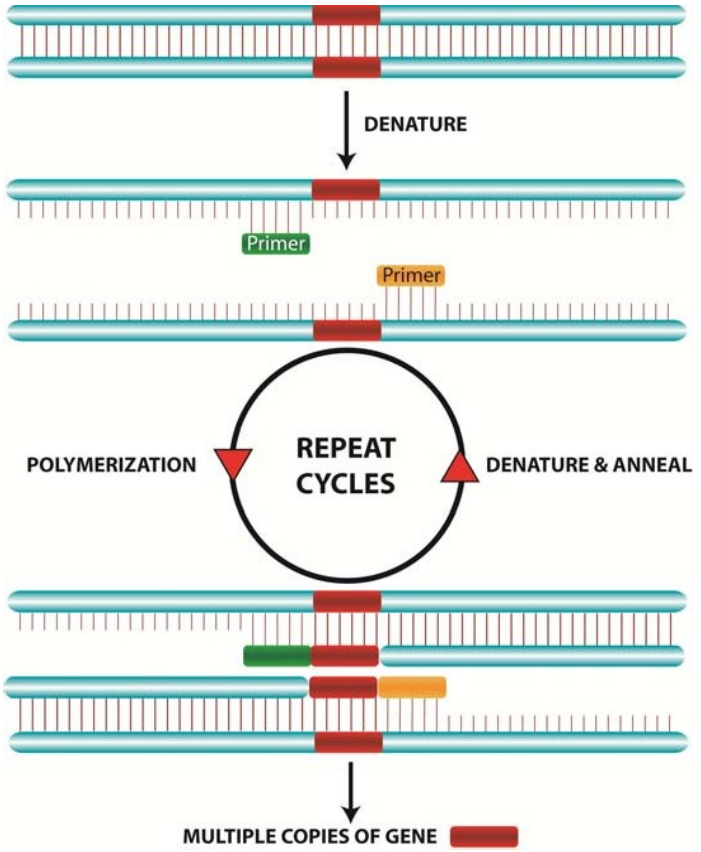
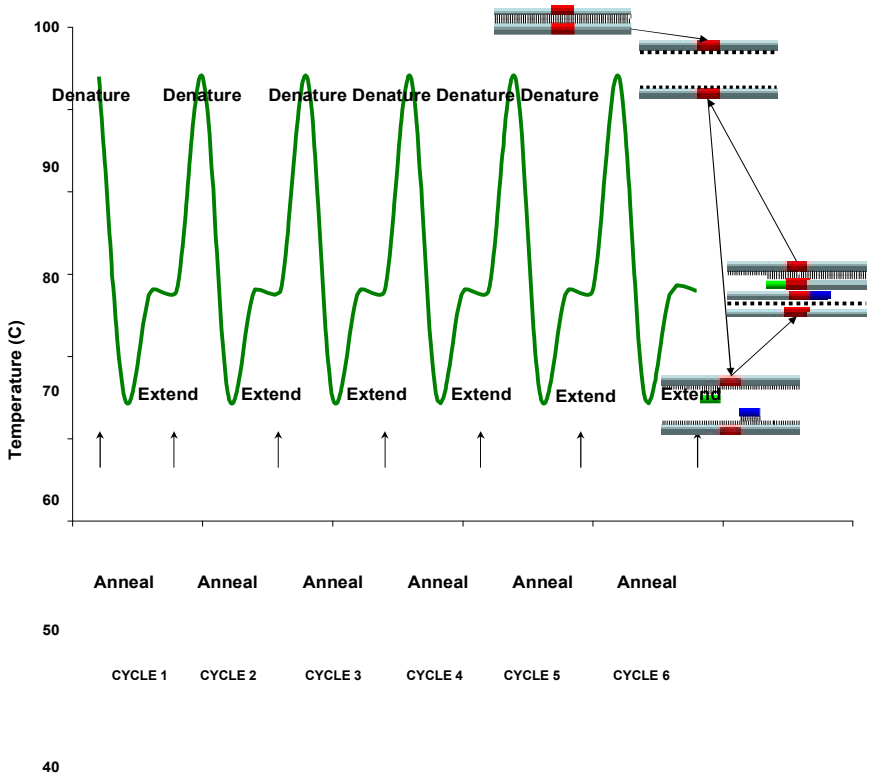


Figure 2: Schematic representation of the Polymerase Chain Reaction

There are three basic steps in PCR (Figure 2). First, the template DNA or genetic material is denatured; the strands of its helix are unwound and separated-by heating to 90-96 ° C. In a normal cell the DNA is unwound by specific enzymes.

The second step is hybridization or annealing. The *Taq* polymerase requires a short piece of RNA to initiate DNA replication, which in a normal cell is synthesized by the RNA polymerase. In the PCR reaction, short complimentary double stranded oligos are added that bind the denatured DNA and act as origins of replications. These double stranded oligos are known as primers and are complimentary to sequences up and down stream of the gene of interest. Two primers are used, one for each strand of DNA. Following denaturation, the reaction mixture is rapidly cooled to a temperature below the melting point of the specific primers (~55°C), below this temperature the primers bind to their complementary bases on the now single stranded DNA.

In the third step, the temperature of the reaction is raised to the optimal temperature for the polymerase (68-72°C). The polymerase synthesizes new DNA, starting from the primer, the polymerase reads a template strand and generates complementary nucleotides very quickly. The result is two new helixes in place of the first, each composed of one of the original strands plus its newly assembled complementary strand.



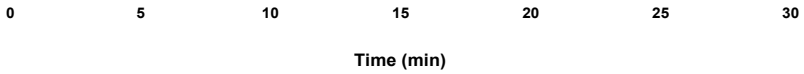


Figure 3: The temperature cycles used during the PCR reaction. The graph depicts the changes in temperature and the resulting effect on the DNA. A schematic of these effects is shown to the right of the graph.

The polymerase chain reaction is able to produce large copies of the genes of interest as the above cycle can be repeated numerous times leading to an exponential increase in the number of new copies (figure1).

The thermocycler is the most important piece of technology for researchers wanting to use PCR. A thermocycler tightly regulates the temperature changes required for denaturation, annealing and extension. It also controls the number of cycles. Today's thermocyclers are fully programmable and allow for rapid heating and cooling and therefore tighter control of the PCR.

In this experiment, students will amplify a nucleotide sequence from chromosome 16 to look for the insertion of a short DNA sequence called Alu within the PV92 locus. DNA from different individuals contain many regions that exhibit a great deal of diversity and these regions are known as polymorphic (many forms) and provide the basis for genetic disease diagnosis, forensic identification and paternity testing*.

The Alu family of short interspersed repeated DNA elements (or SINEs) are distributed throughout primate genomes and over the past 65 million years the Alu sequence has been amplified to a copy number of about 500,000 compromising an estimated 5% of the human genome. The Alu elements are approximately 300bp in length and derive their name from a single recognition site of the AluI endonuclease located in the middle of the Alu sequence. In this experiment, the Alu element being amplified is dimorphic, meaning that it is present in some individuals but not others (Figure 4).

This kit is designed to teach the basics of the polymerase chain reaction and allows students to amplify a gene from their own genome.

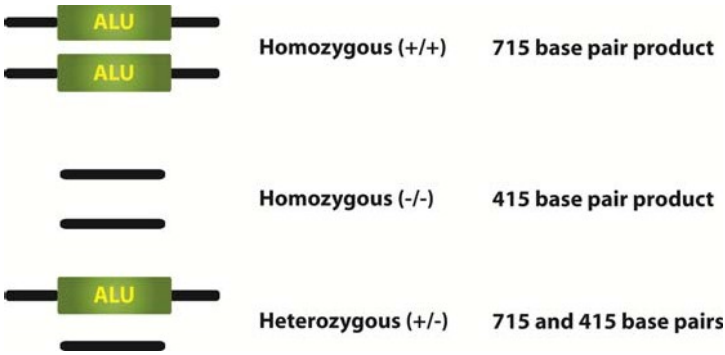


Figure 4: Possible distribution of Alu insert in PV92 locus and PCR fragment sizes.

**The Alu alleles are inherited from parents and can potentially reveal information about family relationships and as a result we caution against generating data from multiple family members. If this is a concern, we recommend mixing up the students samples after the cells have been collected to ensure anonymity.*

TEACHER'S PRE EXPERIMENT SET UP

Genomic Isolation

1. Prior to the commencement of the experiment, add 0.5ml DNA Release Buffer to the vial of dry protease to rehydrate. Mix by inverting the vial several times until a clear solution is visible. This solution can be stored frozen for later use.

Instruct students to stop eating and drinking at least ***one hour*** *before experimentation. This helps prevent loose cells being washed away.*

2. Prepare the DNA wash solution. Label a 15ml tube with "DNA Wash". Transfer 10.5ml Precipitation Solution and 4.5ml ultra pure water to the labeled tube. Invert 5-6 times to mix.

3. Prepare a waterbath or heating block at 50-60°C. Lower temperatures can be used, down to room temperature, however longer digestion times will be required.

4. Tube racks or floats are also required.

Prepare PCR reagents

1. Store all reagents on ice throughout the experiment.

All components used in the polymerase chain reaction should be kept on ice. The students' experiments should all be carried out on ice.

2. Transfer 150μl Sterile Water to the 5' Genomic Primer tube. Resuspend the primer by gently pipetting up and down.

3. Label six tubes with "5' Primer". Transfer 25μl 5' Genomic Primer from step 2 to each tube. Supply each group with a single tube.

4. Transfer 150μl Sterile Water to the 3' Genomic Primer tube. Resuspend the primer by gently pipetting up and down.

5. Label six tubes with "3' Primer". Transfer 25μl 3' Genomic Primer from step 4 to each tube. Supply each group with a single tube.

6. Label six tubes with "*Taq* Buffer". Transfer 40μl 10X PCR Buffer (Mg^{2+} plus) to each tube. Supply each group with a single tube.

7. Transfer 126μl Sterile Water to each vial of dNTPs. Resuspend the dNTPs by gently pipetting up and down.

8. Label six tubes with "dNTP". Transfer 40µl deoxynucleotides to each tube. Supply each group with a single tube.

9. Label six tubes with "Taq". Add 45µl Sterile Water to the vial of Taq DNA Polymerase. Transfer 10µl *Taq* DNA polymerase to each tube. Supply each group with a single tube.

10. Label six tubes with "H_2O". Transfer 0.5ml Sterile Water to each tube. Supply each group with a single tube.

Program Thermocycler

NOTE: This kit is designed so that each student sets up their own PCR reaction, a total of 24. If your thermocycler is unable to accommodate 24 samples then either allow every student to set up the reaction and run a selection or have students work in pairs generating 12 samples. Adjust the volumes aliquoted accordingly.

1. Follow the manufacturer's instructions for your thermocycler. If the thermocycler has a heated lid then the mineral oil is not required. You will need enough space for the number of students in the class (maximum 24).

2. Program the following program:

   A. 1 Cycle of 96°C for 2 minutes.
   B. 33 Cycles 94°C for 1 min, 55°C for 1 min, 68°C for 2 min.
   C. 1 Cycle 68°C for 7mins.
   D. 1 Cycle of 4°C forever.

Visualization of PCR products

1. In order to visualize the PCR products a 2% agarose gel will need to be run. Each student requires 1 well and additional wells are required for reference markers. You may use your own equipment and supplies or use G-Biosciences "Introduction to Agarose Electrophoresis" kit (Cat. # BE-304).

MATERIALS FOR EACH GROUP

Supply each group with the following components. Several components are shared by the whole class and should be kept on a communal table.

- 3 bottles DNA Release Buffer (shared with class)
- 1 vial protease (shared with class)
- 1 bottle DNA Wash (shared with class)
- 1 bottle Precipitation Solution (shared with class)
- 1 bottle DNA Salt Solution (shared with class)
- 4 Cytology Brushes
- 8 centrifuge tubes (1.5ml)
- 1 vial 5' Primer
- 1 vial 3' Primer
- 1 vial *Taq* Reaction Buffer
- 1 vial deoxynucleotides (dNTPs)
- 1 vial *Taq* DNA polymerase
- 1 vial Sterile Water
- 1 vial mineral oil (shared with class)
- 4 PCR tubes

PROCEDURE

Isolation of genomic DNA

1. Label two 1.5ml Centrifuge Tubes with your name.

2. Add 0.4ml DNA Release Buffer to a labeled 1.5ml Tube. This solution contains a detergent that disrupts the cell structure and releases the genomic DNA into the solution.

3. **Collect Cheek Cells**: Use the cytology brush to collect your cheek cells by scraping the inside of your cheek. Scrape the inside of each cheek; work on the area around the gum line 20 times, whilst twirling the brush between your fingers.

 *Instruct students not too scrape too vigorously. The best place for collecting the cells is at the gum line.*

4. Place the brush into the DNA Release Buffer and leave in the solution for 1 minute periodically twirling the brush.

5. Remove the brush from the solution, ensuring that you thoroughly scrape the brush on the side of the tube, releasing the cheek cells into the DNA Release Buffer.

 *The solution should be slightly cloudy and viscous.*

6. Add 0.02ml Protease to the tube to digest and remove the cellular material and protein from the genomic DNA.

7. Close the cap. Briefly mix by inverting the tube 5-6 times and then place in a 50-60°C waterbath or heating block for 20-60mins.

8. After 1 hour, add 90µl DNA Salt Solution to the tube and mix by inverting the tube several times. The salt solution aids in the precipitation of the DNA.

9. Centrifuge the tube for 5 minutes at 10,000xg to pellet the cell debris. Transfer the supernatant to your other labeled tube.

10. Slowly, add 0.95ml Precipitation Solution, close the tube and, whilst watching, slowly invert the tube several times to mix. White DNA strands may appear. *The lack of white strands does not indicate a lack of DNA. Only a tiny amount of DNA is needed for PCR amplification.*

11. To collect the DNA centrifuge the tube at 10,000xg for 10 minutes. A tight white pellet should be visualized in the bottom of the tube. Discard the supernatant and keep the pellet of DNA.
    The white pellet may be very hard to visualize. To aid in locating the pellet, make a note of the tube's orientation within the centrifuge.

12. Add 0.2ml DNA Wash to the pellet and centrifuge at 10,000xg for 10 minutes.
    The DNA Wash is a mixture of alcohol and water that allows for the removal of contaminating salts, without dissolving the DNA.

13. Remove all the DNA Wash with a pipette tip. Add 50μl Sterile Water to the DNA pellet. Incubate at room temperature for 10-15 minutes with periodic mixing.

If necessary, this is a convenient stopping point. Store the DNA at -20ºC in a freezer. The DNA is stable for up to a month.

14. *Optional: To visualize the genomic DNA, remove 10μl genomic DNA and visualize on a 1% agarose gel.*

Polymerase Chain Reaction

 *All the components of the PCR reaction and the setting up the reaction should be done on ice.*

1. Label the side of a PCR tube with your initials and place the tube on ice.

2. As a group prepare a "Master Mix" of the PCR reaction components. In a 2ml centrifuge tube, add the following components. Use a different pipette tip for each component. Pipette each component into the bottom of the tube. On addition of each component, gently mix by pipetting up and down.

    - 20μl 5' Genomic Primer
    - 20μl 3' Genomic Primer
    - 40μl *Taq* Reaction Buffer
    - 40μl dNTPs
    - 10μl *Taq* polymerase
    - 250μl Sterile Water

3. Transfer 5μl of your diluted genomic DNA (from step 13) to your labeled PCR tube.

4. Add 95μl "Master Mix" from step 2 to your PCR tube. Gently mix by pipetting up and down 4-5 times.

5. If using a thermocycler without a heated lid, add 50μl Mineral oil to prevent evaporation.

 *Inform your students if they require mineral oil.*

6. The Thermocycler should be programmed as follows

    - 1 Cycle of 96°C for 2 minutes
    - 33 Cycles 94°C for 1 min, 55°C for 1 min, 68°C for 2 min
    - 1 Cycle 68°C for 7mins
    - 1 Cycle of 4°C forever.

7. The following day, after the reaction has finished, remove the tubes and remove 30μl to be analyzed by agarose electrophoresis.

The PCR reaction can be stored at -20°C until required.

See pre-experiment set up for information on agarose electrophoresis. The PCR reaction should be run on a 2% agarose gel as the gene is 415 and or 715bp.

STOP the gel when the blue dye front is more than 3cm from the gel bottom. If run further the PCR product will be lost as it runs ahead of the dye front.

RESULTS, ANALYSIS & ASSESSMENT

1. What was the size of the PCR product that was visualized on the agarose gel?

   415bp, 715bp or both.

2. Explain your result below in terms of your Alu elements?

   If a single 415bp band was seen then the individual is homozygous negative, meaning they do not have the Alu element.

   If a single 715bp band was seen then the individual is homozygous positive, meaning they have the Alu element on both DNA strands.

   If both a 415 and 715bp band was seen then the individual is heterozygous, meaning that one DNA strand has the Alu element and the other does not.

3. Explain the various steps of the PCR program, explaining the relevance of the temperatures and times.

   The first long 96 ℃ cycle is to allow complete denaturation of the genomic DNA

   The 33 cycles are the main part of the PCR reaction and these consist of a short denaturation stage at 94 ℃, followed by an annealing step. The low temperature allows the primers to attach to the denatured DNA, higher temperatures may prevent annealing. The 68 ℃ temperature is an optimal temperature for the polymerase to synthesize new copies of the DNA. The longer times and increasing incubation times allow the polymerase to synthesize the complete gene. Longer genes would require longer times.

4. Discuss the role of each component in the reaction mixture.

   Primers: Bind to the denatured DNA and act as the origins of replication for the Taq polymerase to bind to.

   dNTPs: A source of nucleotides (adenosine, guanine, cytosine, thymidine) triphosphates (NTP) required for the generation of new DNA.

   Genomic DNA: Acts as the template for new gene synthesis. Taq

   polymerase: Crucial enzyme required for DNA synthesis.

Polymerase Chain Reaction (PCR)

OBJECTIVES

- Isolate your own DNA genome.
- Amplify a specific gene from your genome.
- Understand the principles of the polymerase chain reaction.

BACKGROUND

The Polymerase chain reaction (PCR), first envisaged in 1984 by Kary Mullis, has revolutionized life sciences and has become an essential technique in many aspects of science, including clinical diagnostics, forensics and genetic engineering. Kary Mullis eventually received the Nobel Prize in Chemistry in 1993.

PCR allows scientist to make unlimited copies of DNA fragments and genes from a single copy of initial DNA. Each cycle of the polymerase chain reaction doubles the number of copies of the gene of interest, so for this experiment, which has 33 cycles, over 17 billion copies of your gene of interest will be made for each starting template (see figure 1).

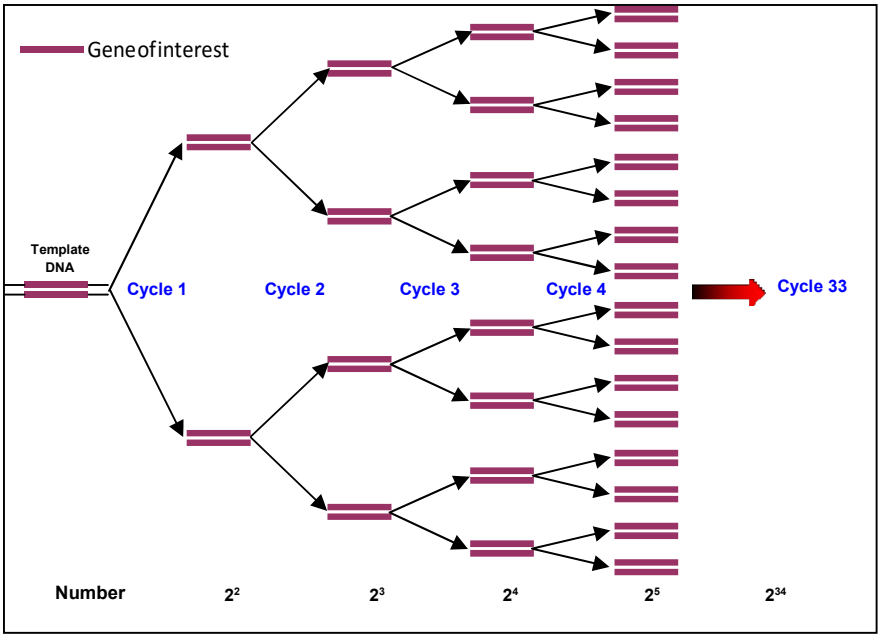


Figure 1: The exponential copying of a gene of interest during the polymerase chain reaction.

PCR utilizes the natural function of polymerase enzymes. In a normal dividing cell, the copying of the genes requires a series of enzyme mediated reactions:

1. The DNA strands are unwound (denatured) by enzymes to form two single strands.

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The key to the polymerase chain reaction was first discovered in 1976. The key is the *Taq* polymerase that was purified from the thermophile *Thermus aquaticus*. A thermophile is an organism that grows at extreme temperature (>100°C). The importance of the *Taq* polymerase being purified from a thermophile is that the enzyme will not be destroyed at high temperatures required to denature the DNA and allow PCR to begin.

A schematic of the PCR reaction is shown in figure 2 and a representation of the critical temperature cycles is shown in the graph in figure 3.

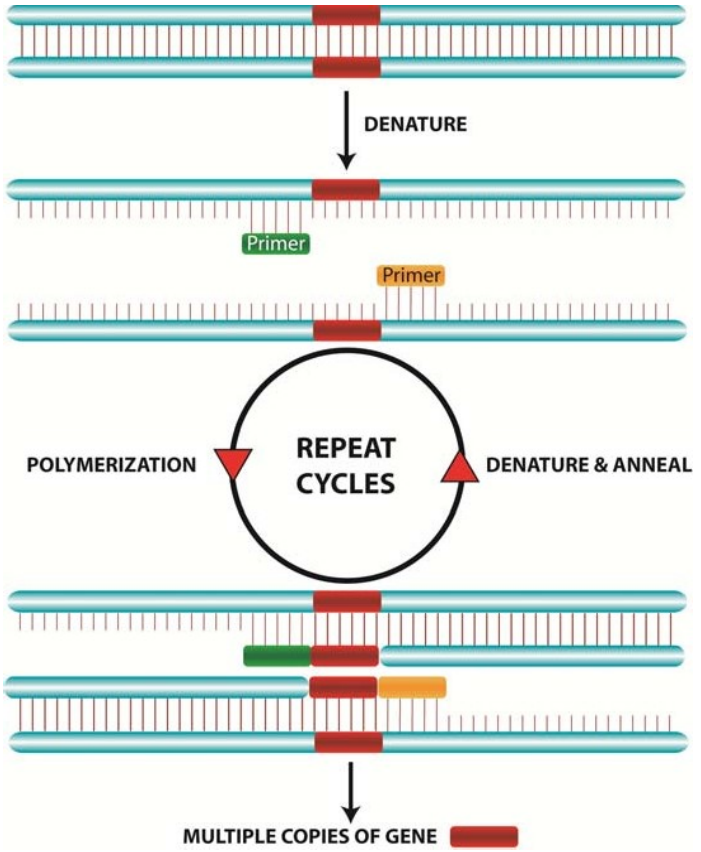

Figure 2: Schematic representation of the Polymerase Chain Reaction

There are three basic steps in PCR (Figure 2). First, the template DNA or genetic material is denatured; the strands of its helix are unwound and separated-by heating to 90-96 ° C. In a normal cell the DNA is unwound by specific enzymes.

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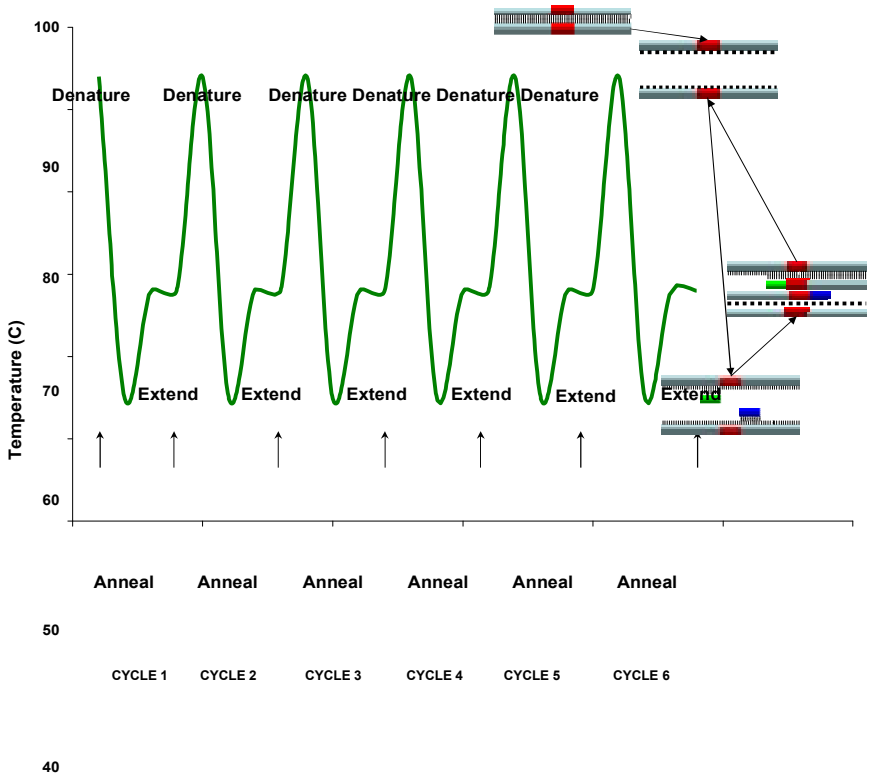

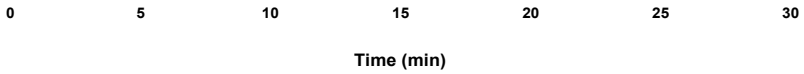

Figure 3: The temperature cycles used during the PCR reaction. The graph depicts the changes in temperature and the resulting effect on the DNA. A schematic of these effects is shown to the right of the graph.

The polymerase chain reaction is able to produce large copies of the genes of interest as the above cycle can be repeated numerous times leading to an exponential increase in the number of new copies (figure1).

The thermocycler is the most important piece of technology for researchers wanting to use PCR. A thermocycler tightly regulates the temperature changes required for denaturation, annealing and extension. It also controls the number of cycles. Today's thermocyclers are fully programmable and allow for rapid heating and cooling and therefore tighter control of the PCR.

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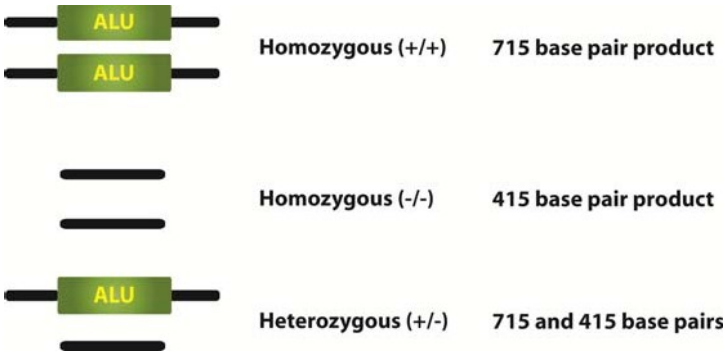

Figure 4: Possible distribution of Alu insert in PV92 locus and PCR fragment sizes.

**The Alu alleles are inherited from parents and can potentially reveal information about family relationships and as a result we caution against generating data from multiple family members. If this is a concern, we recommend mixing up the students samples after the cells have been collected to ensure anonymity.*

MATERIALS FOR EACH GROUP

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- 1 bottle DNA Wash (shared with class)
- 1 bottle Precipitation Solution (shared with class)
- 1 bottle DNA Salt Solution (shared with class)
- 4 Cytology Brushes
- 8 centrifuge tubes (1.5ml)
- 1 vial 5' Primer
- 1 vial 3' Primer
- 1 vial *Taq* Reaction Buffer
- 1 vial deoxynucleotides (dNTPs)
- 1 vial *Taq* DNA polymerase
- 1 vial Sterile Water
- 1 vial mineral oil (shared with class)
- 4 PCR tubes

PROCEDURE

Isolation of genomic DNA

1. Label two 1.5ml Centrifuge Tubes with your name.

2. Add 0.4ml DNA Release Buffer to a labeled 1.5ml Tube. This solution contains a detergent that disrupts the cell structure and releases the genomic DNA into the solution.

3. **Collect Cheek Cells**: Use the cytology brush to collect your cheek cells by scraping the inside of your cheek. Scrape the inside of each cheek; work on the area around the gum line 20 times, whilst twirling the brush between your fingers.

 *Instruct students not too scrape too vigorously. The best place for collecting the cells is at the gum line.*

4. Place the brush into the DNA Release Buffer and leave in the solution for 1 minute periodically twirling the brush.

5. Remove the brush from the solution, ensuring that you thoroughly scrape the brush on the side of the tube, releasing the cheek cells into the DNA Release Buffer.

 *The solution should be slightly cloudy and viscous.*

6. Add 0.02ml Protease to the tube to digest and remove the cellular material and protein from the genomic DNA.

7. Close the cap. Briefly mix by inverting the tube 5-6 times and then place in a 50-60°C waterbath or heating block for 20-60mins.

8. After 1 hour, add 90µl DNA Salt Solution to the tube and mix by inverting the tube several times. The salt solution aids in the precipitation of the DNA.

9. Centrifuge the tube for 5 minutes at 10,000xg to pellet the cell debris. Transfer the supernatant to your other labeled tube.

10. Slowly, add 0.95ml Precipitation Solution, close the tube and, whilst watching, slowly invert the tube several times to mix. White DNA strands may appear. *The lack of white strands does not indicate a lack of DNA. Only a tiny amount of DNA is needed for PCR amplification.*

11. To collect the DNA centrifuge the tube at 10,000xg for 10 minutes. A tight white pellet should be visualized in the bottom of the tube. Discard the supernatant and keep the pellet of DNA.
    The white pellet may be very hard to visualize. To aid in locating the pellet, make a note of the tube's orientation within the centrifuge.

12. Add 0.2ml DNA Wash to the pellet and centrifuge at 10,000xg for 10 minutes.
    The DNA Wash is a mixture of alcohol and water that allows for the removal of contaminating salts, without dissolving the DNA.

13. Remove all the DNA Wash with a pipette tip. Add 50μl Sterile Water to the DNA pellet. Incubate at room temperature for 10-15 minutes with periodic mixing.

If necessary, this is a convenient stopping point. Store the DNA at -20ºC in a freezer. The DNA is stable for up to a month.

14. *Optional: To visualize the genomic DNA, remove 10μl genomic DNA and visualize on a 1% agarose gel.*

Polymerase Chain Reaction

 *All the components of the PCR reaction and the setting up the reaction should be done on ice.*

1. Label the side of a PCR tube with your initials and place the tube on ice.

2. As a group prepare a "Master Mix" of the PCR reaction components. In a 2ml centrifuge tube, add the following components. Use a different pipette tip for each component. Pipette each component into the bottom of the tube. On addition of each component, gently mix by pipetting up and down.

    - 20μl 5' Genomic Primer
    - 20μl 3' Genomic Primer
    - 40μl *Taq* Reaction Buffer
    - 40μl dNTPs
    - 10μl *Taq* polymerase
    - 250μl Sterile Water

3. Transfer 5μl of your diluted genomic DNA (from step 13) to your labeled PCR tube.

4. Add 95μl "Master Mix" from step 2 to your PCR tube. Gently mix by pipetting up and down 4-5 times.

5. If using a thermocycler without a heated lid, add 50μl Mineral oil to prevent evaporation.

 *Inform your students if they require mineral oil.*

6. The Thermocycler should be programmed as follows

    - 1 Cycle of 96°C for 2 minutes
    - 33 Cycles 94°C for 1 min, 55°C for 1 min, 68°C for 2 min
    - 1 Cycle 68°C for 7mins
    - 1 Cycle of 4°C forever.

7. The following day, after the reaction has finished, remove the tubes and remove 30μl to be analyzed by agarose electrophoresis.

The PCR reaction can be stored at -20ºC until required.

The PCR reactions should be run on a 2% agarose gel as the gene is 415 and or 715 bp. STOP the gel when the blue dye front is more than 3cm from the gel bottom. If run further the PCR product will be lost as it runs ahead of the dye front.

RESULTS, ANALYSIS & ASSESSMENT

1. What was the size of the PCR product that was visualized on the agarose gel?

2. Explain your result below in terms of your Alu elements?

3. Explain the various steps of the PCR program, explaining the relevance of the temperatures and times.

4. Discuss the role of each component in the reaction mixture.


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