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Mutation Detection & Analysis

Teacher's Guidebook

(Cat. # BE-314)

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MATERIALS INCLUDED WITH THE KIT

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

CHECKLIST

- 1 vial DNA X
- 1 vial DNA Y
- 1 vial PCR: 5' Plasmid Primer
- 1 vial PCR: 3' Plasmid Primer
- 1 vial PCR: Deoxynucleotides (dNTPs)
- 1 vial 10X PCR Buffer (Mg^{2+} plus)
- 1 vial *Taq* DNA polymerase
- 1 vial Sterile Water
- 1 vial PCR: Mineral Oil
- 12 PCR: PCR tubes
- 1 vial Res. Enz.: R.E. Buffer 3 (6X)
- 6 vials HindIII Enzyme
- 90 Centrifuge Tubes (1.5ml)

SPECIAL HANDLING INSTRUCTIONS

- Store DNA X and Y, 5' and 3' Plasmid Primers, *Taq* Reaction Buffer, dNTPs, *Taq* DNA polymerase, R.E. Buffer 3 (6X) and HindIII *Longlife*™ enzyme at -20°C.
- All other reagents can be stored at room temperature.
- Briefly centrifuge all small vials before opening to prevent waste of reagents.

ADDITIONAL EQUIPMENT REQUIRED

- PCR Machine (Thermocycler)
- Agarose Electrophoresis Reagents and Equipment
- Waterbath or beaker and thermometer

TIME REQUIRED

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

OBJECTIVES

- Use polymerase chain reaction to amplify wild type and mutant gene
- Use restriction enzymes to analyze the amplified genes.
- Identify the mutant gene.

BACKGROUND

A major challenge for molecular biologists and genetic engineers is to easily detect and analyze genetic mutations that occur naturally, causing diseases, or during genetic engineering or cloning, whether deliberate or accidental. For genes there are four main types of mutations that can occur. The deletion, insertion or duplication of bases, ranging from single base pairs to large numbers of base pairs, or the direct substitution of specific base pairs.

If deletions/ insertions/ duplications result in a frameshift, the number changed is not a multiple of three, the usually result in no protein product or truncated protein product. If deletions/ insertions/ duplications remain in frame, this results in the loss or gain of amino acid(s) depending on the size of the deletion/ insertion/ duplication and may give rise to altered protein product with changed properties, i.e. abnormal size. If deletions/ insertions/ duplications occur in an untranslated region, then transcription/ expression and/or stability of the message may become affected.

For substitutions, nonsense mutations can occur, where amino acid codons are converted into stop codons, resulting in no protein production or truncated proteins. Missense mutations can occur and these involve the changing of one amino acid codon to that of another. The effect on the protein is dependent on the changed amino acid, its charge, hydropathy, size and whether it's conserved or not. If the substitutions occur in untranslated regions they may affect the binding sites for transcription factors affecting the expression of the proteins.

The most accurate method for detecting mutations is still direct sequencing of the DNA, however this is still relatively expensive and time consuming, especially if a large number of mutants need to be screened. The advent of the polymerase chain reaction (PCR) (described below) has led to alternative and reliable methods being developed to identify genetic mutations. Several techniques used to detect mutants are briefly described below. In each case a source of DNA is required and this is generated by PCR:

Single strand conformational polymorphism (SSCP) is one of the more popular techniques for scanning for mutations due to its relative simplicity. SSCP uses non-denaturing gels that allows for the separation of nucleic acids by their size *and* shape. The principle is based on the fact that single stranded DNA is highly flexible and adopts a conformation that is determined by intramolecular interactions and is uniquely dependent of the sequence. The conformation of the DNA can be altered by a single base change and these changes can be visualized empirically on non denaturing gels.

Heteroduplex analysis is based on the retardation of the heteroduplex compared with the corresponding homoduplex on a non-denaturing gel. A heteroduplex is a double-stranded DNA molecule or a DNA/RNA hybrid where each strand is from a different source. The heteroduplexes will migrate slower than the corresponding homoduplexes due to a more open configuration caused by the mismatched bases. Basically, heteroduplexes are formed by mixing wild type and mutant DNA amplified by PCR.

Denaturing gradient gel electrophoresis (DGGE) utilizes the melting (denaturation) properties of DNA in solution. When DNA denatures, due to temperature or denaturant concentration, the molecules come apart a specific segments known as melting domains. The denaturing temperatures of these domains are dependent on the sequence. DNA fragments are run on a denaturing gradient gel and mutations are detected by the way the change the denaturation point.

Some known mutations are easily detected by restriction enzyme mapping. Restriction enzymes are described below. The mutant that occurs either generates a new restriction enzyme site or destroys and old one. When the mutant is digested with a particular restriction enzyme a different restriction map is generated. This is the mutation detection and analysis that is utilized in this kit. Two DNA samples are supplied; a wild type and a mutant, using PCR to amplify the gene and restriction mapping students can identify the wild type and mutant sample.

Polymerase Chain Reaction

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).

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.

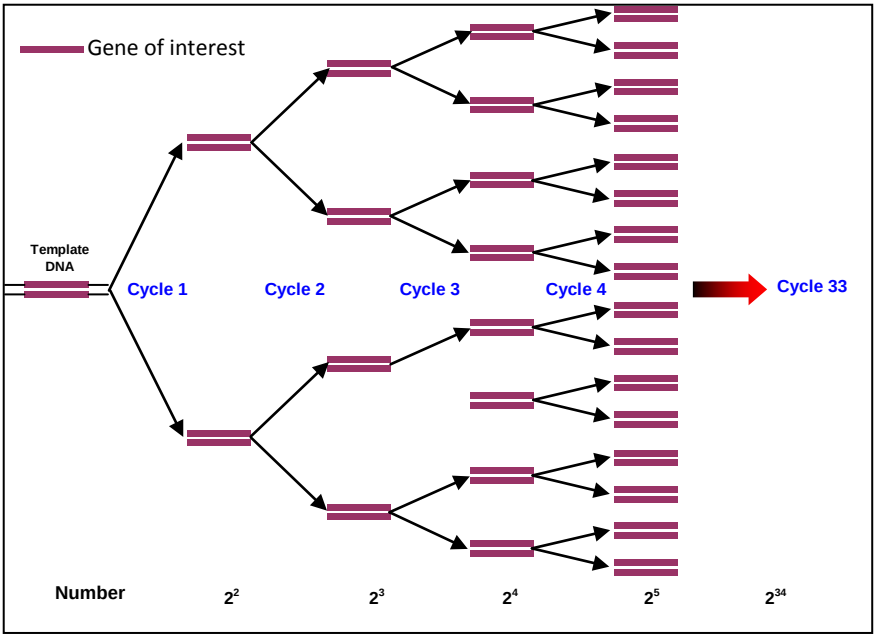


Figure 1: The exponential copying of a gene of interest during the polymerase chain reaction.

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°F). 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.

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.

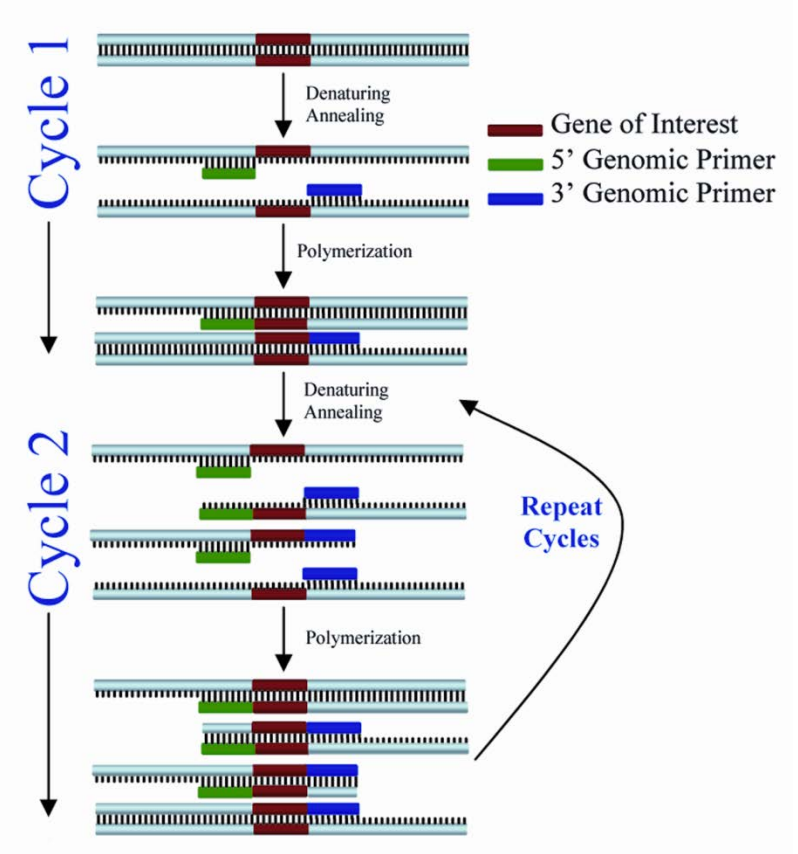


Figure 2: Schematic representation of the Polymerase Chain Reaction

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.

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.

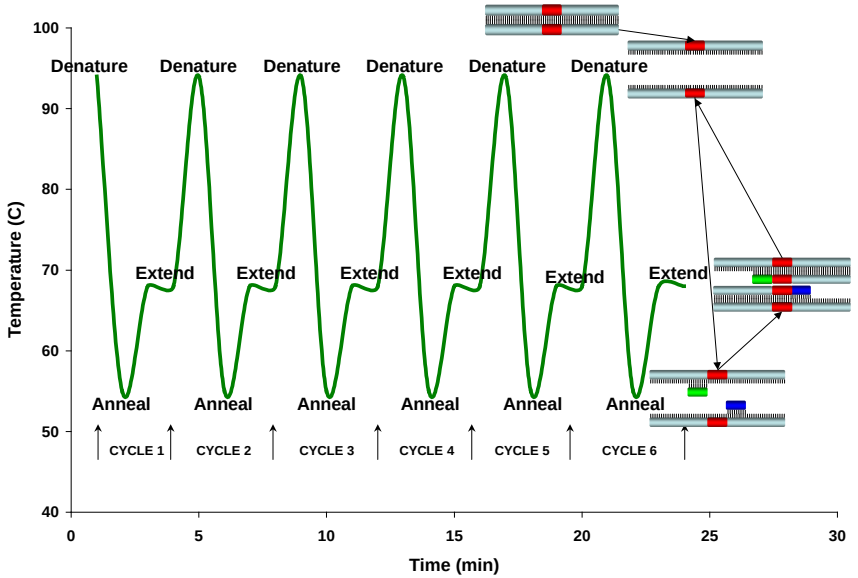


Figure 3: The temperature cycles used during the PCR reaction. The graph depicts the changes in temperature and the resulting effect on the DNA. A schematic of these effects is shown to the right of the graph.

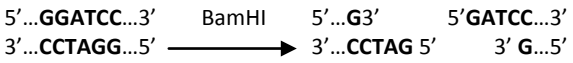
Restriction Enzymes

In 1969, Hamilton Smith discovered and purified the first restriction enzyme, or endonuclease, HindII. Restriction endonucleases are important enzymes that cleave the backbone of DNA molecules at specific sites or sequences. The use of restriction endonucleases has proved invaluable in molecular biology, cloning, genetic engineering and a multiple of other scientific disciplines.

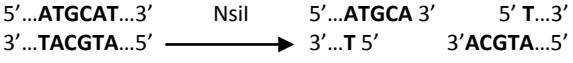
The majority of restriction endonucleases are isolated from bacteria, where they are thought to play a role in host defense by cutting and destroying foreign DNA, for example, of invading viruses. To ensure the bacteria's own genome is not degraded, each restriction endonuclease has a complementary methylating enzyme that methylates the restriction endonuclease site on the genomic DNA preventing digestion. The combined role of these two enzymes is known as the restriction:modification system.

Each restriction endonuclease recognizes a specific sequence of nucleotides, normally ranging from 4-8 base pairs in length. The shorter the recognition sequence the more frequently the site will appear in the DNA sequence. For example a six base pair sequence will appear, on average, every 4^6 or 4096bp, where as a four base pair site will appear every 296bp. Some restriction endonucleases share the same sites as other restriction endonucleases, these are known as isoschizomers.

There are three major patterns of digestions that are achieved by restriction endonucleases. The first is the generation of a 5′ overhang. This occurs when the enzyme cuts at a different place on each strand of DNA leaving one of the strands longer than the other producing a sticky end. This is seen when DNA is digested with BamHI and EcoRI.



The second pattern is the 3′ overhang, as generated by the restriction enzyme NsiI:



The 5′ and 3′asymmetric ends generated by enzymes are known as sticky ends or cohesive ends as they readily stick, or anneal, together with their complimentary base pairs.

The third pattern is known as the blunt ends, these occur when the enzyme cuts the both strands of DNA in the same place resulting in no overhang. An example is the restriction enzyme EcoRV:

Another feature of restriction endonucleases is that some are unambiguous and others are ambiguous. For example, BamHI is ambiguous as it recognizes 6 specific, defined nucleotides (GGATCC); HinFI, an ambiguous enzyme, recognizes a 5 base pair sequence, which starts with GA and ends in TC, but can have any base as the middle base pair.

There are several important factors to consider when using restriction enzymes. These include buffer composition, incubation temperature, DNA methylation and star activity.

There is no universal digestion buffer for restriction enzymes as different enzymes have different preferences for ionic strength (salt concentration) and major cations (sodium or potassium). There are 3-4 commonly used buffers that are generally suitable for most enzyme conditions. For pH, enzymes commonly work around pH8.0, however some enzymes are more particular and have specific buffers. Use of the wrong buffer leads to poor digestions.

Most of the restriction enzymes available have optimal activity at 37°C, but as with the buffers there are many exceptions. Enzymes that have been isolated from thermophiles, bacteria that grow in high temperature environments, have optimal activity at 50-65°C, whereas some enzymes have very short half lives at 37°C and are used at 25°C.

DNA methylation has an inhibitory effect on some restriction enzymes. DNA methylation has two functions: 1) Protection from host restriction enzymes that target foreign (i.e. viral DNA) in an immune response; 2) A gene regulation control step. Almost all strains of *E.coli* contain two site-specific DNA methylases that methylate specific sequences. Researchers must check information of their restriction enzyme of choice and the effects of methylation.

A final consideration is that some enzymes under *non-standard conditions* will cleave DNA at sites different from their specific recognition sequence. Non-standard conditions include high pH (>8.0) or low ionic strength, high glycerol concentrations (enzymes usually supplied in 50% glycerol), extremely high enzyme concentration or the presence of organic solvents (ethanol, DMSO) in the reaction.

TEACHER'S PRE EXPERIMENT SET UP

The digested PCR products are analyzed by agarose gel electrophoresis We recommend using the "Introduction to Agarose Electrophoresis" kit (Cat. # BE-304)

Prepare PCR and Restriction Digestion reagents

For Day 1

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 35µl sterile water to the DNA X tube. Resuspend the DNA by gently pipetting up and down.
3. Label six tubes with "DNA X". Transfer 5µl DNA X from step 2 to each tube. Supply each group with a single tube.
4. Transfer 35µl sterile water to the DNA Y tube. Resuspend the DNA by gently pipetting up and down.
5. Label six tubes with "DNA Y". Transfer 5µl DNA Y from step 4 to each tube. Supply each group with a single tube.
6. Transfer 65µl sterile water to the 5' Plasmid Primer tube. Resuspend the primer by gently pipetting up and down.
7. Label six tubes with "5' Primer". Transfer 10µl 5' Plasmid Primer from step 6 to each tube. Supply each group with a single tube.
8. Transfer 65µl sterile water to the 3' Plasmid Primer tube. Resuspend the primer by gently pipetting up and down.
9. Label six tubes with "3' Primer". Transfer 10µl 3' Plasmid Primer from step 8 to each tube. Supply each group with a single tube.
10. Label six tubes with "*Taq* Buffer". Transfer 20µl 10X PCR Buffer (Mg^{2+} plus) to each tube. Supply each group with a single tube.
11. Transfer 126µl sterile water to the dNTPs. Wait 5 minutes and then resuspend the dNTPs by gently pipetting up and down.
12. Label six tubes with "dNTP". Transfer 20µl Deoxynucleotides (dNTP) to each tube. Supply each group with a single tube.
13. Label six tubes with "Taq". Add 105µl Sterile Water to the vial of Taq DNA Polymerase. Transfer 10µl *Taq* DNA polymerase to each tube. Supply each group with a single tube.
14. If the thermocycler does not have a heated lid then label six tubes "Mineral Oil". Transfer 50µl Mineral Oil to the bottom of each tube. Supply each group with a single tube.
15. Label six tubes with "H_2O". Transfer 200µl sterile water to each tube. Supply each group with a single tube. *This is used for both days.*

For Day 2

16. Label six tubes with “6X Buffer”. Transfer 20µl R.E. Buffer 3 (6X) to the bottom of each tube. Supply each group with a single tube.
17. Supply each group with a single tube of HindIII enzyme.

Program Thermocycler

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 with 5 sec extensions.
    c. 1 Cycle 68°C for 7mins.
    d. 1 Cycle of 4°C forever.

Visualization of Digested PCR products

1. In order to visualize the digested PCR products an agarose gel will need to be run. Each group of students requires 4 wells 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.

Day 1

5µl DNA X
5µl DNA Y
10µl 5’ Plasmid Primer
10µl 3’ Plasmid Primer
20µl *Taq* Reaction Buffer
20µl Deoxynucleotides (dNTPs)
10µl *Taq* DNA polymerase
120µl sterile water (*This is used on both days)*
50µl Mineral Oil (only required if thermocycler lacks a heated lid.)
2 PCR tubes

Day 2

20µl R.E. Buffer 3 (6X)
15µl HindIII
50µl DNA Loading Buffer (6X)
4 1.5ml Centrifuge Tubes

PROCEDURE DAY 1

Polymerase Chain Reaction

 *All the components of the PCR reaction and the setting up the reaction should be done on ice. Everything remains on ice until the PCR reaction is placed in the thermocycler.*

1. Divide your group of four students into two pairs. Each pair takes either DNA X tube or DNA Y tube.
2. Each pair labels the side of a PCR tube with their initials and the DNA letter (X or Y). Place the tube on ice.
3. Each pair sets up the following reaction in the supplied PCR tube on ice with their DNA. Use a clean sterile pipette tip for each reaction to avoid cross contamination of the reagents.
    a. 5µl 5' Plasmid Primer
    b. 5µl 3' Plasmid Primer
    c. 10µl *Taq* Reaction Buffer
    d. 10µl dNTPs
    e. 5µl *Taq* DNA Polymerase
    f. 65µl sterile distilled water
4. Transfer 5µl of your DNA to your labeled PCR tube. Mix by gently tapping the tube.
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

    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.

PROCEDURE DAY 2

The following day, after the reaction has finished, remove the tubes and place on ice.

Restriction Digestion Analysis

1. Each pair labels two 1.5ml centrifuge tubes with their initials and the DNA letter from step 1. Label one tube "U" for undigested and the other "H" for HindIII digested.
2. Place the tubes in the ice bucker containing the rest of the components for the experiment.

 *All the components and the reactions must remain on ice during the set up of the experiment.*

3. Using a clean pipette tip for every reagent or solution to avoid cross contamination, each pair sets up the following reactions on ice. If mineral oil was used, ensure the tip is placed below the mineral oil when pipetting the PCR reaction:

	Tube U	**Tube H**
PCR reaction	20µl	20µl
HindIII	None	5µl
Sterile Water	5µl	None
6X Buffer	5µl	5µl

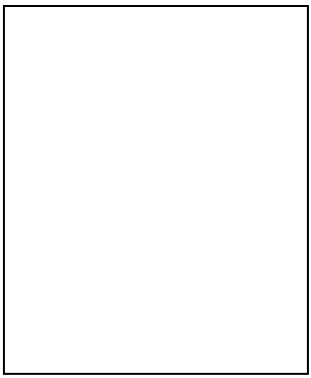
4. Mix the contents of each tube by gently pipetting 4-5 times.
5. Place each tube in a waterbath or incubator at 37°C for one hour.
6. After the one hour incubation, store the digest in a freezer until required.
7. Add and appropriate amount of Loading Buffer and load 20-30µl on the 2% agarose gel and 5µl 1kb Ladder. Migrate the bands at 75 volts. Visualize and record your results.

 *Ensure that agarose gels have been prepared in advance and that you have adequate DNA loading buffer and reference markers. A 1.5 -2% agarose gel is required.*

 *Following electrophoresis, the gels are stained to visualize the DNA. The wild type gene is a single band of ~750bp; the mutant gene is two bands of ~550bp and 200bp.*

RESULTS, ANALYSIS & ASSESSMENT

1. Sketch a representation of your gel in the box below.



2. What was the size of the PCR products and the digested PCR products visualized on the agarose gel?
   DNA X undigested: *~750bp*
   DNA X digested: *~750bp*
   DNA Y undigested: *~750bp*
   DNA Y digested: *~550bp, ~200bp*
3. The wild type gene does not contain a Hind III restriction site. Which DNA is the wild type? Explain a possible mutation that occurred to introduce the new site in the mutant.
   DNA X is the wild type DNA. The mutation is a point mutation that results in the formation of the new site. An insertion or deletion is also possible, if it is a small insertion. A truncation is not possible.

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Polymerase Chain Reaction

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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.

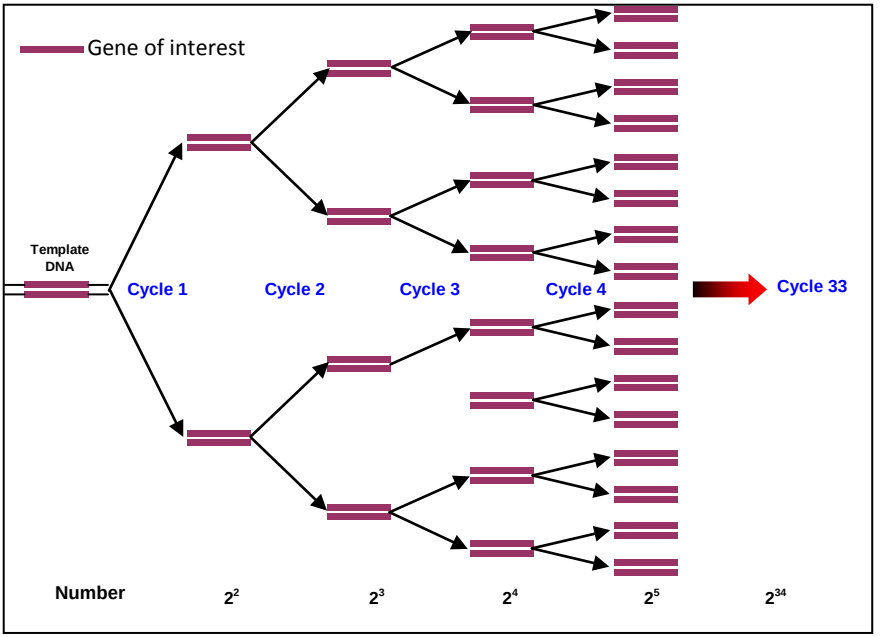


Figure 1: The exponential copying of a gene of interest during the polymerase chain reaction.

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°F). 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.

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.

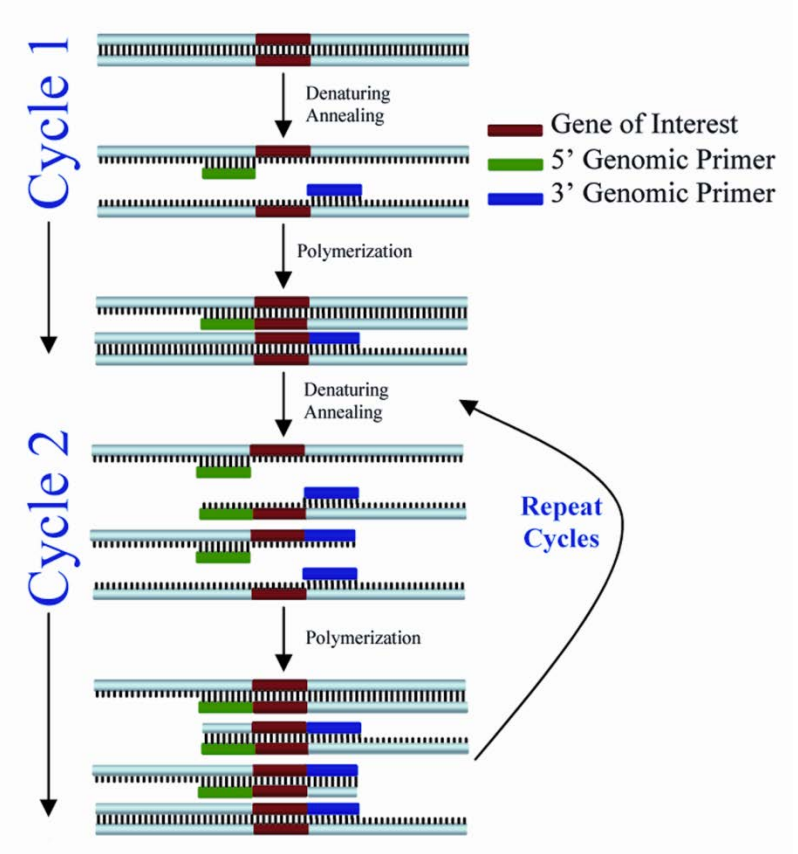


Figure 2: Schematic representation of the Polymerase Chain Reaction

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.

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.

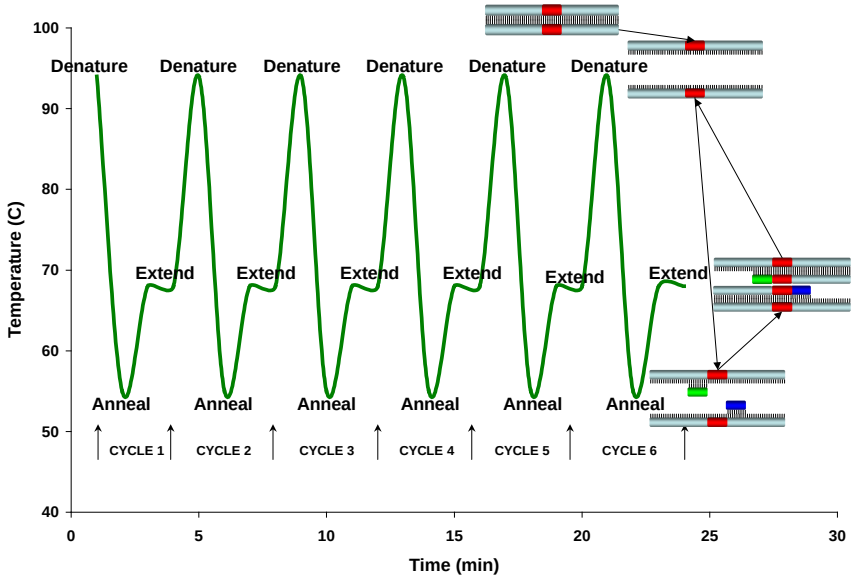


Figure 3: The temperature cycles used during the PCR reaction. The graph depicts the changes in temperature and the resulting effect on the DNA. A schematic of these effects is shown to the right of the graph.

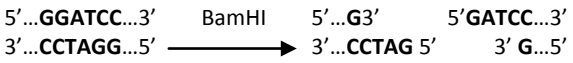
Restriction Enzymes

In 1969, Hamilton Smith discovered and purified the first restriction enzyme, or endonuclease, HindII. Restriction endonucleases are important enzymes that cleave the backbone of DNA molecules at specific sites or sequences. The use of restriction endonucleases has proved invaluable in molecular biology, cloning, genetic engineering and a multiple of other scientific disciplines.

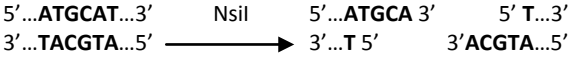
The majority of restriction endonucleases are isolated from bacteria, where they are thought to play a role in host defense by cutting and destroying foreign DNA, for example, of invading viruses. To ensure the bacteria's own genome is not degraded, each restriction endonuclease has a complementary methylating enzyme that methylates the restriction endonuclease site on the genomic DNA preventing digestion. The combined role of these two enzymes is known as the restriction:modification system.

Each restriction endonuclease recognizes a specific sequence of nucleotides, normally ranging from 4-8 base pairs in length. The shorter the recognition sequence the more frequently the site will appear in the DNA sequence. For example a six base pair sequence will appear, on average, every 4^6 or 4096bp, where as a four base pair site will appear every 296bp. Some restriction endonucleases share the same sites as other restriction endonucleases, these are known as isoschizomers.

There are three major patterns of digestions that are achieved by restriction endonucleases. The first is the generation of a 5′ overhang. This occurs when the enzyme cuts at a different place on each strand of DNA leaving one of the strands longer than the other producing a sticky end. This is seen when DNA is digested with BamHI and EcoRI.



The second pattern is the 3′ overhang, as generated by the restriction enzyme NsiI:



The 5′ and 3′asymmetric ends generated by enzymes are known as sticky ends or cohesive ends as they readily stick, or anneal, together with their complimentary base pairs.

The third pattern is known as the blunt ends, these occur when the enzyme cuts the both strands of DNA in the same place resulting in no overhang. An example is the restriction enzyme EcoRV:

Another feature of restriction endonucleases is that some are unambiguous and others are ambiguous. For example, BamHI is ambiguous as it recognizes 6 specific, defined nucleotides (GGATCC); HinFI, an ambiguous enzyme, recognizes a 5 base pair sequence, which starts with GA and ends in TC, but can have any base as the middle base pair.

There are several important factors to consider when using restriction enzymes. These include buffer composition, incubation temperature, DNA methylation and star activity.

There is no universal digestion buffer for restriction enzymes as different enzymes have different preferences for ionic strength (salt concentration) and major cations (sodium or potassium). There are 3-4 commonly used buffers that are generally suitable for most enzyme conditions. For pH, enzymes commonly work around pH8.0, however some enzymes are more particular and have specific buffers. Use of the wrong buffer leads to poor digestions.

Most of the restriction enzymes available have optimal activity at 37°C, but as with the buffers there are many exceptions. Enzymes that have been isolated from thermophiles, bacteria that grow in high temperature environments, have optimal activity at 50-65°C, whereas some enzymes have very short half lives at 37°C and are used at 25°C.

DNA methylation has an inhibitory effect on some restriction enzymes. DNA methylation has two functions: 1) Protection from host restriction enzymes that target foreign (i.e. viral DNA) in an immune response; 2) A gene regulation control step. Almost all strains of *E.coli* contain two site-specific DNA methylases that methylate specific sequences. Researchers must check information of their restriction enzyme of choice and the effects of methylation.

A final consideration is that some enzymes under *non-standard conditions* will cleave DNA at sites different from their specific recognition sequence. Non-standard conditions include high pH (>8.0) or low ionic strength, high glycerol concentrations (enzymes usually supplied in 50% glycerol), extremely high enzyme concentration or the presence of organic solvents (ethanol, DMSO) in the reaction.

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.

Day 1

5µl DNA X
5µl DNA Y
10µl 5' Plasmid Primer
10µl 3' Plasmid Primer
20µl *Taq* Reaction Buffer
20µl Deoxynucleotides (dNTPs)
10µl *Taq* DNA polymerase
120µl sterile water (*This is used on both days)*
50µl Mineral Oil (only required if thermocycler lacks a heated lid.)
2 PCR tubes

Day 2

20µl R.E. Buffer 3 (6X)
15µl HindIII
50µl DNA Loading Buffer (6X)
4 1.5ml Centrifuge Tubes

PROCEDURE DAY 1

Polymerase Chain Reaction

 *All the components of the PCR reaction and the setting up the reaction should be done on ice. Everything remains on ice until the PCR reaction is placed in the thermocycler.*

1. Divide your group of four students into two pairs. Each pair takes either DNA X tube or DNA Y tube.
2. Each pair labels the side of a PCR tube with their initials and the DNA letter (X or Y). Place the tube on ice.
3. Each pair sets up the following reaction in the supplied PCR tube on ice with their DNA. Use a clean sterile pipette tip for each reaction to avoid cross contamination of the reagents.
    a. 5µl 5' Plasmid Primer
    b. 5µl 3' Plasmid Primer
    c. 10µl *Taq* Reaction Buffer
    d. 10µl dNTPs
    e. 5µl *Taq* DNA Polymerase
    f. 65µl sterile distilled water
4. Transfer 5µl of your DNA to your labeled PCR tube. Mix by gently tapping the tube.
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

    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.

PROCEDURE DAY 2

The following day, after the reaction has finished, remove the tubes and place on ice.

Restriction Digestion Analysis

1. Each pair labels two 1.5ml centrifuge tubes with their initials and the DNA letter from step 1. Label one tube "U" for undigested and the other "H" for HindIII digested.
2. Place the tubes in the ice bucker containing the rest of the components for the experiment.

 *All the components and the reactions must remain on ice during the set up of the experiment.*

3. Using a clean pipette tip for every reagent or solution to avoid cross contamination, each pair sets up the following reactions on ice. If mineral oil was used, ensure the tip is placed below the mineral oil when pipetting the PCR reaction:

	Tube U	**Tube H**
PCR reaction	20µl	20µl
HindIII	None	5µl
Sterile Water	5µl	None
6X Buffer	5µl	5µl

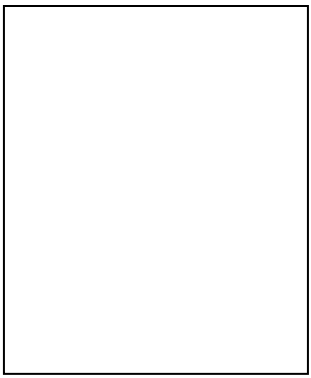
4. Mix the contents of each tube by gently pipetting 4-5 times.
5. Place each tube in a waterbath or incubator at 37°C for one hour.
6. After the one hour incubation, store the digest in a freezer until required.
7. Add and appropriate amount of Loading Buffer and load 20-30µl on the 2% agarose gel and 5µl 1kb Ladder. Migrate the bands at 75 volts. Visualize and record your results.

 *Ensure that agarose gels have been prepared in advance and that you have adequate DNA loading buffer and reference markers. A 1.5 -2% agarose gel is required.*

 *Following electrophoresis, the gels are stained to visualize the DNA. The wild type gene is a single band of ~750bp; the mutant gene is two bands of ~550bp and 200bp.*

RESULTS, ANALYSIS & ASSESSMENT

1. Sketch a representation of your gel in the box below.



2. What was the size of the PCR products and the digested PCR products visualized on the agarose gel?

   DNA X undigested: _____

   DNA X digested: _____

   DNA Y undigested: _____

   DNA Y digested: _____

3. The wild type gene does not contain a Hind III restriction site. Which DNA is the wild type? Explain a possible mutation that occurred to introduce the new site in the mutant.

   _____

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