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The Challenge of a "Knockout" PCR Test

A Thesis

Presented to

the Chancellor's Scholars Council

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the Chancellor's Scholars Program

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by

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PCR Project Goal

This project was divided into three main goals:

(1) To become familiar with the ideas and procedure of a PCR technique,

(2) To verify that primers 3 and 4 will produce a 239 base product in a wildtype and a heterozygote and nothing in the knockout, and

(3) To demonstrate that primers 3 and 6 can show the three genotypes in a single assay by producing a 648 base product in the wildtype and heterozygote and a 2000 base pair product in the knockout.

Note: In examining the gel results for each experiment, the PCR Markers' bands represent the following base pairs:

1,000
750
500
300
150
50

Introduction

Deoxyribonucleic Acid, commonly known as DNA, is one of the, if not the single most important of the molecules of life (Kuo). Found in every living cell, it carries the genetic code that determines all of an organism's characteristics. In humans, these characteristics range from eye color to allergies. Each one of us carries our own unique "signature" of DNA that makes us all different from one another (Marini 100).

A large part of genetic engineering involves identifying tiny units of heredity, called genes, that are found on the DNA. The more DNA that a scientist has to work with, the easier and faster the identification process becomes. In the past, the most common method for making copies of DNA was cloning. In this process, a segment of DNA would be inserted into living bacteria, which would take days or even weeks to produce a sufficient number of copies of the genetic material. Unfortunately, this method was "tedious, time-consuming, and expensive, like copying business documents before Xerox invented the copy machine." (Marini 100)

This is why the PCR technique was created. PCR eliminates this "scientific grunt work." (Marini 100) No longer must a researcher search for genes like looking for a needle in a haystack. With PCR, a single strand of DNA can be copied millions and billions of times, creating as many "needles" of DNA as needed (Marini 100).

DNA Structure

The structure of DNA can be thought of as a ladder. The sides of the "ladder" are composed of phosphate groups attached, through phosphodiester bonds, to 5-carbon sugars called deoxyriboses. The "rungs" of the DNA ladder are composed of bases, attached to the sugars, that are linked together by hydrogen bonds.

There are four bases that are found in DNA. They are adenine, guanine, thymine, and cytosine, which are symbolized A, G, T, and C. These bases bond as complementary base pairs. In other words, adenine only bonds to thymine and guanine only to cytosine.

The special aspect of DNA is that not only does it occur as double-stranded, but these strands are coiled around each other forming what is called a double helix.

One end of a single strand of DNA is called the 5' end where a phosphate group is located. The other end of the strand, known as the 3' end, is where a hydroxide group is found. These ends can be symbolized as 5'P and 3'OH.

The two strands of a DNA molecule exist in a condition known as being antiparallel. This means that at each end of a DNA molecule, one strand possesses a 5'P end and the other strand's end is a 3'OH (Hartl 99).

DNA Replication

DNA replication begins with the action of two enzymes, DNA gyrase and helicase, which move along the DNA molecule in opposite directions in a process called bidirectional replication. These two enzymes relax the DNA coil, and create a "bubble" in the DNA called the replication origin (Kuo).

Once one end of the DNA molecule has been unwound and separated, DNA replication occurs in what is called didirectional synthesis, which is defined as synthesis occurring in opposite directions along the two strands of DNA (Kuo).

DNA synthesis occurs in a 5' to 3' direction. Therefore, the DNA strand to be copied, known as the template, is read in the 3' to 5' direction. A primer, which is a fragment of DNA which is capable of forming hydrogen bonds with the now exposed bases, begins complementary base pairing at the 3' end of the DNA template. This newly forming DNA strand is known as the leading strand. Another primer begins complementary base pairing from the 5' end of the other DNA template. As a result, this newly forming DNA strand forms in fragments and is known as the lagging strand.

Both the lagging and leading strands continue to be formed because of the addition of dNTPs. DNTP, which stands for deoxyribose nucleoside triphosphate, is the form in which the four bases exist in the nucleus of a cell. The nucleotides contain the bases along with a sugar and three phosphates. The

four types are dATP, dCTP, dGTP, and dTTP. These bases form complementary base pairs with the DNA template; A binds to T and C binds to G (Kuo).

Two other types of enzymes are used to aid DNA replication. DNA polymerases help form phosphodiester bonds between the phosphates and sugars and DNA ligase helps join the fragments of the lagging strand together (Kuo).

Once DNA replication ends, there are now two identical copies of the original DNA molecule. Each molecule is now composed of one newly synthesized strand and one original template strand. As a result, DNA replication is also called semiconservative replication since each new molecule "conserves" one of the original strands (Kuo).

Electrophoresis

Electrophoresis is a procedure in which macromolecules are separated in an electric field based on the concepts of charge and mass. When the macromolecules to be separated are molecules of DNA, the electrophoresis process is carried out in a gel; specifically, an Agarose Gel, which is composed of carbohydrates, is used (Woriax).

Because DNA contains phosphate groups, it is negatively charged. Therefore, when placed in an electrophoresis unit, DNA molecules will migrate away from the negative charge and toward the positive charge in an electric field. Usually, 95V-105V is the voltage used in an electrophoresis process because the lower the voltage, the better the DNA samples migrate (Locklear).

When a gel is made, small "pockets" called wells are made in order for the samples to be loaded in. To monitor the electrophoresis process, tracking dye is mixed with the samples before they are loaded into the wells. This allows for the viewing of the samples' migration down the gel (Locklear).

To aid in reading electrophoresis results, the gel usually is stained with Ethidium Bromide to observe the separate "bands" that occur once separation of samples is complete (Locklear).

Electrophoresis is based on the concept that smaller molecules will migrate farther down the gel than larger molecules (Woriax). Therefore, when viewing the gel, it can be determined which DNA sample was longer in length and which was shorter. To

aid in this determination, a gel marker is loaded into one of the empty wells of the gel. This substance will produce bands on the gel in which the length that each band represents is already known. Therefore, a comparison can be made between the gel marker and DNA sample bands to estimate the length in base pairs of the DNA sample molecules of interest (Locklear).

The PCR Process

PCR, which stands for Polymerase Chain Reaction, "works by mimicking the natural DNA replication that occurs whenever a cell divides." (Marini 101) The PCR process, like normal DNA replication, is composed of three main steps: (1) DNA Denaturation, (2) Primer Annealing, and (3) DNA Synthesis.

In DNA denaturation, heat is used to unwind a specific piece of DNA into two separate strands. In primer annealing, primers, which are usually twelve or more nucleotides in length, have a sequence specific for a particular site on the piece of DNA. These primers attach to the DNA strand and signal the DNA polymerase to begin the final stage of PCR, which is DNA synthesis.

The type of DNA polymerase used in PCR is called TAQ Polymerase. TAQ is named for the bacterium, Thermus Aquaticus, from which it is extracted. These bacterium are found in hot springs. As a result, their DNA polymerase can withstand high temperatures, a trait that is necessary during the heating stage of PCR (Woriax).

During DNA synthesis, the nucleotides, in the form of dNTPs, begin complementary base pairing with the DNA template strands until two complete DNA molecules are formed. Each time this process occurs is known as a cycle. As each subsequent cycle is completed, the number of DNA molecules increases exponentially: two becomes four, four becomes eight, eight becomes sixteen, and

the cycle continues on indefinitely.

Usually the PCR process is carried out in a machine known as a Thermal Cycler. This machine can be programmed to mimic certain temperatures to allow the three main steps of the PCR process to occur. In addition, the PCR process does not occur indefinitely in the Thermal Cycler since the number of cycles to be carried out can be programmed in the machine.

The Genetics

Most genes occur in pairs. The combination of paired genes in a particular individual is called its genotype. This genotype can exist in one of three forms: (1) two dominant (normally expressed) genes, (2) two recessive (usually unexpressed) genes, or (3) one dominant and one recessive gene. For example, two dominant genes could be expressed in a genotype represented by the symbol AA. The symbol aa could represent the genotype containing two recessive genes. An individual carrying a trait based on one dominant and one recessive gene would carry the genotype symbolized as Aa.

The Chancellor's Scholars Project

Scientists at the National Institute of Environmental Health Sciences (NIEHS), have developed what are known as Estrogen Receptor "Knockout mice." These mice are called ERKOs and have had both of the genes that code for a receptor for the hormone estrogen, known as the ER gene, replaced by a foreign gene. This foreign gene is called the NEO gene because it is responsible for resistance to neomycin in bacteria (Maxwell).

Like the three genotypes (two dominant, one dominant and one recessive, or two recessive genes) that are normally possible, there are three possible genotypes of the mice that will be used in this project.

The ERKO mice have had both ER genes disrupted and could be thought of as having two recessive genes. Wildtype mice have had neither of their two ER genes disrupted and therefore resemble the genotype composed of two dominant genes. Finally, the heterozygotes, which contain one dominant and one recessive gene, have had only one of their ER genes disrupted (Maxwell).

Primers 3 and 4 have been used to amplify DNA sequences that indicate the presence of the ER gene, while primers 5 and 6 have been used to amplify DNA sequences containing the presence of the NEO gene. Unfortunately, no protocol has yet been developed in which both primer pairs could be used in a single assay. As a result, this often means that several PCR tests be done to determine the genotype of one individual organism.

Therefore, it would be advantageous in both time and cost management to determine a way to determine the presence of both genes in a single assay. Therefore, the purpose of this project is to develop a way to determine the genotype of wildtype, heterozygote, and knockout mice in a single assay (Maxwell).

PCR Protocol

The overall PCR procedure is composed of four main steps, (A-D), each containing their own individual sub-procedures.

Step A: Extraction of DNA from the Mice

1. A piece of the tail of each mouse is snipped.
2. This cutting is then subjected to enzymatic degradation.
3. Finally, the DNA is extracted from the small piece of tail by chloroform-phenol.

(Note: For the samples used in these experiments, step A had already been completed by the technicians at the NIEHS.)

Step B: Preparation of Reaction Mixtures

(Note: Before beginning step B, label each PCR tube with the number of the sample it is to contain.)

1. Prepare the Lower Buffer Mixture in a 1.5 mL tube.
2. Prepare the Upper Buffer Mixture without the TAQ Polymerase in another 1.5 mL tube.

(Note: At this point, the Thermocycler Machine is turned on so that it can begin its warming up process.)

3. Place 13 microliters of the Lower Buffer into all PCR tubes.
4. Add 2 microliters of the appropriate sample to the appropriately labeled tubes.
5. Add the TAQ Polymerase to the Upper Buffer Mixture.

6. Add 10 microliters of the Upper Buffer to one of the tubes, pipet in a small amount of mineral oil, about 70 microliters, and place the tube in the Thermocycler Machine.
7. Repeat step 6 for the remaining tubes.

Step C: Setting Up and Running the PCR

(Note: The "Setting Up" of the Thermocycler involves programming in the specific procedure of cycles prior to beginning step B.)

Once all of the sample tubes have been loaded into the Thermocycler, the lid is closed, the start mechanism engaged, and the following program occurs:

1. DNA Denaturation
   (95 degrees Celsius for one minute)
2. Primer Annealing
   (55 degrees Celsius for two minutes)
3. DNA Synthesis
   (72 degrees Celsius for thirty seconds)

After steps 1-3 are completed 40 times (40 cycles), the Thermocycler will hold the samples at 4 degrees Celsius indefinitely.

Step D: Separation of PCR Products by

Agarose Gel Electrophoresis

On the same day that the PCR Run is conducted, a 2.8% metaphor gel containing 7 microliters of ethidium bromide and with wells is made and stored in a "coldroom" at least overnight.

On the day for Electrophoresis, the samples are thawed and 3 microliters of tracking dye (6X TCD) are added to each sample. Twenty microliters of each sample containing tracking dye is loaded into its appropriate well of the gel. In addition, one well should contain PCR Markers.

For example, the following guide can be used:

Lane	Sample
1	Wildtype
2	Heterozygote
3	Knockout
4	PCR Markers
5	Wildtype (duplicate)
6	Heterozygote (duplicate)
7	Knockout (duplicate)
8	Empty

The gel is subjected to a current of approximately 100 volts for one hour. After removal, the gel is viewed using an Ultraviolet Light Transluminator.

Materials

1. 1.5 mL Tubes
2. Micropipetters (P20 and P200) with tips
3. Lower Buffer Mixture
4. Upper Buffer Mixture
5. Thermocycler
6. PCR Tubes
7. DNA Samples
8. Mineral Oil
9. 1X TBE
10. Metaphor Agarose
11. Ethidium Bromide Solution
12. Tracking Dye (6X TCD)
13. PCR Markers
14. Electrophoresis Unit
15. UV Transluminator with Camera
16. Safety Glasses

Recipes

1. Lower Buffer Master Mixture

   48 microliters of PCR water

   32 microliters of Buffer J

   4 microliters of Primer 3

   4 microliters of Primer 4 or Primer 6

   16 microliters of 10X dNTPs

2. Upper Buffer Master Mixture

   62 microliters of PCR water

   16 microliters of Buffer J

   2 microliters of TAQ Polymerase

3. 10X TBE

   108 grams of Tris Base

   55 grams of Boric Acid

   40 mLs of 0.5M EDTA (pH=8.0)

   Add in enough water to make the final volume equal to one liter.

4. 1X TBE

   100 mLs of 10X TBE

   900 mLs of distilled water

5. Ethidium Bromide Solution

   100 mgs of Ethidium Bromide

   10 mls of distilled water

6. 2.8% Metaphor Gel

   2.8 grams of Metaphor Agarose

   100 mLs of 1X TBE

Tiffany Nicol Quick
Dates of Experiment: October 24 and 25, 1995
(Note: Chad Locklear aided on the first day of this experiment.)

Experiment #1

I. Purpose

The purpose of this lab was to verify the "known" genotypes of the samples and to verify whether primers 3 and 4 can or cannot be used to determine the three genotypes in a single assay. This lab was also used to gain experience performing the PCR technique.

II. Procedure

(Note: The exact procedure can be found in the section titled "PCR Project Protocol" on page 13.)

III. Data

The samples were loaded as follows:

Lane 1 - Wildtype (Sample 34-15)

Lane 2 - Heterozygote (Sample 34-30)

Lane 3 - Knockout (Sample 39-24)

Lane 4 - PCR Markers

Lane 5 - Wildtype (Sample 34-15)

Lane 6 - Heterozygote (Sample 34-30)

Lane 7 - Knockout (Sample 39-24)

Lane 8 - Empty

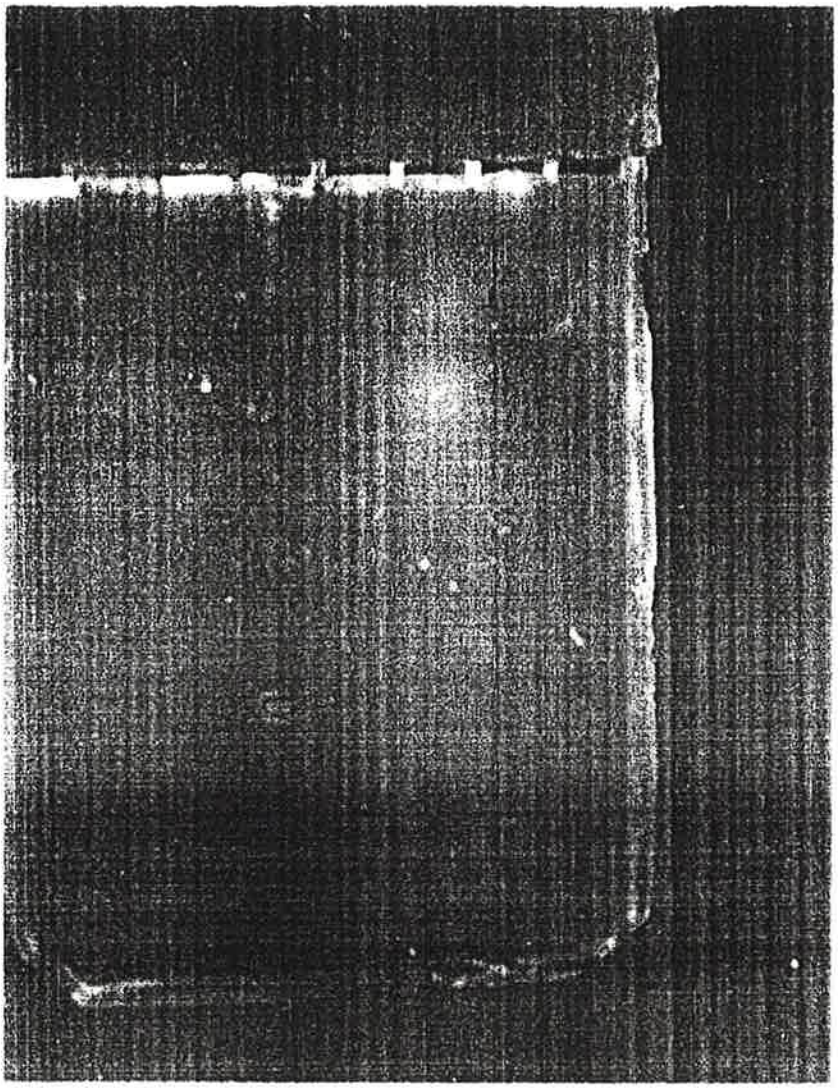
IV. Data Analysis

(Note: A copy of the gel results for this experiment are found on page 20.)

V. Conclusion

For some unknown reason, the DNA stayed in the wells and did not migrate properly.

Experiment # 1



Tiffany Nicol Quick
Dates of Experiment: October 31 and November 1, 1995

Experiment #2

I. Purpose

The purpose of this experiment was to again verify the "known" genotypes of the samples and to verify whether primers 3 and 4 can or cannot be used to determine the three genotypes in a single assay.

II. Procedure

(Note: The exact procedure can be found in the section titled "PCR Project Protocol" on page 13.)

III. Data

(Note: Unfortunately, there were many problems while loading the gel. For example, some of the wells developed holes during the loading of the samples. As a result, the samples were loaded in a way that is described below.)

Lane 1 - Wildtype (Sample 34-51)

Lane 2 - Wildtype (Due to migration of Sample 34-51)

Lane 3 - Empty (An effort was made to load Sample 44-01)

Lane 4 - PCR Markers

Lane 5 - PCR Markers (These are a backup for Lane 4.)

Lane 6 - Heterozygote (Sample 44-01)

Lane 7 - Empty (Trial #2 for loading Sample 44-01.)

Lane 8 - Knockout (Sample 44-14)

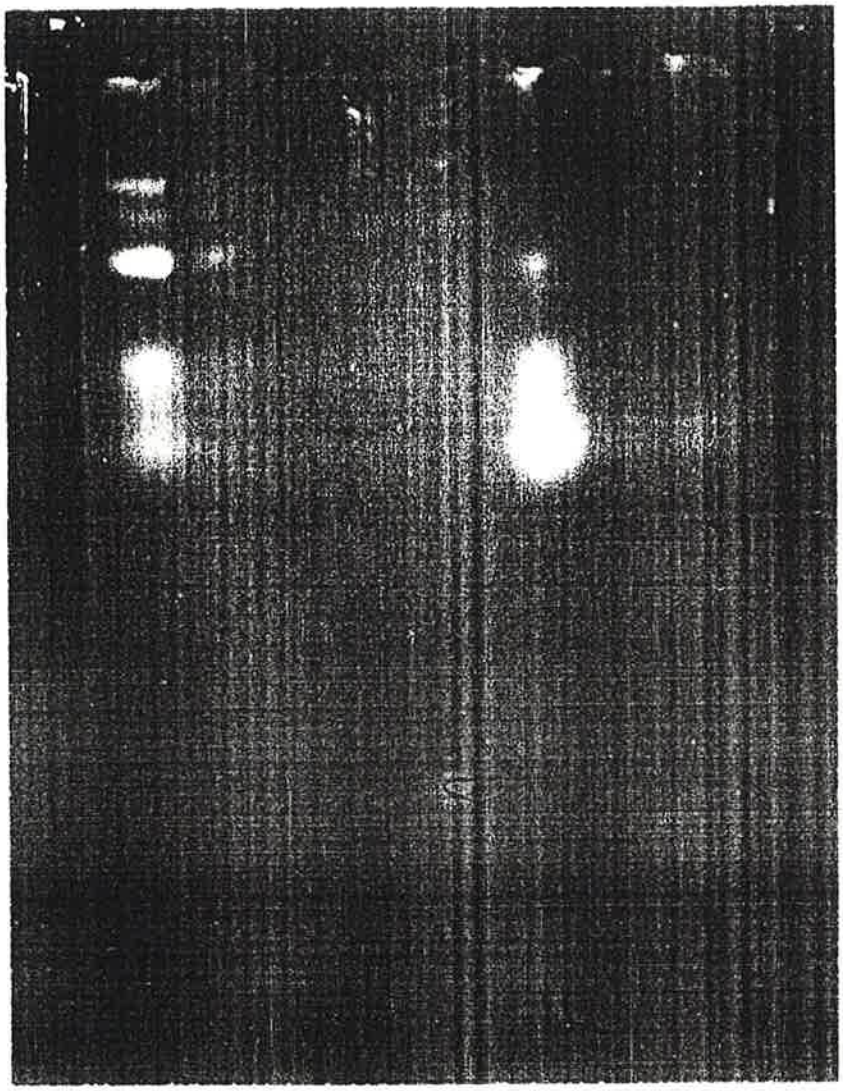
IV. Data Analysis

(Note: A copy of the gel results for this experiment can be found on page 23.)

V. Conclusion

The wildtype and heterozygote found in wells 1 and 6 produced bands as approximately a 239 base product. In addition, nothing occurred in the knockout in well 8. Therefore, it was verified that primers 3 and 4 cannot be used to determine the three genotypes in a single assay. This PCR run was successful.

Experiment # 2



Tiffany Nicol Quick
Dates of Experiment: November 14 and 15, 1995

Experiment #3

I. Purpose

The purpose of this lab was to determine whether primers 3 and 6 can or cannot be used to determine the three genotypes in a single assay.

II. Procedure

(Note: The exact procedure can be found in the section titled "PCR Project Protocol" on page 13.)

III. Data

The samples were loaded as follows:

Lane 1 - Wildtype (Sample 39-27)

Lane 2 - Heterozygote (Sample 45-13)

Lane 3 - Knockout (Sample 46-03)

Lane 4 - PCR Markers

Lane 5 - Wildtype (Sample 39-27)

Lane 6 - Heterozygote (Sample 45-13)

Lane 7 - Knockout (Sample 46-03)

Lane 8 - Empty

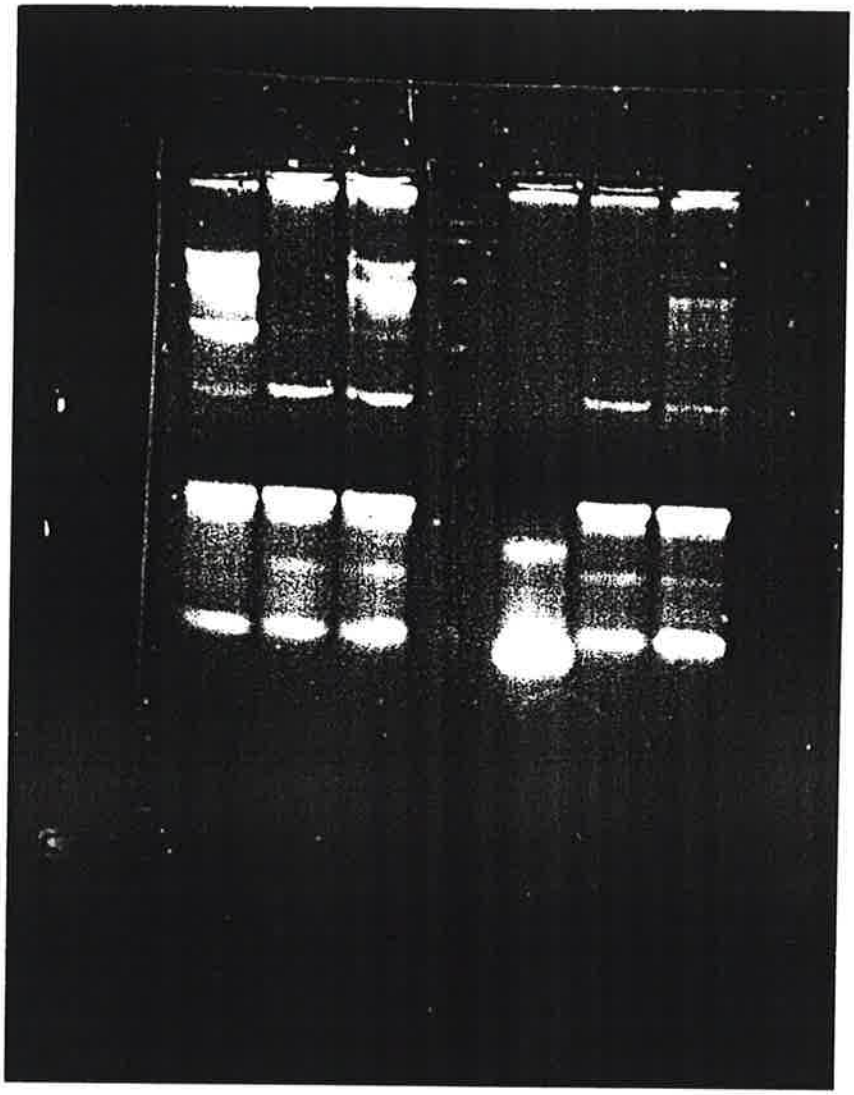
IV. Data Analysis

(Note: A copy of the gel results of this experiment is found on page 26.)

V. Conclusion

Unfortunately, there was inconsistency in the bands produced by the heterozygote and the knockout. The results indicate that there is a possibility that these two samples were either contaminated or labeled incorrectly.

Experiment # 3



Tiffany Nicol Quick
Dates of Experiment: December 6, 7, and 8, 1995

Experiment #4

I. Purpose

The purpose of this lab was to again run an assay using primers 3 and 6 using samples whose genotype was verified in experiment #2.

II. Procedure

(Note: The exact procedure can be found in the section titled "PCR Project Protocol" on page 13.)

III. Data

The samples were loaded as follows:

Lane 1 - Wildtype (Sample 34-51)

Lane 2 - Heterozygote (Sample 44-01)

Lane 3 - Knockout (Sample 44-14)

Lane 4 - PCR Markers

Lane 5 - Wildtype (Sample 34-51)

Lane 6 - Heterozygote (Sample 44-01)

Lane 7 - Knockout (Sample 44-14)

Lane 8 - Empty

IV. Data Analysis

(Note: A copy of the gel results of this experiment can be found on page 29.)

V. Conclusion

Unfortunately, as had occurred before, the DNA did not migrate properly and remained in the wells.

Experiment # 4

Tiffany Nicol Quick
Dates of Experiment: January 22 and 24, 1996

Experiment #5

I. Purpose

Due to the odd results obtained in the third experiment, this lab's purpose is to repeat experiment #3 to determine if primers 3 and 6 can or cannot be used to to determine the three genotypes in a single assay.

II. Procedure

(Note: The exact procedure can be found in the section titled "PCR Project Protocol" on page 13.)

III. Data

The gel was loaded as follows:

Lane 1 - Wildtype (Sample 39-27)

Lane 2 - Heterozygote (Sample 45-13)

Lane 3 - Knockout (Sample 46-03)

Lane 4 - PCR Markers

Lane 5 - Wildtype (Sample 39-27)

Lane 6 - Heterozygote (Sample 45-13)

Lane 7 - Knockout (Sample 46-03)

Lane 8 - Some of the Knockout diffused in

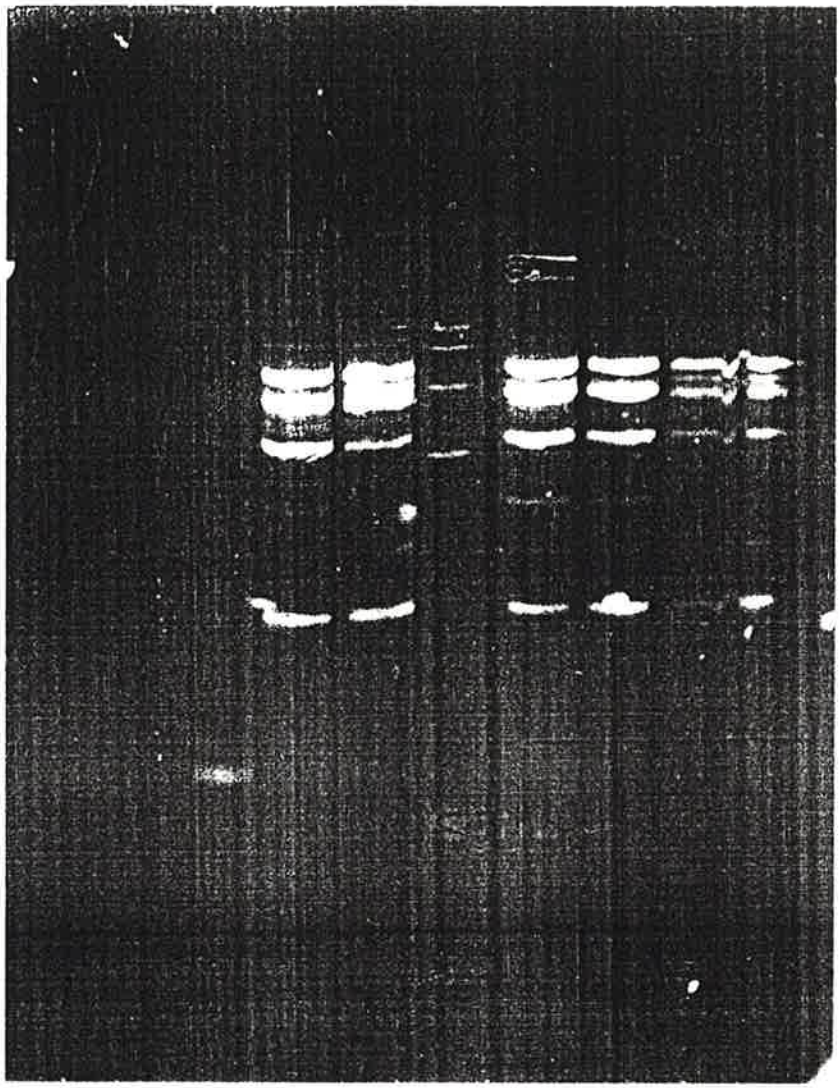
IV. Data Analysis

(Note: A copy of the gel results for this experiment can be found on page 32.)

V. Conclusion

The wildtype and heterozygote samples produced successful results by producing bands in the range of 648 base pairs. Unfortunately, the knockout samples also produced bands in this range instead of in the 2000 base pair range. Therefore, there is a very strong possibility that the knockout sample is not really a knockout.

Experiment # 5



Tiffany Nicol Quick
Dates of Experiment: January 31 and February 2, 1996

Experiment #6

I. Purpose

The purpose of this lab was to determine whether or not Sample 46-03, one of the "Knockout" DNA Samples, is really a Knockout in the hopes of explaining the odd results produced in experiment #5.

II. Procedure

(Note 1: The exact procedure can be found in the section titled "PCR Project Protocol" on page 13.)

(Note 2: Primers 5 and 6 were used in this experiment instead of primers 3 and 4.)

III. Data

The gel was loaded as follows:

Lane 1 - Some Knockout migrated in

Lane 2 - Knockout (Sample 46-03)

Lane 3 - PCR Markers

Lane 4 - Knockout (Sample 46-03)

Lane 5 - Empty

Lane 6 - Empty

Lane 7 - Empty

Lane 8 - Empty

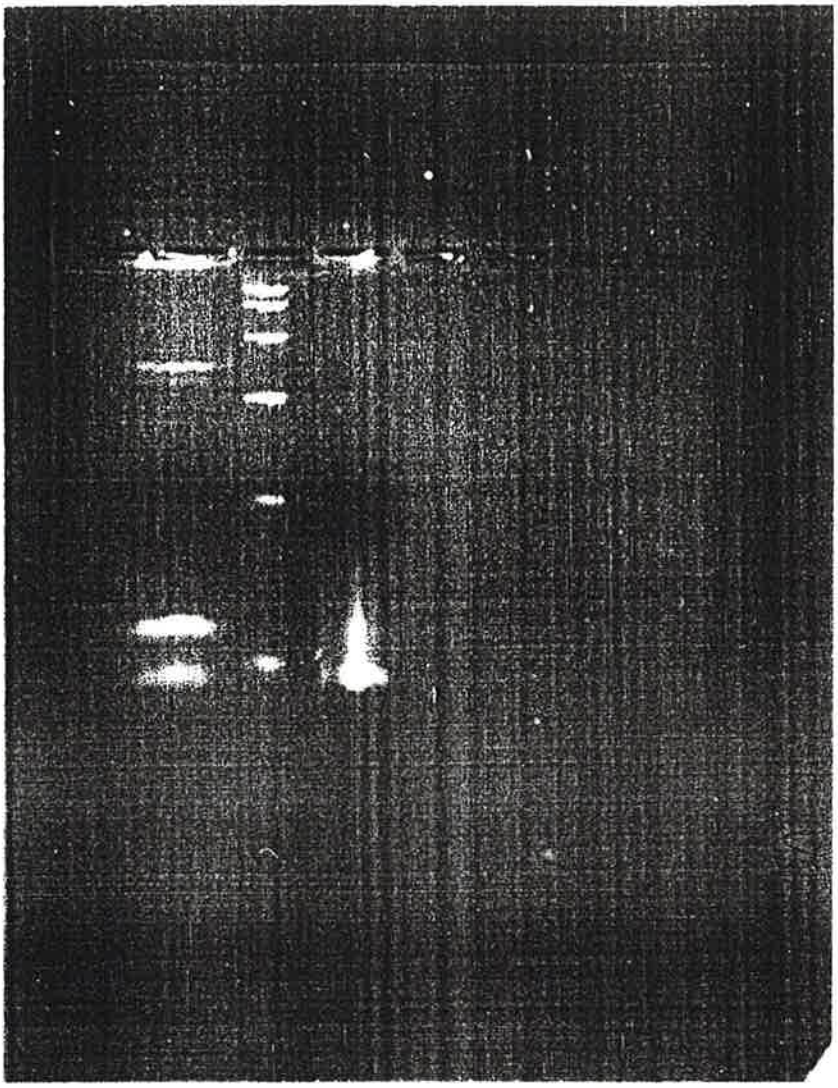
IV. Data Analysis

(Note: A copy of the gel results for this experiment can be found on page 35.)

V. Conclusion

Although a minute amount of DNA remained in the wells, it does appear that Sample 46-03 is not a "Knockout" sample due to the absence of a 649 base pair product which results when primers 5 and 6 are used. Therefore, this experiment was successful in explaining the results that were obtained in experiment #5.

Experiment # 6



Tiffany Nicol Quick
Dates of Experiment: March 21 and 22, 1996

Experiment #7

I. Purpose

The purpose of this lab was to determine if primers 3 and 6 can or cannot be used to determine the three genotypes in a single assay.

II. Procedure

(Note: The exact procedure can be found in the section titled "PCR Project Protocol" on page 13.)

III. Data

The samples were loaded as follows:

Lane 1 - Wildtype (Sample 44-10)

Lane 2 - Heterozygote (Sample 47-18)

Lane 3 - Knockout (Sample 47-03)

Lane 4 - PCR Markers

Lane 5 - Wildtype (Sample 44-10)

Lane 6 - Heterozygote (Sample 47-18)

Lane 7 - Knockout (Sample 47-03)

Lane 8 - Empty

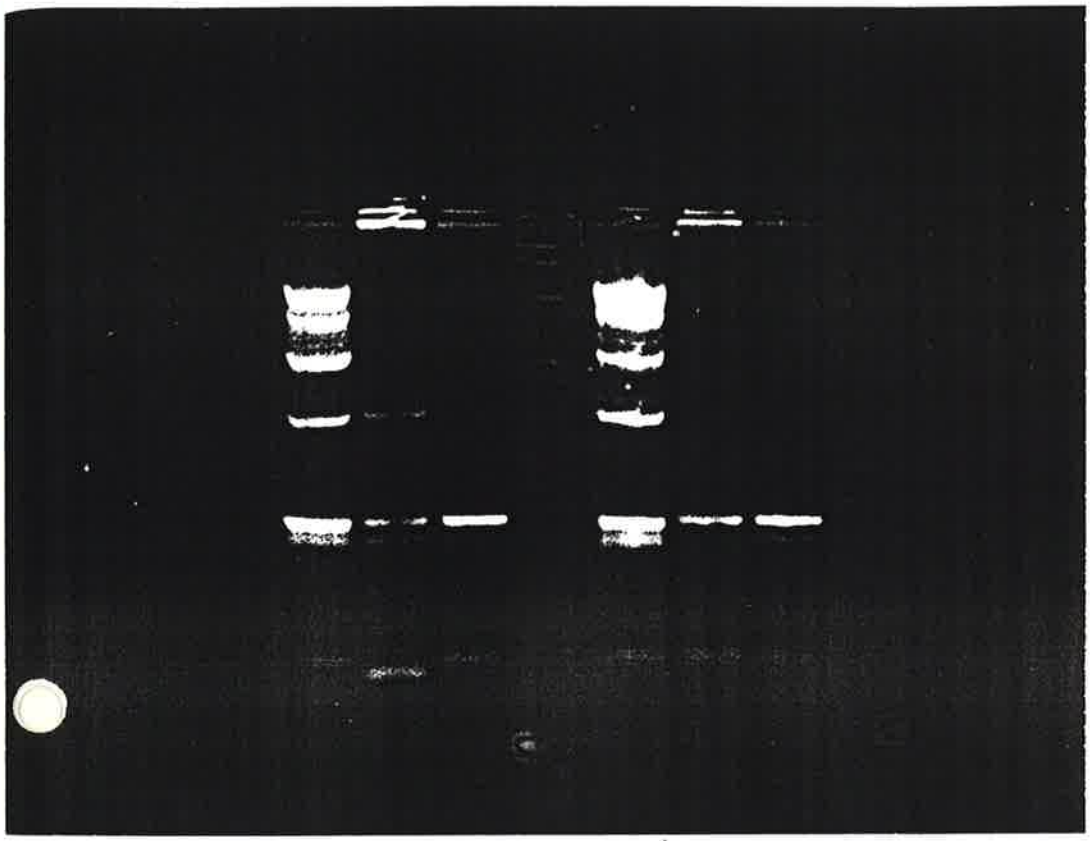
IV. Data Analysis

(Note: A copy of the gel results for this experiment can be found on page 38.)

V. Conclusion

The wildtype, with its band near 648 base pairs, and the knockout, with its band near 2000 base pairs, appear to have produced successful results. Unfortunately, the heterozygote did not.

Experiment # 7



Tiffany Nicol Quick
Dates of Experiment: March 28 and 29, 1996

Experiment #8

I. Purpose

The purpose of this lab was to determine whether primers 3 and 6 can or cannot be used to determine the three genotypes in a single assay by using three samples that appear to have produced successful results.

II. Procedure

(Note: The exact procedure can be found in the section titled "PCR Project Protocol" on page 13.

III. Data

The samples were loaded as follows:

Lane 1 - Wildtype (Sample 39-27)

Lane 2 - Heterozygote (Sample 45-13)

Lane 3 - Knockout (Sample 47-03)

Lane 4 - PCR Markers

Lane 5 - Wildtype (Sample 39-27)

Lane 6 - Heterozygote (Sample 45-13)

Lane 7 - Knockout (Sample 47-03)

Lane 8 - Empty

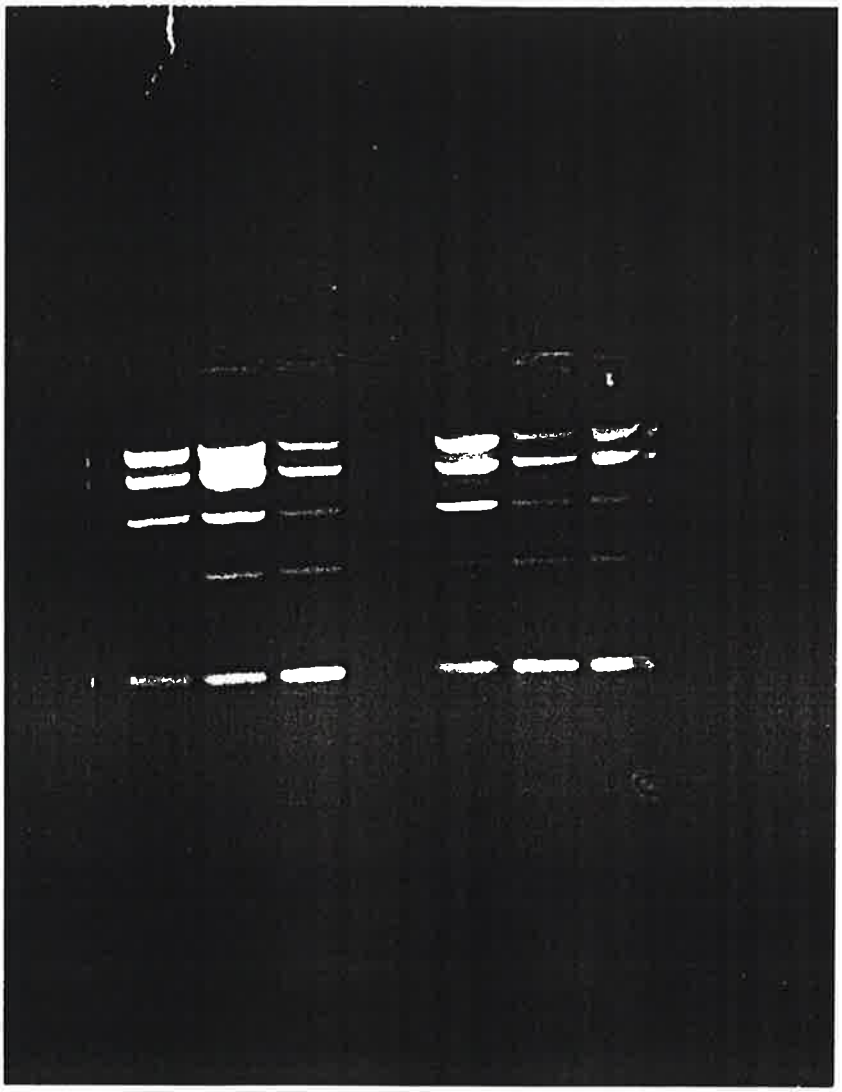
IV. Data Analysis

(Note: A copy of the gel results of this experiment can be found on page 41.)

V. Conclusion

Due to the odd bands produced in lanes 3 and 7, it appears that there may have been some contamination of the once successful knockout sample during this run. However, the wildtype and heterozygote did produce successful results once again with bands in the 648 base pair range.

Experiment # 8



Summary of Results

This project was successful in three main ways:

(1) Familiarity with the ideas and procedures of a PCR technique were gained,

(2) Verification, through experiment #2, that primers 3 and 4 will produce a 239 base product in a wildtype and heterozygote and will produce nothing in the knockout and therefore cannot be used to visualize the three genotypes in a single assay, and

(3) Demonstration, although not shown in one gel, through the gel results of the knockout in experiment #7 and of the wildtype and heterozygote in experiment #8, that primers 3 and 6 can produce a 648 base product in the wildtype and heterozygote and a 2000 base pair product in the knockout.

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